TROUBLESHOOTING

Chromatographic Theory as a Problem-Isolation Aid: Part I

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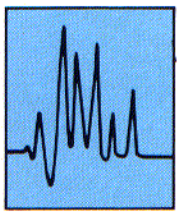

Many chromatographers shy away from the use of theory to help them isolate problems that arise in their chromatographic systems. It is unfortunate that this "chromatophobia" places one of the most powerful problem-isolation tools out of the chromatographer's reach. This month's discussion will cover the use of column theoretical plate number (N), capacity factor (k'), and column selectivity (α) as tools for measuring the performance of a chromatographic system. Note that the use of these chromatographic parameters as discussed here is limited to isocratic conditions. If you are using gradient elution for routine assays and observe a degradation in system performance, you should return to standard isocratic conditions to perform the tests described here in order to isolate the problem.

COLUMN PLATE NUMBER

The column parameter most frequently evaluated is the column theoretical plate number (N), which is often referred to as *column efficiency*. Column manufacturers use this unit of comparison as proof of the quality of their columns. Commercially available columns that are packed with 5-μm particles typically have column plate numbers in the range of 60,000 plates/m to 100,000 plates/m. Columns packed with 3-μm particles may have as many as 150,000 plates/m. Manufacturers frequently include column performance measurements with the column when it is shipped. In some cases, these measurements are the actual results of testing of the column that is shipped; in other cases, the results are simply typical figures for columns made from a particular packing lot. All column manufacturers will replace a new column if, upon receipt, it does not perform as claimed on the test certificate. It is therefore good practice to repeat the column test *before* the column is used for sample analysis. The manufacturer's test conditions are generally included on the column test certificate; toluene, for example, may be used to determine the value of N for a C18 column with a 70:30 methanol/water mobile phase.

If, after repeating the column manufacturer's recommended test, you find that the column demonstrates less than about 90% of the claimed efficiency, you should try to isolate the problem. Poor column efficiency can be caused by a bad column, extracolumn factors, or a combination of the two. The easiest way to isolate the problem is to exchange the test column with another new column or with one that you know is performing well. If the results are satisfactory with the substituted column, you should call the manufacturer to arrange for a replacement of the faulty column. If the efficiency is still low, however, you should try to isolate the problem by using some of the methods discussed below.

In order to gain some insight into the cause of low column efficiency, it is necessary to understand the factors that determine the column plate number. One common formula for calculating N is provided in Equation 1:

$$N = 16(t_r/t_w)^2 \qquad [1]$$

where

t_r = retention time
t_w = peak width at the baseline between tangents drawn to the peak.

N is inversely related to system variance, σ_{tot}^2. Hence, larger values of N indicate a better column, and smaller values of σ_{tot}^2 are desired for more efficiency. The width of a Gaussian band at the baseline is 4σ, so $4t_r/t_w$ is used to calculate the value of N. Squaring this expression produces the right half of Equation 1. Calculating N with Equation 1 requires drawing tangents to the peak and accurately determining the position of the baseline. Determining the tangents may be difficult if the peak fronts or tails significantly. For ease of measurement, Equation 1 can be converted to Equation 2, which uses the peak width at one-half of the peak height, $t_{w1/2}$, instead of peak width at the baseline:

$$N = 5.54\,(t_r/t_{w1/2})^2 \qquad [2]$$

In this case, measure the retention time from injection to the peak maximum for t_r and the width of the peak at one-half of its height for $t_{w1/2}$. Other methods, such as statistical moments (which are best performed by a computer), exist for calculating N, but the manual method that is based on Equation 2 is quite satisfactory for this application. It was mentioned above that N is related to the total *system* variance, σ_{tot}^2. This definition implies that other factors besides column performance contribute to the total variance. The major factors appear in Equation 3:

$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{inj}^2 + \sigma_{det}^2 + \sigma_{tub}^2 \qquad [3]$$

where the total variance is a sum of the contributing variances from the column (σ_{col}^2), injector (σ_{inj}^2), detector (σ_{det}^2), and tubing (σ_{tub}^2). For a well-designed system with a 25-cm column, 5–20 μl injections, an 8–10 μl detector cell, and 0.010-in. i.d. tubing, the column is usually the major factor in band spreading. If, however, you switch from the 25-cm column to a 5-cm column with the same internal diameter and efficiency (with all other factors remaining constant), the column will contribute only one-fifth as much to σ_{tot}^2. Extracolumn volumes now become significant if the system is to be operated at maximum efficiency. Switching to a 25 cm × 1 mm microbore column reduces the band spreading and, as a result, reduces the column contribution to total variance by a factor of about 20. It is easy to see why it is difficult to operate 1-mm microbore columns with conventional liquid chromatographs. More in-depth discussions of system performance with short columns (1) and microbore columns (2) may be found in recent issues of *LC Magazine*.

If you do use columns that contribute less to σ_{tot}^2 than conventional columns, you must take precautions to minimize the contributions from the other elements of Equation 3: injection volumes must be minimized, smaller detector cells and time constants must be used, and 0.010-in. i.d. or smaller connecting tubing must be used between the injector and column and between the column and detector. Carefully assembled fittings are also important because they can add extracolumn volume to the system if they are not assembled properly (3). A more detailed discussion of the influence of these parameters may be found in a good reference text such as Snyder and Kirkland's *Introduction to Modern Liquid Chro-*

matography (4). An example of the application of column efficiency measurements to size-exclusion columns is given in an article by Ekmanis (5).

These comments should support the advice given in a previous Troubleshooting column: keep good records of the performance of your chromatograph under standard conditions (6). With a good system record, you can tell if the column efficiency dropped gradually with use, as is expected, or if deterioration was sudden, as is often the result when system components are changed.

CAPACITY FACTOR

The capacity factor (k') is a measure of retention and is often a more valuable parameter to monitor than retention time for early detection of system problems. Manufacturers usually specify k' for selected compounds under specific conditions. The capacity factor can be calculated by Equation 4:

$$k' = (t_r - t_0)/t_0 \qquad [4]$$

where

t_r = retention time (or retention volume)

t_0 = column dead time (or dead volume).

Because all of the units in Equation 4 cancel out, you can measure t_r and t_0 from a chromatogram with a ruler or by using the time values printed on a data integrator. Retention time (t_r) is measured from the moment of injection to the center of the peak of interest. Column dead time (t_0) may be measured as the elution time of an unretained marker such as uracil in a reversed-phase system, or as the point at which the first baseline disturbance occurs. Column dead volumes of about 3 ml and 2 ml are typical for analytical columns with dimensions of 250 mm × 4.6 mm and 150 mm × 4.6 mm, respectively. Examination of Equation 4 shows that k' is simply a measurement of the retention corrected for dead time ($t_r - t_0$) expressed in units of t_0. It should be noted that k' is independent of flow rate and column dimensions. In other words, if you change the flow rate from 1 ml/min to 3 ml/min, the retention time decreases by a factor of 3, but the k' value does not change. Similarly, the addition of a guard column or a reduction in column length from 25 cm to 5 cm will not change the k' value as long as there is no change in the mobile phase and the packing material.

Once again, careful record keeping, which includes recording k' values under specific conditions, is a great aid in identifying the causes of changes in system performance. Did the k' value change when you changed columns, made a new batch of mobile phase, or modified your sample preparation technique? Good records greatly simplify problem solving.

COLUMN SELECTIVITY

Column selectivity (α) is a measure of the separation of adjacent peaks in a chromatogram. The manufacturer usually provides the value of α with other data obtained from column performance tests. Column selectivity is the ratio of the capacity factors of two adjacent bands:

$$\alpha = k'_2/k'_1 \qquad [5]$$

where k'_1 and k'_2 are the capacity factors for the first and second bands of the pair of interest. The α value is one of the most important parameters because the separation of two adjacent bands is the basic goal of liquid chromatographic separations. The α value is often the parameter that varies the most between similar columns from different manufacturers. This variation is one reason why it is so difficult to repeat a separation on one manufacturer's column that was developed for a different manufacturer's column. The α value is also much more difficult to control than N or k'. The plate number (N) can be increased or decreased in a predictable manner by changing the column length or particle diameter. The value of k' can also be changed predictably by altering the mobile phase strength. On the other hand, the value of α is based on specific chemical interactions among the sample molecules, the mobile phase, and the stationary phase. These interactions are not completely understood, and it is this lack of understanding that leads to the trial-and-error approach to methods development that is used by many chromatographers. Even sophisticated optimization schemes such as Simplex optimization and the Sentinel system (Du Pont Co., Wilmington, Delaware) are merely regimented search routines that help to reduce the time involved in analyzing a large number of conditions for a given separation.

An observed change in the value of α may be gradual or sudden. Gradual variations in α are generally expected as the column ages. The buildup of contaminants at the head of the column or on a guard column is probably the most common cause of changing α values. If adverse conditions such as high pH are used that result in the slow dissolution of column packing or other irreversible damage to the column, changing α values are often seen. Sudden variations in α that occur upon changing a column may result from selecting the wrong stationary phase, changing column brands, or replacing a deteriorated column with a new one. Modifying the mobile phase components and/or composition will also change α. Although temperature has less of an effect on α than on N, large temperature variations will alter α values. If you have kept a good logbook, identifying the system modification that affected the α value should be relatively simple.

CONCLUSION

The three parameters of chromatographic performance — N, k', and α — are useful in two contexts. They are used to verify the claimed performance of a new column when it is received from the manufacturer. This procedure involves repeating the manufacturer-specified column test conditions. If you find significant deviations from the specifications that cannot be explained by extracolumn factors such as the use of large-bore connecting tubing, a replacement column should be requested from the manufacturer.

These parameters are also useful for maintaining a quantitative operation record of system performance in your logbook. By keeping a daily record of N, k', and α values for a given separation to use as a reference, you will find that the isolation of system performance problems is greatly simplified.

Despite their usefulness in these cases, N, k', and α do not provide all the information that is needed to determine the quality of the separation of interest; for example, α measures only the distance between two band centers. The bandwidth is also important in determining how well two peaks are separated. In other words, for a given α value, a column with a large value of N (narrow peaks) may produce a good separation; however, another column that has the same α value but lower efficiency (broader peaks) may produce an unacceptable band overlap.

The three parameters, N, k', and α, are conveniently combined in a fourth parameter, resolution (R_s), which provides much more information about the quality of the separation than any of the individual components. The November "Troubleshooting" column will contain a discussion of resolution, the influence of each parameter on resolution, and some suggestions for optimizing resolution by making selected changes in the individual parameters.

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Readers are invited to contribute their troubleshooting tips to this column or to submit topics or questions for discussion in future articles. Write to: The Editor, *LC Magazine*, P.O. Box 50, Springfield, OR 97477.

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