Troubleshooting

Starting Out Right, Part V — Changing Column Conditions

Resolution, pressure, and run time — choose any two.

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In the four preceding installments of "LC Troubleshooting" (1–4), I provided guidelines that progressed from initial injection to using the mobile phase to obtain a successful liquid chromatography (LC) separation. Hopefully, by the time these steps in the method development process have been completed, the method will separate all the peaks in the sample. When the chemical factors in the chromatographic process have been fully exploited, the separation often can be improved further by adjusting the column conditions — flow rate, column size, and packing particle diameter. This month's "LC Troubleshooting" will focus on selecting column conditions to increase resolution or to improve sample throughput.

Back to the Basics

In February's column (3), I introduced the fundamental resolution equation as a guide for the method development process:

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

where resolution (R_s) is a function of the column plate number (N), the *i* portion of the equation; the selectivity factor (α), the *ii* portion of the equation; and the retention factor (k), the *iii* portion of the equation. The February and March installments (3,4) discussed adjusting solvent strength and mobile-phase chemistry to control k and α and obtain the best possible separation for one or more pairs of peaks in the sample. After these parameters are optimized, chromatographers can do nothing else using that column, solvent system, or temperature to improve the separation. However, at this point in the method development process, the column plate number has not been optimized.

There are several schools of thought about the best column size to use when starting method development. Some workers contend that a short, 30–50 mm column packed with 3-µm particles should be used because it allows very fast runs and, thus, the chance to explore many different mobile-phase conditions quickly. At the other extreme, others would choose a 250-mm, 3-µm d_p column, so that they could obtain the maximum resolution from every run. I tend to sit in the middle of the pack — my first choice would be a 150-mm, 5-µm d_p or 75-mm, 3.5-µm d_p column. I feel that although the short columns allow more separations to be run in a shorter time, the plate numbers aren't large enough to provide reasonable separations of anything but simple samples. The long, small-particle columns require low flow rates for reasonable back pressures, so the run times are excessive. The intermediate column lengths generate sufficient separation power for most separations, yet they can be operated at flow rates of 2 mL/min or higher without excessive back pressure.

No matter which column size you select for the initial work, you may improve the overall separation with a change in the column conditions, including the mobile-phase flow rate, column size, and packing particle size. Sometimes these changes can transform a marginal separation into a satisfactory one. At other times, changes in column conditions can trade excess resolution for improved sample throughput. For many samples, the separation is good enough already, so it isn't worth trying to adjust the column conditions. If you have access to chromatography modeling software, you can model changes in column conditions to help you decide if this avenue of method improvement is worthwhile.

Flow Rate — Simple Things First

As it is with mobile-phase parameters, analysts always have a choice of which column parameter to vary first. I usually choose to vary flow rate first, although flow rate is not likely to make a large change in resolution, because it is easy and it will not change selectivity due to the absence of potential chemical changes. The flow rate's impor-

tance as a parameter influencing resolution is decreased as the column packing's particle size is reduced. Figure 1 illustrates this relationship by comparing 10-, 5-, and 3-μm d_p column packings. Compare the results of a change in flow rate for each packing size. In these comparisons, it is easy to focus on the resolution, which is expressed as the depth of the valley between the two peaks. With the 10-μm d_p material, a dramatic improvement in resolution occurs as the flow rate is reduced from 4 mL/min ($R_s \approx 0.8$) to 2 mL/min to 1 mL/min ($R_s \approx 1.1$). The change for the 5-μm d_p column is less obvious, and the resolution with the 3-μm d_p column changes only slightly. Another way to look at this relationship is that increasing the flow rate from 1 mL/min to 4 mL/min will reduce the run time by a factor of four in all cases, but it costs less than 10% in resolution for the 3-μm d_p column, whereas resolution drops by 25% with the 10-μm d_p column.

For practical purposes, flow rate is not a very important factor with the 3–5 μm d_p columns commonly used today. Most workers choose to adjust the flow rate for the shortest run time while keeping the system pressure within reasonable limits. In cases in which resolution is marginal, a drop in flow rate sometimes can be advantageous, but the effect will be small, as is illustrated in the examples of Figure 1. However, changes in flow rate are easy to make by resetting the LC pump, and, because no chemical change is made, the relative peak spacing will remain unchanged.

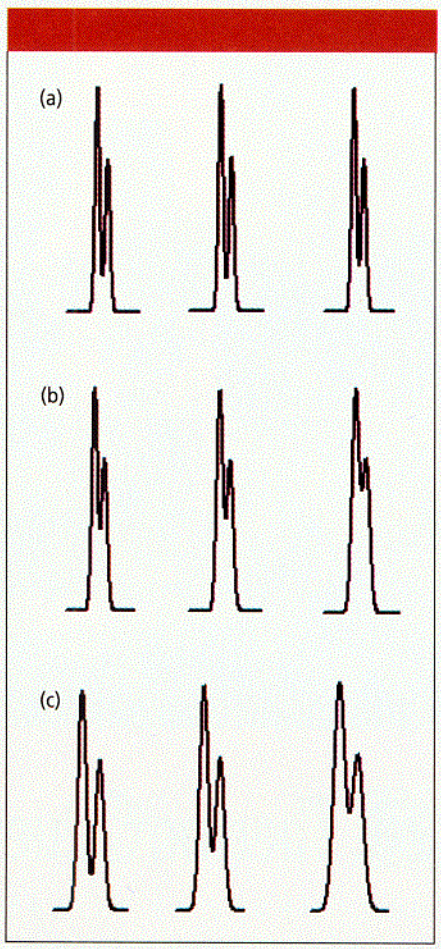

Figure 1: Separations showing the effect of flow rate on resolution for columns packed with (a) 3-, (b) 5-, and (c) 10-μm d_p particles. Flow rates (from left to right) are 1, 2, and 4 mL/min.

Column Size — Too Many Choices

My next choice of column parameters to change is the column size. I choose the column size before the particle diameter because the likelihood of system chemistry changes is minimal. That is, if I switch column lengths for the same type of column from the same manufacturer, it is likely that only the column length will change. On the other hand, if I change the particle size, I know that the base silica was prepared using a slightly different process, and although the column chemistry allegedly is the same, the possibility of minor chemistry changes exists.

Where do you start when considering column size changes? Most manufacturers have a large variety of column configurations from which to choose. For example, I made a quick check of the catalog on the Waters Corp. (Milford, Massachusetts) web site (www.waters.com) for the XTerra columns packed with the company's MS phase. The listing includes 60 separate part numbers distributed between combinations of 2.5-, 3.5-, and 5-μm d_p packings in columns of 20-, 30-, 50-, 100-, 150-, and 250-mm lengths and 2.1-, 3.0-, 3.9-, and 4.6-mm inner diameters. The assumption here is that chromatographers could freely change between any of these columns and have identical selectivity, as long as the mobile-phase composition and column temperature didn't change. Let's take a look at the influence of column diameter and length on the separation; particle size is covered in the next section.

Column inner diameter should have no effect on the separation, if workers take two important factors into account. First, if the linear velocity of the mobile phase is kept constant, the separation should be identical for columns of the same length and packing content. Therefore, if the column diameter is changed, the flow rate must be adjusted according to the square of the diameter change. For example, changing from a 4.6-mm to a 2.1-mm i.d. column

results in approximately a fivefold change in cross-sectional area, so the flow rate must be reduced by fivefold to maintain the same linear velocity.

Second, a potential problem related to column diameter occurs when a column configuration generates very narrow peaks. Because extracolumn volume is more detrimental to small-volume peaks, early eluted peaks from a 50 mm × 2.1 mm column are much more likely to be adversely affected than well-retained peaks on a 150 mm × 4.6 mm column packed with the same particles. For this reason, it is much easier to operate a larger column near its theoretical efficiency than a small, narrow-bore column. When using narrow-diameter columns, chromatographers must take care to use short lengths of narrow-bore connecting tubing and take other precautions to minimize extracolumn band broadening.

Column length, on the other hand, is a convenient tool to adjust the method either to increase the resolution or to reduce the run time. Consider an increase in resolution. The second two peaks in Figure 2a are marginally separated with a resolution of roughly 1.4. Let's assume that our method goal is resolution of at least 1.7. By increasing the column length from 150 mm to 250 mm, resolution is improved to 1.8 (Figure 2b). (Table I lists conditions for these separations.) According to equation 1, resolution increases with the square root of the plate number, and the present case demonstrates that relationship. The plate number increases in proportion to the column length, so $(250/150)^{0.5} \approx 1.3$, and resolution improves by $(1.8/1.4) \approx 1.3$. However, the increase in resolution is not without cost. Note that the pressure increases in proportion to column length, as does the run time. If you want to keep the system pressure near 2000 psi, the flow rate must be reduced by one-third, which would further increase the run time by one-third to approximately 10 min. Because of the square-root relationship between N and R_s (equation 1), you can make only minor gains in resolution by changing column length — perhaps 30–40% gain is possible in favorable conditions.

The runs of Figure 3 illustrate another case in which adjustment of the column length will be useful. In this case, the initial separation (Figure 3a) used a 150-mm long column operated at 1 mL/min. The desired separation for the second two peaks was a resolution of at least 1.7; the actual resolution is 2.1. Although many workers would leave the separation as is, if you need a resolution of only 1.7, this separation is wasting time. Reducing the column length from 150 mm to 100 mm (Figure 3b) reduces the resolution to the 1.7 target, but it also shortens the run time and results in lower back pressure. Doubling the flow rate has no practical effect on the resolution, yet the run time is halved while maintaining a reasonable system pressure (Figure 3c). Thus, in this example, shortening the column by one-third allows the run time to be reduced by two-thirds.

To take advantage of adjusting column length to throw away excess resolution, you must start with a separation that has excess resolution. This is one argument for doing the extra work to get the maximum possible resolution with a separation through adjustment of the mobile-phase chemistry — any excess resolution can be traded for shorter run times by adjusting the column conditions.

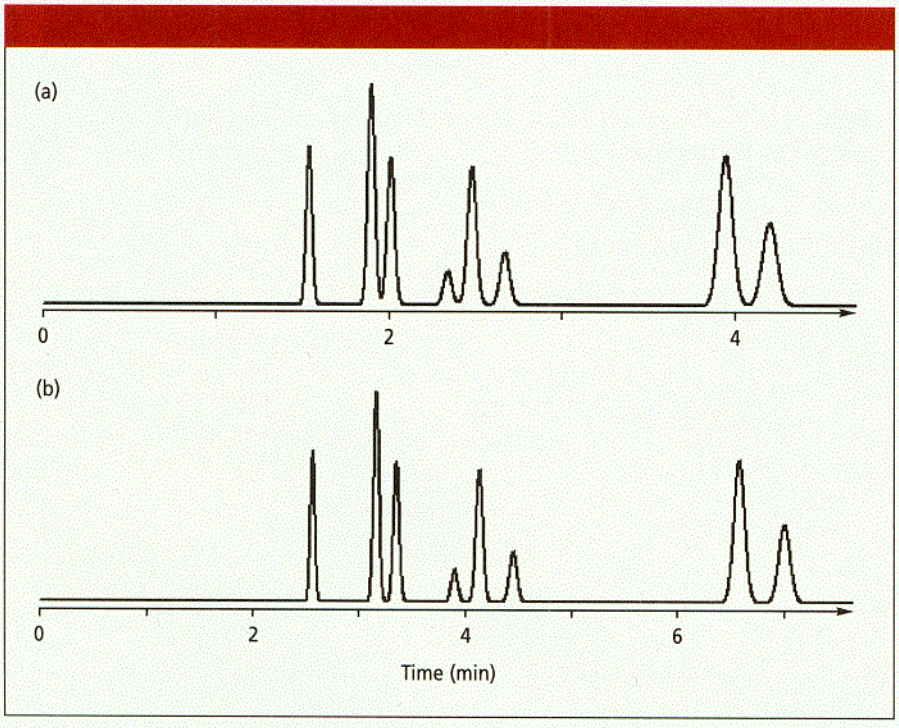

Figure 2: Simulated separations generated using (a) 150- and (b) 250-mm long columns. Pressures were (a) 1800 and (b) 3000 psi. Other conditions are summarized in Table I. The computer simulations are based on data from reference 5.

Particle Size — Is Smaller Better?

It is tempting to think of reducing packing particle size as a universal way to improve separations. A satisfactory separation almost always is a compromise between resolution, run time, and back pressure; therefore, reduced particle size may not provide the best results. By examining Fig-

Table I: Summary of the data from Figures 2–4

Figure	Length (mm)	Particle Size (μm)	Flow Rate (mL/min)	Resolution	Pressure (psi)	Run Time (min)
2a	150	5	4	1.4	1800	5
2b	250	5	4	1.8	3000	8
3a	150	3	1	2.1	1250	18
3b	100	3	1	1.7	825	12
3c	100	3	2	1.7	1650	6
4a	100	1.5	1	2	3300	12
4b	75	1.5	0.7	1.6	1750	13

ures 2–4 and the data of Table I you can see some examples of the advantages and disadvantages of changing particle size. The resolution for the run of Figure 2a using a 150-mm long, 5-µm d_p column definitely improved by changing to a 3-µm packing material, as shown in Figure 3a. However, the smaller particles generated higher back pressure, so the flow rate was reduced and the run time was increased. When the 3-µm d_p column used in Figure 3b was replaced with the 1.5-µm d_p one generating the chromatogram of Figure 4a, resolution again increased but at the cost of trebling the pressure. Shortening the column and adjusting the flow rate (Figure 4b) moved the separation close to the target resolution with a reasonable back pressure, but this separation has no improvement in run time.

My observations from my laboratory and those I visit lead me to believe that the 5-µm particles still are the workhorse packing for routine use. Many times analysts can improve resolution or shorten run times by using 3-µm packing material. When available, I prefer the 3.5-µm particles over the 3.0-µm ones because they allow use of column hardware that is less prone to blockage and provide nearly the same chromatographic performance as 3.0-µm particles. Smaller particles, such as 1.5-µm d_p, are available, but they are best used for specialty applications such as separation of proteins or fast runs when a short, low-plate number column can be traded for speed.

Putting It All Together

You can think of resolution, pressure, and run time as representing three corners of a triangle, as shown in Figure 5. To make gains in one parameter, you must sacrifice the performance of one or both of the other parameters. Figures 2–4, as summarized in Table I, illustrate these tradeoffs. If resolution is held constant, run time can be reduced only at the expense of pressure (compare Figures 3b and 3c). If pressure is kept approximately constant, resolution

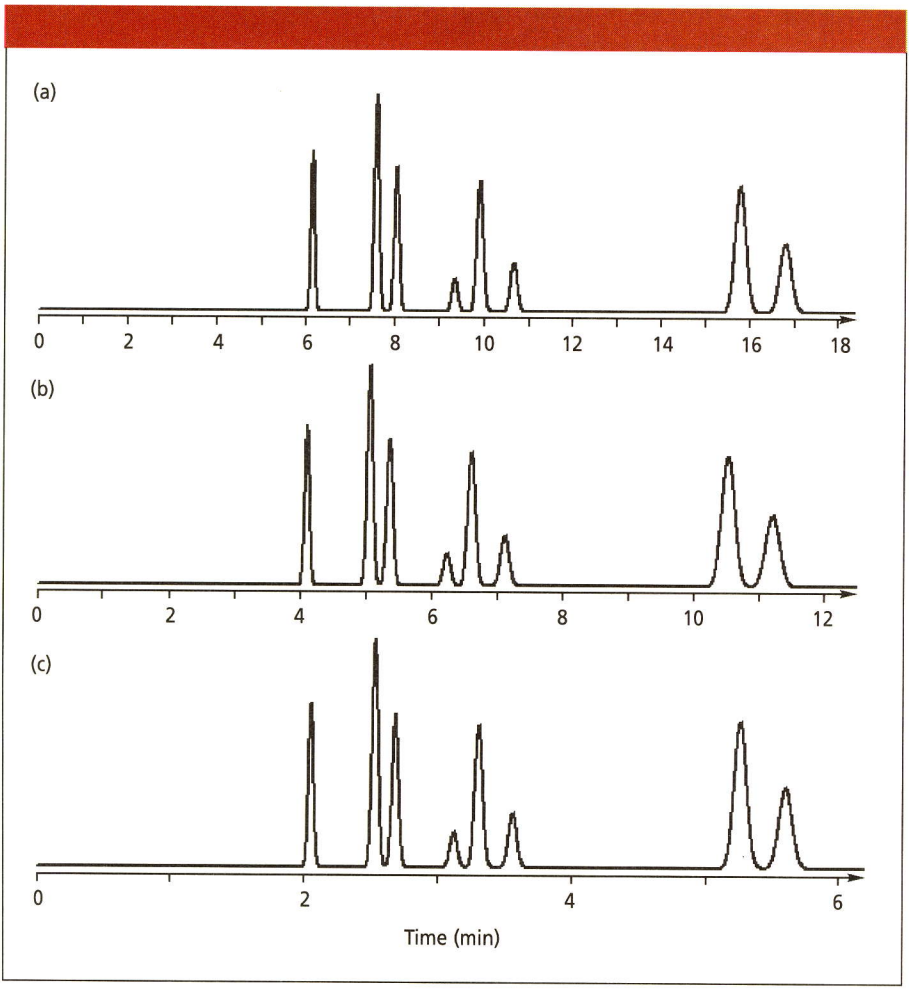

Figure 3: Influence of packing particle size, column length, and flow rate on resolution. Conditions: (a) 150-mm long, 3-µm d_p column operated at a 1-mL/min flow rate; (b) 100-mm long, 3-µm d_p column operated at a 1-mL/min flow rate; (c) 100-mm long, 3-µm d_p column operated at a 2-mL/min flow rate. Other conditions are listed in Table I. Same sample as in Figure 2.

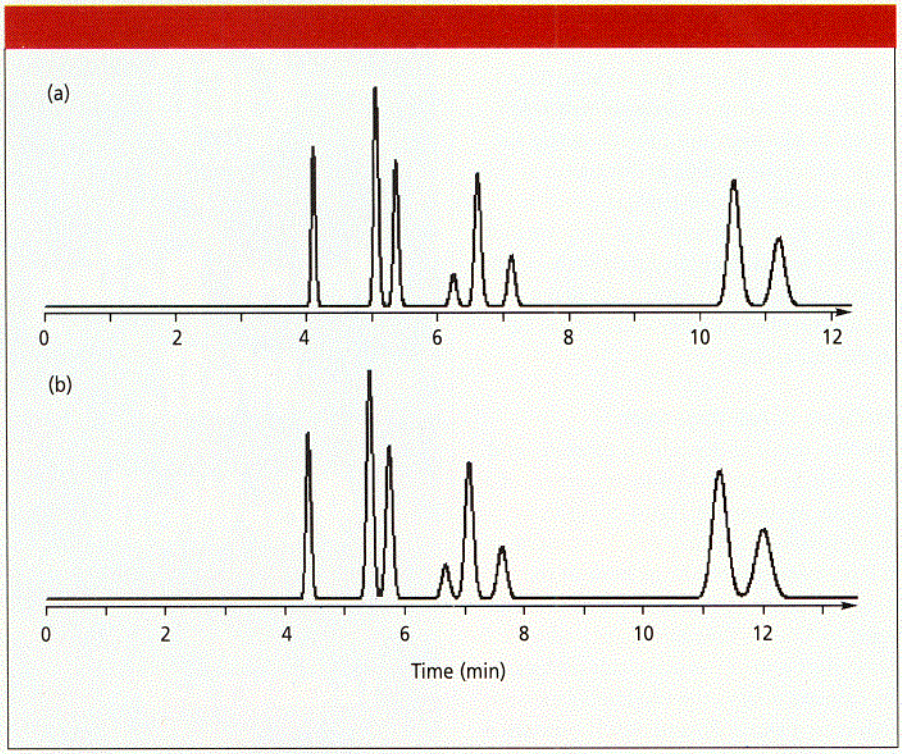

Figure 4: Influence of packing particle size, column length, and flow rate on resolution. Conditions: (a) 100-mm long, 1.5-μm d_p column operated at a 1-mL/min flow rate; (b) 75-mm long, 1.5-μm d_p column operated at a 0.7-mL/min flow rate. Other conditions are listed in Table I. Same sample as in Figure 2.

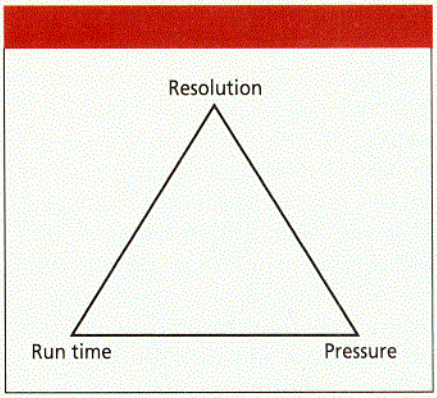

Figure 5: Illustration of the relationship between resolution, run time, and system pressure.

can be increased only at the expense of run time (compare Figures 2b and 4a). If run time is held constant, resolution can be increased only at the expense of pressure (compare Figures 3b and 4a). So you can set goals for resolution, run time, and pressure before starting the method development process, but in most cases, you must make compromises before the final method is complete.

You can fine-tune a separation by making adjustments in the flow rate, column dimensions, and packing particle size. My preference is to start with the flow rate, because it is easy and leaves the selectivity unchanged. Next, I'd change the column length, because it is unlikely that any significant column chemistry changes will occur between columns from the same manufacturer packed with the same size and description of packing material. Finally, I'd look at changing the packing particle size. Although particle size is a powerful way to change the plate number, it carries the most risk of selectivity changes because the different particle sizes were made in a slightly different synthesis process. Fortunately, today's column manufacturers work very hard to give chromatographers a variety of column configurations that have as little variability in column chemistry as possible.

Next month's "LC Troubleshooting" will wrap up the "Starting Out Right" series by considering the use of gradient scouting runs to speed the method development process.

References

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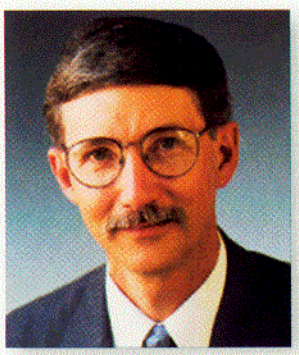
(5) L.R. Snyder, J.W. Dolan, and M.P. Rigney, *LCGC Mag.* **4**(9), 921–929 (1986).



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