LC Troubleshooting

Starting Out Right, Part II — Measuring Satisfaction

Just how good is good enough?

Last month's "LC Troubleshooting" discussed selecting appropriate starting conditions when developing a new liquid chromatography (LC) method (1). Choosing the proper column and mobile phase is an important step in increasing the likelihood of achieving a successful separation. Another important part of the method development process is obtaining the tools that quantitatively measure the quality of the separation. Although most chromatographers can look at a chromatogram and provide a qualitative opinion about the quality of the separation, it is important to be able to measure the separation quality. This measurement ability is especially important because it allows chemists to assess the separation when intentional or unintentional changes occur.

This month, I will cover the measurement of retention, peak shape, and resolution. These quantitative measurements of separation quality are important from the first injection through method validation and on into the application stage of an LC method.

Retention

The retention time (t_R) is measured as the time between sample injection and the apex of the peak (Figure 1). Retention time probably is the most used chromatographic parameter. Retention is said to be characteristic of a compound but not unique. It is characteristic because if all conditions are held constant, an analyte will be eluted at the same time in every run. For this reason, retention time often is used as a qualitative

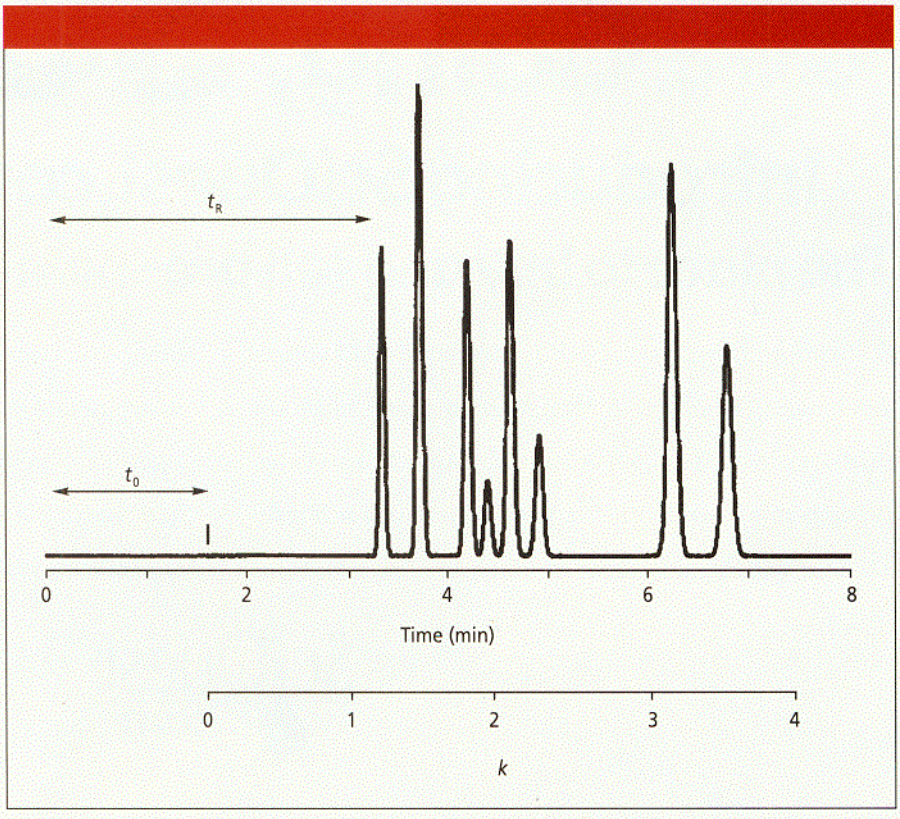

Figure 1: Chromatogram illustrating retention time, column dead time, and retention factor.

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tool to identify a compound. If a standard of the compound is injected, and the retention time is the same as a peak in the sample, it is likely that the two compounds are the same. However, retention is not unique because more than one compound can be eluted at the same retention time. This potential for coelution is the basis of the separation challenge in LC.

Retention Factor

Although retention time is a very useful measurement, the retention factor (k) often is a more useful parameter for method development. The retention factor provides analysts with information about the quality of the separation. For example, chromatographic conditions that generate values of $1 < k < 20$, or better yet $2 < k < 10$, are more likely to yield an acceptable separation than those that generate k values outside these limits. Chromatographers know from experience that if k is too small, the retention is short and the peaks of interest tend to have problems with interferences at the injection front; if k is too large, peaks become broad and hard to detect and the run time is excessive.

The k value can be calculated from equation 1

$$k = \frac{t_R - t_0}{t_0} \qquad [1]$$

where t_0 is the column dead time, usually noted by the first disturbance in the baseline or the elution of a solvent peak (Figure 1). Although k can be calculated, usually it is easier to estimate the value of k, and an estimate is good enough for method development purposes. Note that the numerator of equation 1 is the corrected retention time — the time after t_0 required for the peak to be eluted. The denominator defines the units for k — units of t_0. So to estimate t_0, just start measuring at t_0 and see how many t_0 units the peak requires to be eluted. Figure 1 illustrates this measurement below the time axis. The peak eluted at 6 min comes out three t_0 units after t_0, so the k value is approximately 3. For method development purposes, if k is estimated within 0.5–1 units, it will be satisfactory. It should be noted that k, as defined in equation 1, is useful only for isocratic separation.

Let's pause for a moment and compare t_R and k. Retention is directly affected by changes in the flow rate and column size, whereas k remains constant when either of these parameters changes. Both t_R and k change when the mobile-phase composition or the column temperature changes. Thus, anything that affects t_0 and t_R proportionally will not affect k. The practical implication of this situation? After you obtain a separation that you like on one column, you can change to a column of different dimensions, even if the flow rate is changed, and the k value will remain unchanged, which means that the quality of the separation, in terms of retention, is unchanged.

So when examining a chromatogram, you should make a quick estimate of the k values of the first and last peaks to see if they fall within the $1 < k < 20$ range. If everything is bunched at the beginning (small values of k) or is strongly retained (large k values), a change in the mobile-phase composition probably will be necessary to obtain a satisfactory separation.

Peak Shape

One of the first things experienced chromatographers notice when looking at chromatograms is the shape of the peaks. Ideally, chromatographic peaks are Gaussian shaped, but in practice, most peaks show some peak tailing. Peak tailing is measured using the asymmetry factor (A_s) or the U.S. Pharmacopeia (USP) tailing factor (T_f) as defined in Figure 2. The pharmaceutical industry uses the USP tailing factor as a standard, whereas most other chemical applications use A_s to measure peak shape. From a practical standpoint, it doesn't matter which measurement you use, as long as you use one technique to measure peak tailing. The two measures are roughly comparable if minimal tailing is present, as Table I shows (2).

Tailing peaks indicate that more than one retention mechanism is present in the interaction of that peak with the stationary phase, a situation that is undesirable. A more practical concern is that when tailing peaks are present, analysts must use longer run times for baseline separations, and because some of the area is under the tail, the peak heights are smaller, which can compromise detection limits. Usually peak tailing can be minimized by using a column based on Type B silica and operated with a low-pH mobile phase, as was discussed in last month's "LC Troubleshooting" (1). Sometimes mobile-phase additives such as triethylamine can be used to reduce peak tailing.

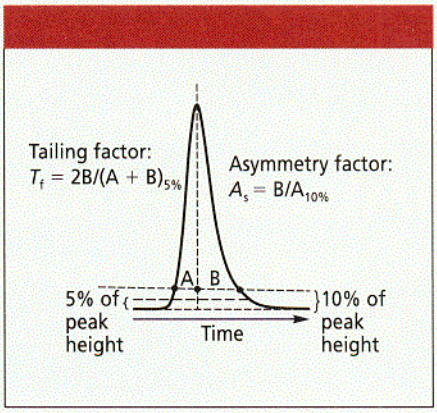

Figure 2: Measurement of peak tailing using the USP tailing factor and peak asymmetry factor.

Table I: Peak Asymmetry and Peak Tailing Factor Relationship*

Peak Asymmetry Factor (at 10%)	Peak Tailing Factor (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

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Column Plate Number

In addition to peak shape, it is important to examine the peak width to determine whether the column is performing in a reasonable manner. Although peak width can be expressed in time units, the plate number (N) is a more useful measure of peak width. Most analysts prefer to calculate the plate number as shown in Figure 3 using the half-height method, in which they use the width at half the peak's height to calculate N. It is easier to determine the width at half-height than at the baseline if the base-

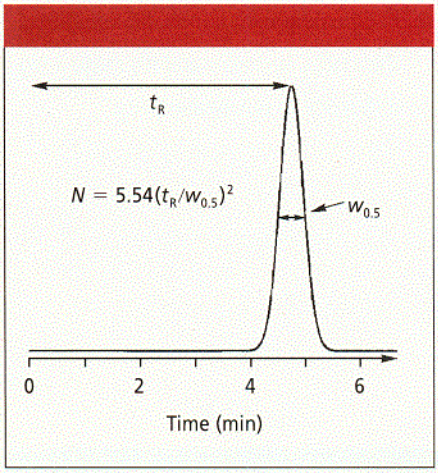

Figure 3: Calculation of the column plate number using the half-height method.

line is noisy or if the peak tails or is not fully separated from neighboring peaks.

A new column containing 5-µm particles will generate roughly 80,000 plates/m using the column manufacturer's test conditions, whereas a 3-µm particle column will yield approximately 100,000 plates/m. These measurements under standardized test conditions are useful when the column is new to determine if it passes initial quality tests, but this testing is inconvenient when the column is in routine use. Furthermore, the test compounds used by column vendors often provide much higher N values than real compounds under normal operating conditions. It is much more convenient to test the column during each use by using a system-suitability sample to verify acceptable performance. For these purposes, the plate number can be estimated as

$$N = \frac{3000L}{d_p} \qquad [2]$$

where L is the column length in centimeters and d_p is the particle diameter in micrometers. Thus, a 150-mm column containing 5-µm particles should have a plate number of approximately 9000 under practical operating conditions. Using this guideline, you should examine peaks in the chromatogram to see if they have reasonable plate numbers. Chromatographers should consider corrective action if the plate number for a peak of interest is more than about 20% below the guideline of equation 2.

Putting It Together

So now you have three tools to help you examine the quality of a chromatogram. First, you will want to estimate k values for the first and last peaks in the chromatogram to see if retention is in the region most likely to yield a good separation. Next, examine the individual peaks to see if they are well shaped and if the peak widths are reasonable. This set of measurements will determine if the peaks are well behaved, increasing the likelihood of a successful separation. However, these observations of the chromatogram are secondary to the real goal of chromatography — obtaining a reasonable separation. To determine if the separation is acceptable, you must make one more measurement — resolution.

Resolution

Resolution is the separation between two peaks in a chromatogram, defined as

$$R_s = \frac{t_2 - t_1}{0.5\left(w_1 + w_2\right)} \qquad [3]$$

where t_1 and t_2 are the retention times and w_1 and w_2 are the baseline widths of the two peaks. An alternative formula uses the half-height peak widths

$$R_s = \frac{t_2 - t_1}{\left(1.7\right)\left(0.5\right)\left(w_{0.5,1} + w_{0.5,2}\right)} \qquad [4]$$

where $w_{0.5,1}$ and $w_{0.5,2}$ are the half-height peak widths. For Gaussian shaped peaks, the valley between two peaks hits the baseline at $R_s \approx 1.5$. To have a safety margin that allows for some deterioration of a method, most workers strive for separations with a minimum resolution of 1.7–2.0. When resolution gets much larger than 2, no particular separation improvement is achieved for most applications and run times can be excessive.

Although equations 3 and 4 are good tools for measuring resolution, during method development resolution is more usefully defined using

$$R_s = \underbrace{0.25N^{0.5}}_{i}\ \underbrace{(\alpha - 1)}_{ii}\ \underbrace{[k/(k+1)]}_{iii} \qquad [5]$$

where α is k_2/k_1, which is the ratio of k values for two adjacent peaks. Equation 5 comprises three major components. Part *i* relates to the column quality. Columns with larger plate numbers result in better resolution. However, resolution improves only as the square root of the plate number, so to double the resolution, chromatographers must increase N fourfold. This change is impractical. For example, connecting four columns in series would mean a fourfold increase in run time and pressure plus a significant financial investment for a twofold increase in resolution. The plate number is easily calculated from first principles, and it is easy to predict how a change in particle size, column length, or flow rate will affect N. Because plate number changes are so easily predicted, it is best to leave these changes to the end of the method development process. Start with a column that will generate a

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reasonable number of plates for most separations. Last month I suggested that a 150-mm, 5-μm d_p or 75-mm, 3.5-μm d_p column was a good starting point.

Part *ii* of equation 5 relates to selectivity — the ability of the column–mobile phase combination to separate two peaks. Selectivity is related primarily to chemical factors, but, as I will describe next month, selectivity and retention are closely related as well. Whereas plate number changes are readily predicted, changes in selectivity are more difficult to anticipate.

Part *iii* of equation 5 is the retention term (in *iii* k is the average k value of the two peaks under consideration). As retention (or k) increases, resolution also improves. An interplay between the retention and selectivity terms exists, because both contain k; that is, a change in k (*iii*) generally will change α (*ii*) as well.

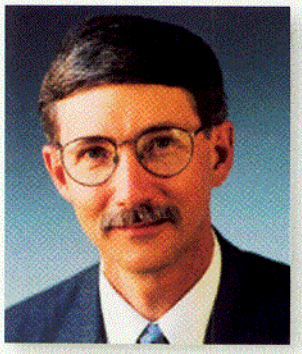
Putting the Tools to Work

Last month, I looked at the importance of selecting a column and the mobile-phase conditions that were most likely to provide a successful separation. Although success under those conditions is not guaranteed, the chances are improved for obtaining an acceptable separation with a minimum investment in method development time.

This month, I presented the tools for measuring the quality of the chromatogram. These tools allow you to look at a chromatogram, make a few calculations, and determine if the separation is satisfactory or not. An unsatisfactory peak shape or column plate number indicates problems with the basic chromatographic process, and they should be addressed before further method development. Generally, chromatographers can adjust retention (k value) and resolution by changing either the mobile- or stationary-phase conditions. Now that you have these tools at your disposal, you can move into the method development process and use them to move quickly to an adequate separation. Next month I'll examine how to control retention and selectivity, using equation 5 to help guide us.

References

1) J.W. Dolan, *LCGC* **17**(12), 1094–1097 (1999).
2) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997), p. 210.



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