LC TROUBLESHOOTING

The Perfect Method, Part I: What Is Your Goal?

Different methods require different strategies.

John W. Dolan
LC Troubleshooting Editor

Nearly every chromatographer needs to do some kind of method development at one time or another. Whether your job is running a routine liquid chromatography (LC) method that needs an occasional "tweak," you need to develop a one-use method to support chemical synthesis, or you need a robust method to monitor a production process, a good understanding of the principles of LC method development are valuable to know. I have titled this series "The Perfect Method," a little tongue-in-cheek, because, at least in my experience, there is no such thing as a "perfect" method — every method I have seen can always be made better. Herein lies the first principle of method development: "better is the enemy of good enough." You can always make the method just a little better, but it comes at a cost of time that you might not be able to afford. Develop a method that is adequate for the job at hand, then stop.

Over the next several months, we'll look at the subject of LC method development in detail. A few years ago (December 1999–May 2000), I covered this topic with a little different emphasis. In terms of reader feedback, the series was one of the most popular discussions in this column. So we'll look at method development again with a little different twist. Before we start, though, let me caution you that this will not be the final, authoritative treatment on LC method development. If method development is a part of your life in the laboratory, your personal library should include reference 1, which I think is the best book ever written on the subject.

Where Are You Going?

We've all heard Lewis Carroll's quote:

"If you don't know where you are going, any road will take you there."

This seems to be the attitude many chromatographers take when they start a method development project. There doesn't seem to be a goal in mind, and even if there is one vaguely formulated, it is felt that a trial-and-error approach will eventually get the job done. Trial-and-error ends up more commonly as error-and-error, which wastes valuable time and money. I think that Laurence J. Peter's take on this subject is much more apropos for method development:

"If you don't know where you are going, you will probably end up somewhere else."

And most of us don't have the luxury of extra time to spend exploring possibilities that lead us away from our goal.

So we need a goal. But that can vary widely. If you desire that method mentioned earlier to use as a quick check of the purity of your synthetic product, a 30-min generic gradient will probably do the job — no need for anything fancy. On the other hand, if your method will need to support a 10,000-sample clinical study, the energy spent in reducing the run time from 6 min to 4 min can well be worth the investment. You could think of a number of different criteria that you might use to help define your goals. Here's a list that we use in one of our method development classes at LC Resources:

- Number of samplers
- Run time
- Number of analytes

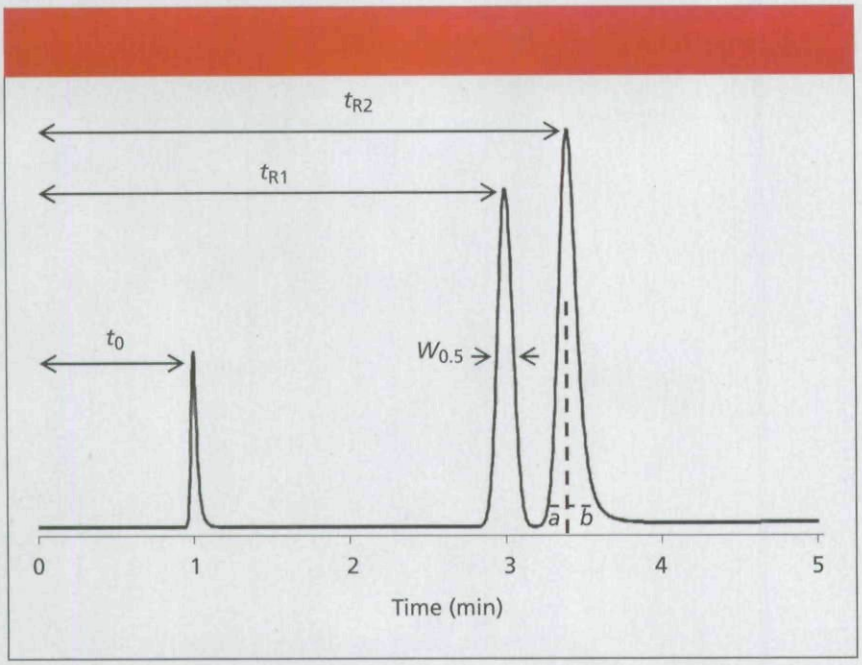

Figure 1: Simulated chromatogram to illustrate calculation of retention factor, tailing factor, resolution, and column plate number.

- Number of matrices
- Sensitivity
- Reproducibility
- Precision and accuracy
- Concentration range
- Qualitative or quantitative
- Equipment or operator limitations
- Sample preparation requirements
- Validation requirements

The list could go on and on. There is no need to discuss each of these criteria in detail, and some will be more important than others for your specific method. Rarely can you answer all the questions, but you can make a good guess in most cases. For example, if you have two active ingredients to quantify in a dissolution experiment at microgram-per-milliliter concentrations, you can be much more specific about your answers than if you are looking at a stability-indicating assay or impurity profile, where force-degraded samples can generate 5–30 peaks, some of which can be in the 0.05–0.1% peak area range relative to the major component. In the latter case, you know that the separation is going to be more challenging than the former, so you can start with an experimental setup that has higher resolving power. A formal document listing the answer to each of the criteria questions might not be required, but it is a good idea to write out a list of as many of the method characteristics as you can think of. If the new method modifies a previous one or is similar to another method, you might be

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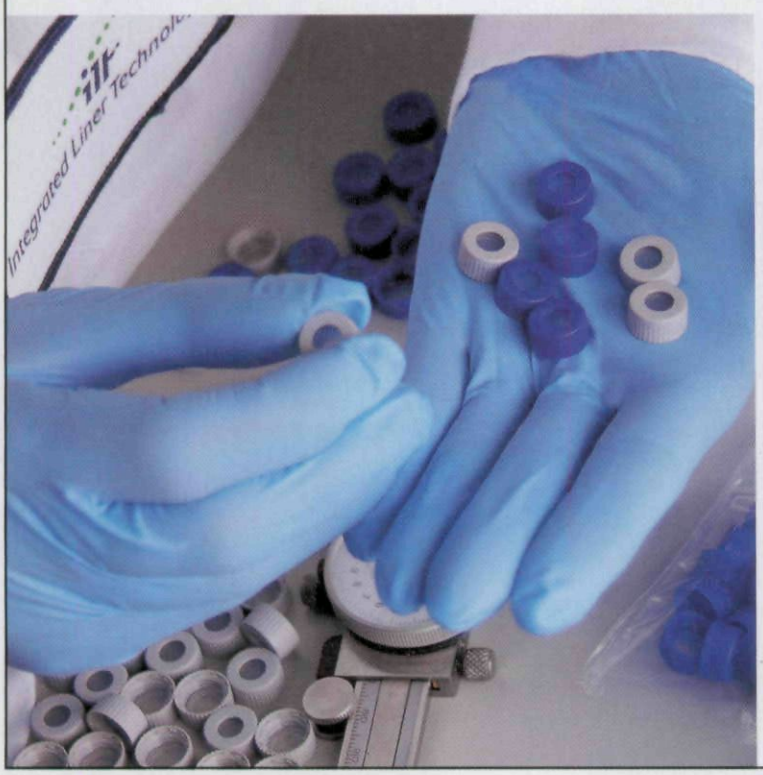

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able to use the performance criteria of the existing method as a starting place.

When Are You Done?

You need to have a way to quantify the endpoint of your development efforts so that you don't fall prey to the "just one more experiment" trap that can needlessly extend the method development process. You need a some quantitative measurements to go with the qualitative "feel" that your method development is ready to use. One way to do this is to follow the recommendations of regulatory agencies. For example, the US Food and Drug Administration's Center for Drug Evaluation and Research (FDA-CDER) publishes "reviewer guidance" documents designed to help their staff in the review of chromatographic methods for adequate performance relative to the regulations. One of these is a guidance for the validation of chromatographic methods (2). This is not law or policy, but gives us a good idea of what the inspectors will look for in our methods. Four of the quantitative criteria are the retention factor, k, (referred to as capacity factor, k', in the document), tailing factor, T_f, (T in the document), resolution, R_s, and the column plate number, N. These are good measurements to make for the evaluation of any separation, and can form the core of a system suitability test that is run before running each batch of samples with a method.

Retention factor is a measure of the distribution of the sample between the mobile phase and the stationary phase, but from a practical standpoint is another way to measure retention:

$$k = (t_R - t_0) / t_0 \quad [1]$$

where t_R and t_0 are the retention time and column dead time, respectively. These are defined as illustrated in Figure 1. The dead time usually is determined either by injecting an unretained substance or identifying the first baseline disturbance in the chromatogram, often referred to as the solvent peak. Retention time is measured from the time of injection to the peak maximum. Ideally, you would like all peaks to be eluted in $2 < k < 10$ for the best chromatographic performance, but $1 < k < 20$ is acceptable, especially for more complex samples. With $k < 2$, peaks can be poorly resolved from the unretained material at t_0 in most chromatograms, and retention is more sensitive to small changes in the mobile phase composition than when $k > 2$. The FDA (2) recommends $k > 2$. For Figure 1, $t_0 = 1.00$ min, $t_{R1} = 3.00$ min, and $t_{R2} = 3.40$ min, so $k_1 = (3.00 - 1.00)/1.00 = 2.00$ and $k_2 = 2.4$.

Tailing factor is sometimes referred to as asymmetry factor (with a slightly different method of calculation), and measures the amount that a peak fronts or tails:

$$Tf = (a + b) / 2a \quad [2]$$

where a and b are defined as shown in Figure 1. A vertical line is dropped from the peak apex and the front and back half-width of a peak at 5% of the peak height are measured. The FDA (2) recommends $T_f < 2$, but you will have better looking chromatograms, improved quantification, and fewer problems separating minor peaks from major ones if you target $T_f \leq 1.5$. For peak 2 of Figure 1, $a = 0.10$ min and $b = 0.16$ min, so $T_f = (0.10 + 0.16)/(2 \times 0.10) = 1.30$.

Resolution measures the separation of two peaks in a chromatogram:

$$R_s = (t_2 - t_1) / 0.5 (w_1 + w_2)$$

where t_1 and t_2 are the retention times of peak 1 and peak 2, respectively, and w_1 and w_2 are the baseline peak widths measured between tangents drawn to the sides of the peak. Determination of the baseline peak width is inconvenient, especially if the baseline is noisy or drifting and if the peaks are not fully separated. Most workers prefer measuring the peak width at half the peak height, $w_{0.5}$, as illustrated in Figure 1, because it is easier and less error-prone. Now equation 3 becomes

$$R_s = (t_2 - t_1) / 1.7 \times 0.5 (w_{0.5,1} + w_{0.5,1}) \quad [4]$$

For well-shaped peaks, the valley between the peaks reaches the baseline for $R_s = 1.5$, but this does not guarantee a complete separation if there is any peak tailing or degradation of the method over time. The FDA (2) recommends $R_s > 2$.

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For Figure 1, $w_{0.5,1} = 0.112$ min and $w_{0.5,2} = 0.126$ min, so $R_s = (3.40-3.00)/1.7 \times 0.5(0.112 + 0.126) = 1.98$.

The column plate number (also called column efficiency) is a measurement of overall column performance. The plate number is influenced most by the packing particle size (smaller particles give larger values of N) and column length (longer columns give larger values of N), as well as many other less important factors, such as flow rate, temperature, mobile phase composition, sample molecular weight, and so forth. The plate number is calculated as follows:

$$N = 16 \left(t_R / w \right)^2 \quad [5]$$

but, as with the measurement of peak width for resolution, it is easier to measure the width at half the peak height, so most workers prefer to use

$$N = 5.54 \left(t_R / w_{0.5} \right)^2 \quad [6]$$

A new 150-mm column packed with 5-mm diameter particles or a 100-mm, 3-mm column will generate $N = 12{,}000$ or more with an well-behaved test compound, but more in the range of $N = 10{,}000$ for real samples. The FDA (2) recommends $N > 2000$. This could be obtained with a poorly performing 50-mm, 5-mm column, so in my opinion, this criteria is not worth much in terms of evaluating the quality of the column. For peak 1 of Figure 1, $N = 5.54(3.00/0.112)^2 = 3975$. One thing to keep in mind is that equations 5 and 6 are for isocratic separations; they will not work for gradient conditions.

Now You Are Ready to Start

You have made a list of the requirements of your method. You have both qualitative (look and feel) and quantitative (R_s, k, run time, and so forth) criteria that you can use to determine if the method is satisfactory. In other words, you know where you are going. In the next installments of this series, we'll look at how to get to that goal. It really is quite simple, again as stated by Lewis Carroll,

"Begin at the beginning and go on until you come to the end: then stop."

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LC TROUBLESHOOTING

The Perfect Method, Part II: Where to Start?

Getting started on the right foot is important for efficient method development.

John W. Dolan
LC Troubleshooting Editor

This is the second installment in a series on how to develop liquid chromatography (LC) methods in an efficient manner. Last month (1), we considered how to set goals for new methods. This month, we will look at some of the factors involved in selecting a starting point for method development. Although our focus in this series is on method development, in the spirit of LC troubleshooting, we need to remember that many of the choices we make during method development will determine some of the problems that may be encountered or avoided with the final method. So each choice of a specific parameter to optimize should be made with a consideration of what kind of problems might occur during method development and with the completed method.

Playing the Odds

The first choice that we have to make in method development is which chromatographic mode we will use. There are reversed-phase, normal-phase, hydrophilic interaction chromatography (HILIC), ion-exchange, size-exclusion, chiral, and other modes from which we can choose. For most of us in the pharmaceutical, environmental, and chemical industries, the choice will be reversed-phase LC. I look to Ron Majors' "Column Watch" reviews of the Pittsburgh Conference each spring as a finger in the wind in terms of favored column technology. Year after year, you'll see that the most common columns, either in terms of overall use or new product introductions, are reversed-phase columns. The reasons are simple — they provide the necessary separation power for a majority of separation problems, are easy to use, and are reasonably robust. If I were a gambling man, I'd lay my money down on the reversed-phase bet every time, unless I had a solid reason to choose otherwise.

Some obvious applications require other chromatographic modes. If your sample contains chiral compounds, you need a chiral column, chiral mobile phase, or chiral derivative to enable the separation — reversed-phase LC just won't work. If you need to maintain biological activity of an enzyme or other biomolecule, you will avoid reversed-phase LC because of its strongly denaturing mobile phases. Separation of ionic compounds, particularly inorganic ions, generally will go better with ion-exchange or ion chromatography. The separation of positional isomers is difficult by reversed-phase LC, but generally straightforward by normal phase. So if your samples have special characteristics that preclude use of reversed-phase techniques, use common sense and go with the chromatographic mode that is most likely to lead to success. But for the vast majority of compounds, reversed phase is the best place to start.

Continuous or Discontinuous?

Now that we've decided upon reversed phase as our starting column type, we need to think a bit about the strategy we will use to get a reversed-phase method. There are several variables that we can use during the development process. We need to choose wisely to make the most out of our investment of time and money. One way to classify the parameters is whether they are continuously variable or not, as listed in Table I. Continuous variables are those that can be changed in infinitely small steps, which gives an advantage in fine-tuning the separation and generally makes them more convenient to use. As the concentration or magnitude of a continuous variable is changed, retention changes in a regular fashion, generally in a linear or

logarithmic manner. Discontinuous variables are those that can be changed only in a stepwise fashion, and as a result, retention does not change in a continuous manner. Let's consider the list in Table I.

Solvent strength: By solvent strength, we mean the amount of the strong solvent in the mobile phase, usually methanol, acetonitrile, or tetrahydrofuran in reversed-phase LC. This also is referred to as percent B-solvent (%B). Of course, we can vary the %B in any increment we want.

Temperature: Temperature can be varied most easily from a few degrees above room temperature to the limit of the column or column oven. This means temperatures in the 30–70 °C range for most systems.

Solvent type: The solvent type can be changed from methanol to acetonitrile to tetrahydrofuran. At first glance, you might think of this as a discontinuous variable, but on closer examination, it is continuous. For example, you can blend methanol and acetonitrile in any combination you desire, making it a continuous variable. In fact, blending solvents can be a very powerful tool so that the characteristics of each solvent can be fine-tuned for maximum separation power.

Additives: The concentration of mobile-phase additives, such as buffers, ion pairing reagents, salts, or amines, can be adjusted in a continuous fashion from not present up to their point of saturation in the mobile phase.

pH: The mobile-phase pH falls in a grey area between continuous and discontinuous variables, so I listed it in parentheses in Table I. Most reversed-phase columns will operate satisfactorily in the $2 < pH < 8$ region, and base-stable columns will operate at higher pHs. The pH can be adjusted in a continuous manner, so in that context, pH is a continuous variable. However, the effect of a change in the pH is not continuous. In the region of ± 2 pH units of the pK_a of a compound, the pH will modify retention in a predictable and regular manner, but once outside this region, additional changes in pH usually have little effect on retention.

Column type: A change in column type, such as C18 to embedded polar phase to cyano to phenyl, comes in discrete steps. For example, you can't move from cyano to phenyl in 1% steps. This discontinuous nature of a change in the column type means that you will not be able to finetune this variable. You can have one column or another, but not some fraction of each. There is one company (Bischoff, Leonberg, Germany) that makes a column product that allows connecting together discrete column segments containing different stationary phases, but even this is limited to stepwise changes.

Which Variable First?

The ability to fine-tune the effect of a variable and the commercial availability of chromatographic retention modeling software (for example, DryLab, Molnar Institute, Berlin) gives us incentive to focus on the continuous variables of Table I before we change column type. Our next decision is which parameter we should focus on first. With many choices, we want to work first with the variable that has a reasonable probability of generating a successful separation. However, at the same time, we want to balance the power of a variable to make a change in the separation with the ease of making adjustments in the variable. That is, we may choose a less powerful variable to pursue first if it is much easier to use than a more powerful one.

Table I: Chromatographic Variables

Continuous Variables
Solvent strength (%B)
Temperature
Solvent type
Additives
(pH)
Discontinuous Variable
Column type

I've classified the variables of Table I in a little different manner in Table II. I have listed some of the characteristics of the variable that will help us make a decision about which one(s) to choose first. We'll look in more detail at solvent strength, then cover the other variables of Table II in less detail, because once the context of Table II is understood, most of the information is simple to understand.

Solvent strength (%B): A change in peak spacing (α) is the desired result of a change in a parameter during method development, so this factor ranks high in selecting our first choice. A change in %B results in a change in α in many cases, but it is not the most powerful variable to elicit a change in peak spacing, so I give it a 0, or neutral rating. A change in the solvent strength works for every compound type and is easy to make — just program a different mobile-phase mixture in the LC system controller — so a + is received for these characteristics. Acetonitrile works well for low-wavelength UV detection (<220 nm); methanol is alright at low wavelengths for isocratic applications, but might not be suitable for gradients; tetrahydrofuran has strong absorbance at <240 nm, but is not used widely. Any of the three solvents will work well for LC–mass spectrometry (MS) applications, although tetrahydrofuran cannot be used when PEEK tubing is present. All in all, detection is not an issue, so another + here. Solvent strength is easy to control and produces robust separations and column equilibration is rapid

Table II: Ranking the variables

Variable	Change in α	Universal	Convenient	Low-UV/ LC–MS	Robustness	Equilibration
%B	0	+	+	+	+	+
Temperature	–	+	+	+	+	+
Solvent type	++	+	+	0	+	0
Ion pair	+	–	+	0	–	–
pH	+++	–	0	0	–	+
Column type	+	+	0	+	+	+

for both isocratic and gradient experiments, so we get another +. You can see that although solvent strength isn't the most powerful variable to change α, it is positive in all other aspects, so it usually is my first choice in a variable to explore during method development.

Temperature: Temperature usually is considered a weak variable in terms of a change in α, and as a result, many workers ignore it. However, it scores well in all other categories, so it might be worth a more serious consideration in light of other data. For example, as was discussed in the May 2007 "LC Troubleshooting" installment (1), in some cases, when ionic samples are present, a change in temperature can have the same effect as a change in pH, yet is much easier to control. As we'll see in a later column, temperature and gradient elution is an especially powerful combination of variables for eliciting selectivity changes.

Solvent type: A change in mobile-phase organic solvent from methanol to acetonitrile to tetrahydrofuran can be a powerful way to change selectivity, it works for all types of samples, and it is an easy change to make. Tetrahydrofuran has strong UV absorbance below about 240 nm and cannot be used with LC–MS when PEEK tubing is present. Gradients with methanol are difficult below about 220 nm because of baseline drift, but the addition of a UV absorber to the A-solvent can allow use of gradients at lower wavelengths. Column equilibration with acetonitrile and methanol is not a problem, but the use of tetrahydrofuran might take a little more time to equilibrate. Blending different solvents, especially a small amount of tetrahydrofuran with acetonitrile or methanol, will create intermediate solvent properties that can be useful for changing peak spacing. On-line blending of solvents under direction of the system controller can allow exploration of many mixtures in unattended operation.

Ion pair: Ion-pair chromatography is a very useful tool for improving retention, especially for hydrophilic, basic compounds and can be effective to change peak spacing. However, ion pairing does not work for nonionic compounds and has many experimental problems, including very slow column equilibration, so most workers consider other variables before ion pairing is explored.

pH: A change in the mobile-phase pH can be the most powerful variable to change peak spacing, but it only works with ionic compounds. It is not difficult to change the pH, but one must make up a new buffer solution — changing pH by online mixing is not reliable. There are buffers that will work well with low UV or LC–MS detection, but many buffers will not work for one or both of these techniques. If the pH is near the pK_a of the analyte, the separation can be very susceptible to small changes in pH, such as by use of buffers outside their buffering region, or changes in temperature or organic solvent concentration. However, most separations will be more consistent if the pH of the mobile phase is controlled, because the pH influences the ionization of the column as well as the sample molecules. For this reason, it is best to control the mobile-phase pH, even if the pH is not being explored as a primary variable. In most cases, a $2 < pH < 3$ is a good default value for mobile-phase pH unless there is a compelling reason to use another value.

Column type: As mentioned earlier, a change in column type can be a very effective way to change selectivity. This is especially true if one can use specific column selectivity comparison tools (for example, see reference 2) to help choose alternate columns. In the absence of such guidance in selecting a column with "orthogonal" selectivity, the ability to successfully choose a column of different selectivity is limited. For example, there might be more difference between two different C18 columns than between a C18 column and an embedded polar phase column. Because the column is a discontinuous variable, changes are less convenient — the column must be removed and replaced or a column switching valve must be used. And finally, because the typical reversed-phase column costs in the $500 range, the expense of changing a column is much greater than changing any of the other variables. For these reasons, most workers prefer to start with a column that will provide a sufficient number of theoretical plates to separate most sample types, and then change the other variables before changing to a different column type.

On Your Mark, Get Set

If we consider the pros and cons of the various parameters discussed previously, we can choose an intelligent starting point. These conditions can be altered based upon specific sample information, but in the absence of other data, they provide a good place to set your starting blocks in the method development race.

I recommend starting development with a C18 or C8 column that will generate enough theoretical plates for a "typical" sample. This generally means a 150 mm × 4.6 mm column packed with 5-μm particles or a 100 mm × 4.6 mm, 3 μm column operated at 1-2 mL/min. For LC–MS and other applications that don't require quite so much resolving power, a 50 mm × 2.1 mm, 3-μm column operated at 0.2–0.5 mL/min is usually the first choice. A temperature a few degrees above room temperature, such as 30 or 35 °C, is a good starting point. Of course, you should choose one of the newer Type-B or high-purity silica columns and use a new column when starting development of a new method.

Silica-based bonded phase columns are most stable in the $2 < pH < 8$ range. Phosphate buffer at pH 2.5-3.0 and 15-25 mM is suitable for UV detection. For LC–MS and other detectors requiring volatile buffers, 0.1% formic acid is a good starting choice. The low pH will suppress ionization of column silanol groups and most acidic sample components. To work above the pK_a of most bases will require a specialty column stable to $pH > 8$, so high-pH operation usually is not the first choice.

Acetonitrile is a good first choice for an organic solvent. It has good UV transparency down to 200 nm and works well with LC–MS. Methanol is a good alternative, but it has stronger UV absorbance at wavelengths below 220 nm. Tetrahydrofuran is less popular because of poor performance at low wavelengths, incompatibility with PEEK, and unfavorable handling characteristics.

These column and mobile-phase conditions are a good place to start most separations. Prior knowledge about separations of a particular sample type might suggest other starting conditions. After the starting conditions are identified, the variables of Table I or Table II can be explored to develop the desired separation. In next month's "LC Troubleshooting," we'll look at the next step in method development — control of retention.

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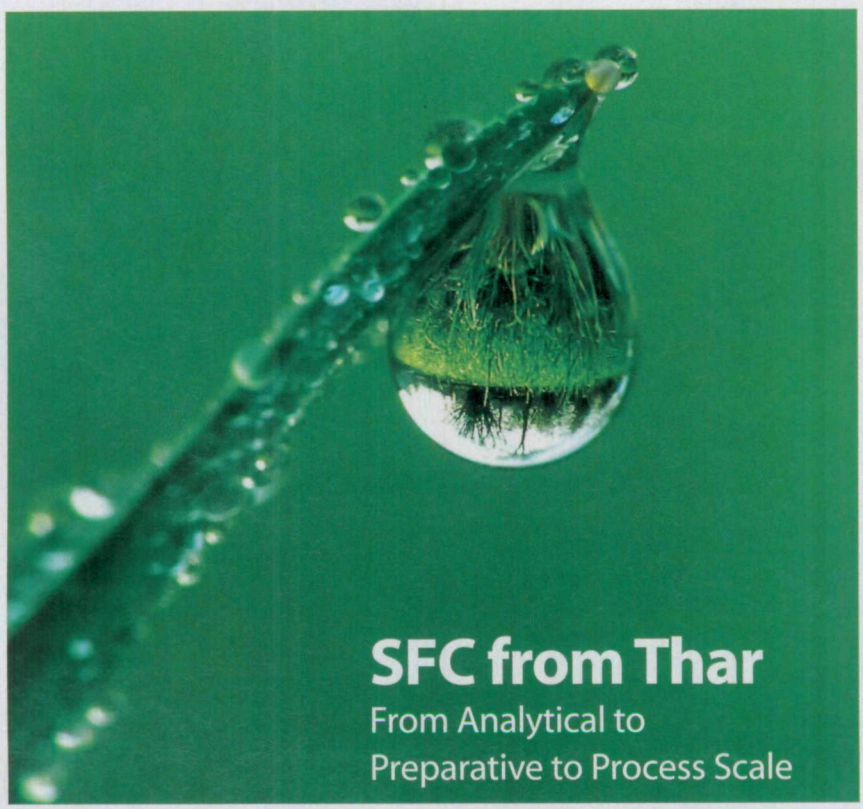

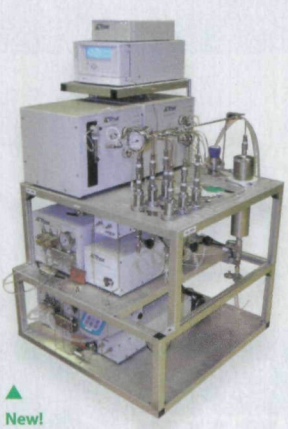

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LC TROUBLESHOOTING

The Perfect Method, Part III: Adjusting Retention

Faster isn't always better

John W. Dolan
LC Troubleshooting Editor

This is the third installment in a series on method development for liquid chromatography (LC). The focus of the series is on developing new reversed-phase methods in a manner that makes for more reliable methods, while at the same time identifying key areas where problems can occur. In the first section (1), we looked at goal setting, and the second (2) considered the selection of starting conditions. This month's installment of "LC Troubleshooting" will focus on selection of mobile phase conditions that will give reasonable retention of our sample compounds.

Last month, we concluded that a good starting place for most methods was to use a 150 mm × 4.6-mm, 5-µm particle column or a 100 mm × 4.6 mm, 3-µm column for most sample types. This configuration gives enough theoretical plates ($N \approx 10{,}000$ for real samples) to separate most sample types and can be run at 1–2 mL/min for fast method development runs. A mobile phase of low-pH buffer (for example, 25 mM phosphate at pH 2.5) blended with acetonitrile, or optionally methanol, was the mobile phase of choice for UV detection unless you have information to suggest otherwise. For LC–mass spectrometry (MS) applications, a 50 × 2.1-mm, 3-µm particle column operated at 0.2–0.5 mL/min is typical, and 0.1% formic acid is used instead of phosphate buffer. The column temperature is controlled, generally at 30 °C or 35 °C as a starting point.

Our Guide

We'll be using equation 1 as our guide through the method development process.

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

where R_s is the resolution, k is the retention factor, α is the separation factor, and N is the column plate number. Recall that the retention factor (sometimes called the capacity factor, k') is

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

where t_R is the retention time and t_0 is the column dead time (sometimes abbreviated t_m). The column dead time is the retention of an unretained sample component, usually determined by the upslope of the "garbage" or "solvent" peak at the beginning of the chromatogram. We are concerned only about retention (part *i* of equation 1) this month. In future installments, we'll consider parts *ii* and *iii*.

Selecting a Target *k*-Value

To get the "best" chromatography, we strive for $2 < k < 10$, but this often is not possible, so $1 < k < 20$ generally is acceptable. When k is in one of these ranges, we'll usually get the best separation, but this is not guaranteed. One of the important reasons we would like $k > 2$ is that resolution is less susceptible to small fluctuations in mobile phase concentration. This is illustrated in Figure 1 and Table I.

In Figure 1, the influence of retention (term *i*, equation 1) is plotted against resolution. If k is infinity, term *i* approaches 1.0, so this is shown as the maximum possible resolution by the dashed line in Figure 1. I like to think of the influence of k on R_s in three ways.

First, let's look at getting the maximum power or leverage out of k as a variable to obtain resolution. If $2 < k < 10$, we have achieved 70–90% of the possible resolution by adjusting k (see Figure 1 and column 2 of Table I). This is a pretty good return on our investment

Table I: Sensitivity of resolution to changes in retention

k	$k/(1 + k)$*	Error in R_s (%)**
0.5	0.3	6.3
1	0.5	4.7
2	0.7	3.1
5	0.8	1.6
10	0.9	0.85

* Surrogate for resolution in Figure 1.
** Error for 1% change in %-organic solvent in mobile phase.

in this variable. Increased retention to $k \approx 20$ will gain only an additional 5% in resolution, but at a cost of twice the run time and result in broader peaks — not a good tradeoff in my opinion. On the other hand, if $k \approx 1$, we're only at 50% of the maximum resolution, so we are not taking full advantage of this variable. For $k < 1$, resolution drops off rapidly. Thus, we can see that the $2 < k < 10$ or $1 < k < 20$ guidelines make sense from the standpoint of taking advantage of the power of k to achieve resolution.

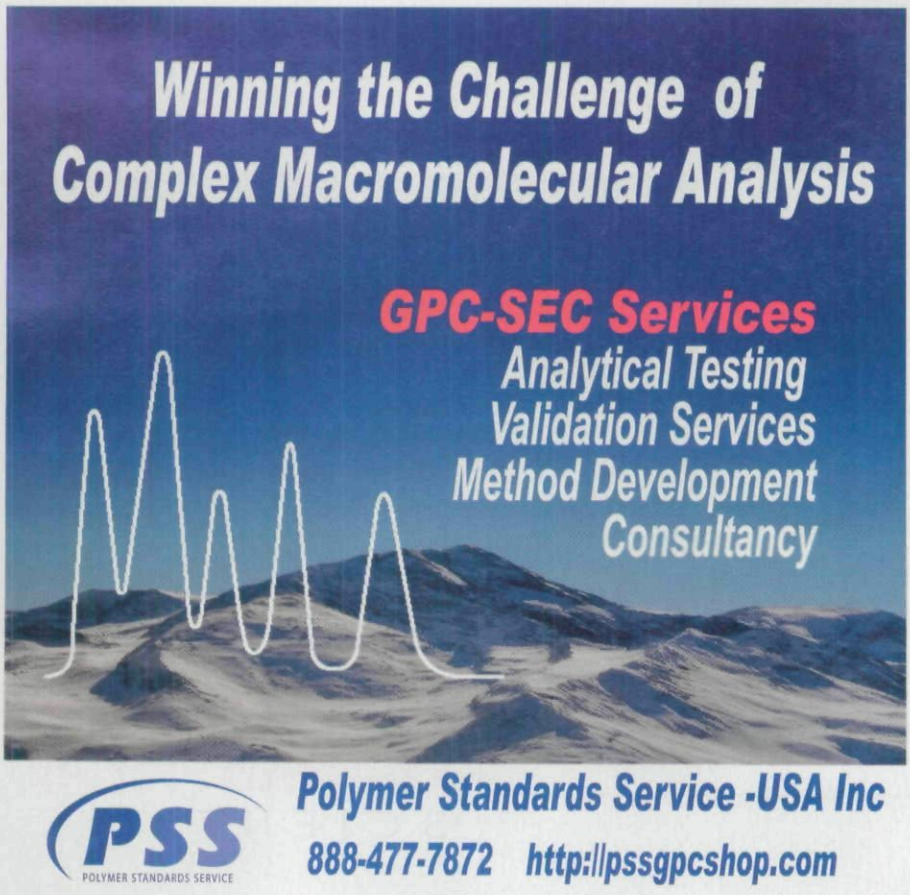
Second, we can consider the susceptibility of a method to small errors in mobile phase composition. For example, if we use a 1% error in mobile phase organic for comparison, we can see that larger k-values are less susceptible to changes in resolution for small changes in percent organic (see Figure 1 and column 3 of Table I). That is, when compared to the error at $k \approx 10$ (0.85%), $k \approx 2$ has about three times the error and $k \approx 0.5$ is more than seven times the error, with a 6.3% change in resolution for a 1% change in organic solvent. So we can see that methods that generate small k-values will be more susceptible to changes in resolution when small changes in mobile phase composition are made, either as a result of normal variation or due to an instrument or operator error.

Finally, real samples almost always generate a large peak at t_0, often called the solvent front or garbage peak, due to unretained materials in the sample. In LC–MS, although a peak at t_0 usually is absent, a general region of ion suppression exists near t_0. In both cases, quantification of analyte peaks can be compromised due to unretained compounds in most samples.

All of these influences support the goal of having $2 < k < 10$ for our samples, or if this is not possible, $1 < k < 20$. One last way to look at these recommendations is to consider them in light of potential method problems. I have a friend who claims that if every problem LC method were adjusted so that k for the first peak was at least 1, 50% of the problems would go away. I think this is a bit of an oversimplification, but the basic premise is valid. We tend to want to make our method run times short, so peaks get pushed up into the $k < 1$ region, where there will be more problems with method variation and more likelihood of interference with unretained materials.

What About Run Time?

There is no denying that for most applications, shorter run times are desirable. This is some of the motivation for the current emphasis on sub-2-µm particle columns operated at pressures greater than 6000 psi (400 bar). However, in any application it seems like we will have to trade run time for an increase in k-values. That is, if we have to increase k to get it within the $2 < k < 10$ target range, the run will be longer. Contrary to popular opinion, this is not necessarily the case. Consider the case in which the current method gives $k = 0.5$ for the first peak and we adjust the conditions so that $k = 2$ for the first peak. For a 150 mm × 4.6 mm column operated at 1 mL/min, $t_0 \approx 1.5$ min, so we can rearrange equation 2 to solve for t_R and figure out the retention time in both



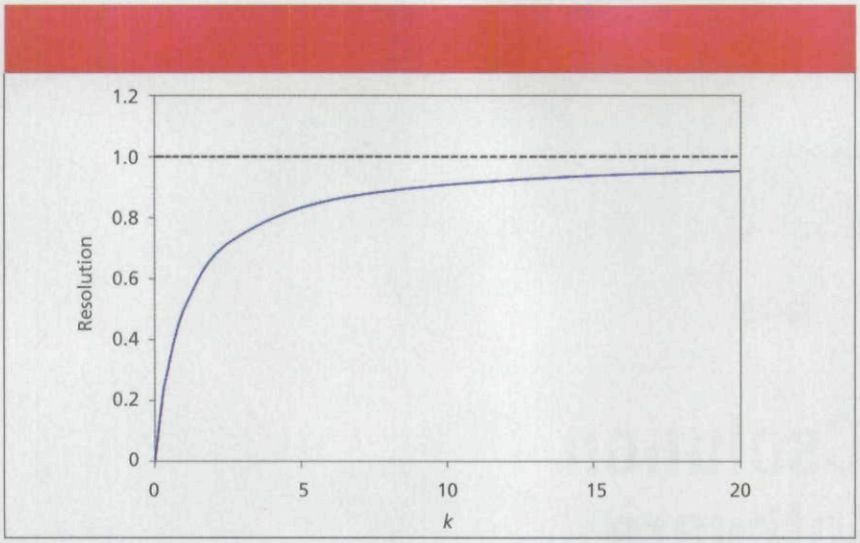


Figure 1: Relationship between resolution and retention. From equation 1, R_s is plotted against $k/(1 + k)$.

cases. For $k = 0.5$, $t_R = 2.6$ min and for $k = 2$, $t_R = 4.5$ min, so the run time nearly doubles. However, if we are willing to increase the flow rate, we can gain back some or all of this time. For example, most of us run conventional methods with pressures in the 2000–2500 psi region, yet the upper pressure limit for most traditional LC systems is 6000 psi. You can see that doubling the flow rate will reduce the retention time proportionally, but according to equation 2, a change in flow rate has no effect on the retention factor, because both t_R and t_0 change in proportion to flow rate. Yes, doubling the flow rate will reduce the column plate number and, thus, resolution, at least in theory. But from a practical standpoint, most methods will not have a noticeable reduction in resolution by a doubling of the flow rate for columns packed with 3- or 5-μm columns. For example, my calculations for a 150 mm × 4.6 mm column packed with 5-μm particles show that a peak pair with $k \approx 2$ and $R_s = 1.7$ will degrade to $R_s = 1.6$ when the flow is changed from 1 to 2 mL/min. And this is with a well behaved system — most of our methods are not as sensitive to a twofold change in flow rate. So for this example, we have a win-win situation — k is increased to give better chromatography and fewer method problems, yet retention time does not increase. Of course, this assumes that the peak spacing does not degrade with a change in k, which might or might not be true, as we will see in next month's discussion.

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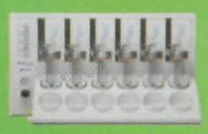

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Circle 23

Getting *k* Right

Ok, now we have a target range for k and justification for it, how do we achieve the desired result? One time-honored approach is to start with a strong mobile phase and decrease the mobile phase strength in steps until the desired retention is observed. For example, make a run at 100% acetonitrile, 90%, 80%, and so forth. Then when you are close to the desired result, make small changes to finetune the separation. This technique works well, and when I worked in an application laboratory for one of the instrument companies, it was the standard procedure.

There is a simpler way. If we make a plot of $\log(k)$ versus percent organic (%B) in the mobile phase, we will see a graph similar to that of Figure 2. One of the most striking observations about this graph is that it is linear, so it can be described as:

$$\log(k) = \log(k_0) - (S)(\%B) \qquad [3]$$

where k_0 is the (extrapolated) value of k at 0% B (100% water or buffer) and S is the slope of the plot. Armed with this relationship, we need only two experimental points to make the plot, not a whole series of 10% steps. This means that once we have made two experimental runs, such as 70% and 50% B in this case, we can predict the k-value of our sample compound under any other

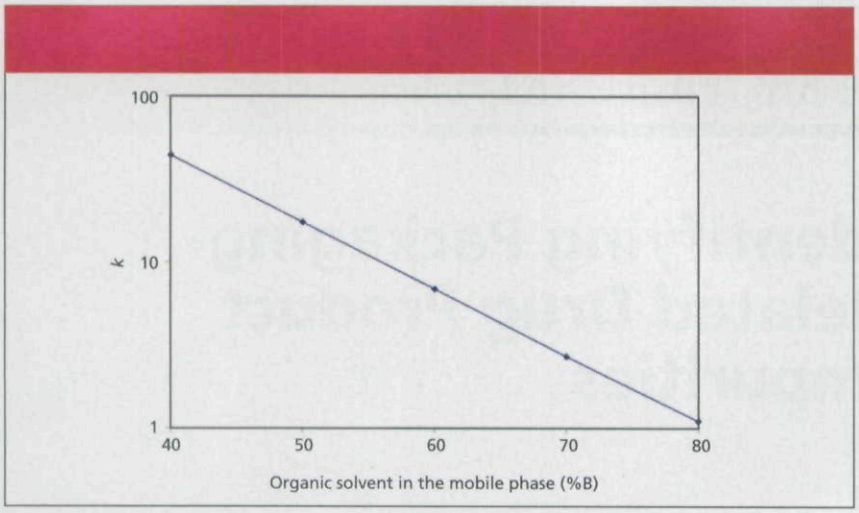

Figure 2: Plot of log(k) versus mobile phase organic solvent (%B). See text for details.

mobile phase concentration. For this example, it is trivial to determine that $k = 1$ at 81% B and $k = 20$ at 48% B, so I should first look within the 48–81% B region for the best k-values, and hopefully the best separation.

As we'll see in next month's "LC Troubleshooting," plots similar to Figure 2 for samples of similar molecular weight will have similar, but not necessarily identical, slopes, or S-values. So even without making experimental plots, we can make a generalization of The Rule of Three, which states that a 10% change in mobile phase organic concentration will change k by a factor of about three. This is not a hard-and-fast rule — it can be 2.5 or 4 for some compounds, and applies for compounds of molecular weights less than ≈ 1000 Da, but it gives us a nice guideline. For example, in the earlier case of a change in k from 0.5 to 2, without any experiments I can guess that the change will require a reduction in mobile phase organic solvent concentration of 10–15%.

Conclusions

We've seen that, from a method development standpoint, it is desirable to adjust retention for that $1 < k < 20$, or even better $2 < k < 10$. This gives us sample retention times that will give more robust methods in terms of sensitivity to small changes in mobile phase composition. From a troubleshooting standpoint, we can understand that when $k < 1$ for most methods, besides excessive sensitivity to mobile phase composition, there is more likelihood of quantification problems due to interferences at t_0.

The regular behavior of retention and mobile phase organic concentration in reversed-phase LC, as illustrated in Figure 2, gives us a tool to use to more quickly find experimental conditions that will give us retention in the desired range. You might have noticed, however, that a change in %B to change k often results in a change in relative retention, or peak spacing, as well. This can be a problem that can create problems when we try to increase k to move peaks away from t_0, but, as we'll see in next month's installment of "LC Troubleshooting," we can use such changes in selectivity to our advantage so as to finetune a separation with very little extra work.

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Circle 24

LC TROUBLESHOOTING

The Perfect Method, IV: Controlling Peak Spacing

How do I get the most out of my efforts?

John W. Dolan
LC Troubleshooting Editor

This is the fourth installment in a series on method development for liquid chromatography (LC), with an emphasis on developing trouble-free methods quickly. We started out by considering some of the goals we might have and some method development strategies (1). Next, we selected starting conditions for reversed-phase separations (2). This was followed by a discussion of how to control retention for good chromatographic performance (3). This month, we'll consider how to pull apart those troublesome peak pairs.

Getting Retention Right

Last month (3), we were introduced to equation 1:

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

as a guide for the method development process. Here R_s is the resolution, k is the retention factor, α is the separation factor, and N is the column plate number. We looked at ways to adjust the retention factor

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

by changing the mobile phase strength. (t_R and t_0 are the retention time and column dead time, respectively.) A retention factor of $2 < k < 10$ is ideal, but $1 < k < 20$ is satisfactory in many cases. There is a regular change in retention with solvent strength for each analyte according to:

$$\log(k) = \log(k_0) - (S)(\%B) \quad [3]$$

where k_0 is the (extrapolated) retention at 0% organic (100% water or buffer), %B is the percent organic solvent in the mobile phase, and S is the slope of the plot. The relationship of equation 3 allows us to predict retention for a given analyte based upon just two experiments at different %B-values, because the plot of $\log(k)$ versus %B is linear in most cases.

Retention of "Regular" Compounds

Compounds that have very similar structures, such as homologs, we will refer to as "regular" compounds. These have very similar plots of $\log(k)$ vs. %B, as seen in Figure 1 for a sample of nine triazine herbicides (4). In such cases, the individual plots tend to fan out, with increasing peak spacing for weaker solvents (lower %B-values). This is what is expected from the fundamental resolution equation (equation 1) — as k is increased, R_s is increased. However, relative peak spacing doesn't change, so in terms of selectivity, there is little to be gained from a change in the mobile phase strength. As such, a simple change in the mobile phase strength is of little help in pulling apart two peaks that are difficult to separate when samples are all related closely in structure.

Retention of "Irregular" Compounds

Fortunately, samples comprising entirely "regular" compounds are much less common than those samples whose components differ in functional group types. Such samples we will refer to as "irregular" samples. An example of the retention behavior of an "irregular" sample is shown

Table I: Ranking the variables

Variable	Change in α	Universal	Convenient	Low-UV/ LC–MS	Robust-ness	Equilibration
%B	0*	+	+	+	+	+
Tempera-ture	–	+	+	+	+	+
Solvent pair	++	+	+	0	+	0
Ion pair	+	–	–	0	–	–
pH	+++	–	0	0	–	+
Column type	+	+	0	+	+	+

* Overall benefit, +++ = excellent, + = good, 0 = neutral, – = unfavorable

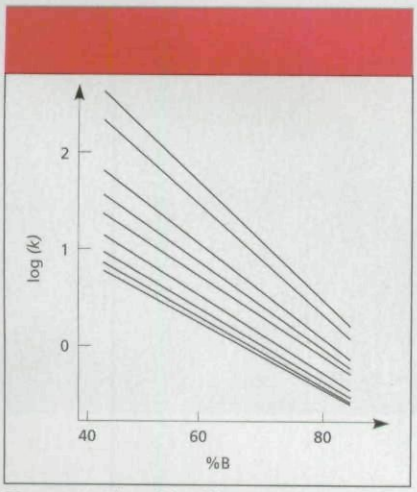

Figure 1: Plot of log(*k*) vs. %B for the "regular" sample of reference 4.

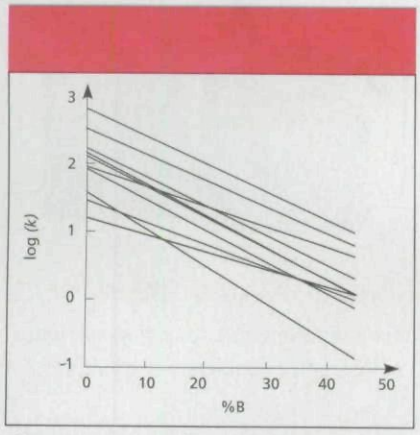

Figure 2: Plot of log(*k*) vs. %B for the "irregular" sample of reference 5.

in Figure 2 for a mixture of substituted benzoic acids (nitro, chloro, fluoro, and so forth) and substituted anilines (5). It is obvious that, although the general slope of the plots is similar to Figure 1, the peak spacing for individual pairs of compounds changes dramatically with a change in the mobile phase strength. In several cases, peak crossovers (retention reversals) occur. It is such changes that we can take advantage of, so that by adjusting the %B, we can adjust the selectivity of the separation and pull specific peaks apart. We also can quickly find conditions to avoid, where the lines cross in the plots and, thus, peaks overlap completely.

Taking Advantage of Selectivity

To quantify selectivity, we use the separation factor α:

$$\alpha = k_2 / k_1 \qquad [4]$$

where k_1 and k_2 are the retention factors of the first and second peak of a given peak pair. If we have retention behavior as in Figure 2, *k*-values will not change in parallel, so the α-related term *ii* of equation 1 will change, resulting in a change in resolution.

In the earlier discussion (2) of Table I, we saw that changing the %B was a great way to change the peak spacing for most samples. Although a change in %B is not the most powerful way to change selectivity, it is very easy, robust, and is compatible with UV and mass spectral detectors. Samples that contain analytes with different functional groups, such as those of the irregular sample of Figure 2, will respond well to %B as a tool to change peak spacing. These reasons support our decision to change the %B first in our efforts to fine-tune the selectivity of a given separation.

The Resolution Map

We can use plots, such as Figures 1 and 2, to calculate *k* for each peak and, thus, α for each peak pair at any %B. Because these plots are on a semilog scale, they can be hard to interpret visually. A more useful approach is to take advantage of the relationship of equation 1. From our log(*k*) versus %B plots, we can get values for the retention (*i*) and selectivity (*ii*) terms of equation 1 for any %B. A value for resolution R_s is much more useful than *k* or α, and this can be obtained by calculating, measuring, or estimating the column plate number *N* (term *iii*). If we chose starting conditions with a 150 mm × 4.6 mm column packed with 5-µm particles, the plate number is approximately 10,000 (2), which is sufficiently close for resolution estimates using equation 1. Now, we can plot R_s versus %B, as shown in Figure 3 for a mixture of six nitroaromatic compounds. This is called a resolution map and is available in the popular retention modeling software packages (for example, DryLab from Molnar Institute, Berlin,

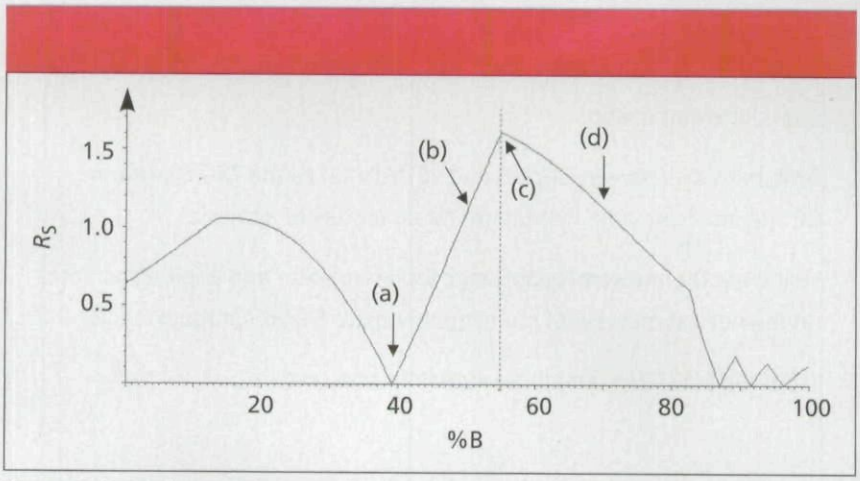

Figure 3: Resolution map for a sample of six nitroaromatic compounds. Resolution values at (a) 40% B, (b) 50% B, (c) 55% B, and (d) 70% B correspond with the chromatograms shown in Figure 4.

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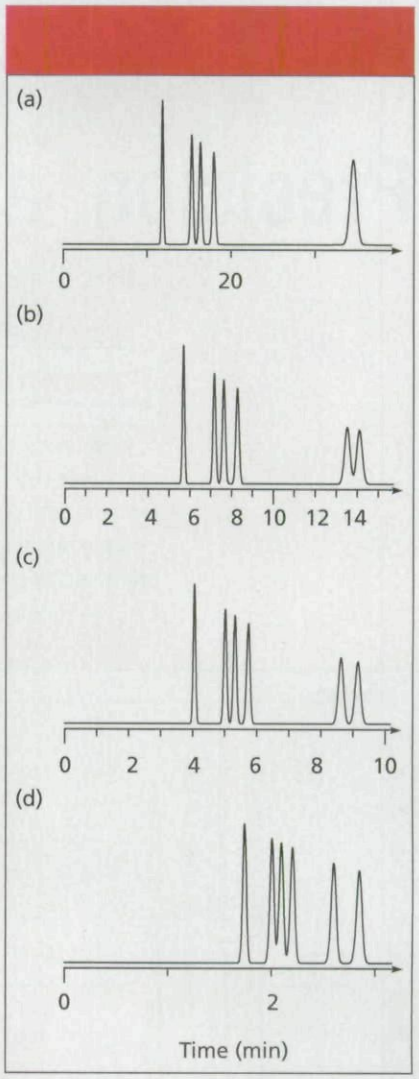

Figure 4: Simulated chromatograms for separations corresponding to conditions indicated by (a-d) in Figure 3.

Germany, ChromSword from Merck, Darstadt, Germany, and ACD/LC Simulator from Advanced Chemistry Development, Toronto, Canada).

The resolution map is a plot of the resolution of the least-resolved, or "critical," peak pair at every %B-value. This is illustrated with the simulated chromatograms of Figure 4 for several points on the resolution map of Figure 3. At 40% B (Figure 4a), the minimum resolution is near zero and we see that the last two peaks are merged into a single peak. These two peaks pull apart as we move to higher %B-values, as for 50% B in Figure 4b. The maximum overall resolution is at the apex of the plot at 55% B (Figure 4c), where the resolution of peaks 2 and 3 is equal to that of peaks 5 and 6. If we continue to move to higher %B-values, peaks 2 and 3 become the critical peak pair and

the overall resolution is reduced (Figure 4d). Note how the retention times (and, thus, *k*-values) change for the different conditions of Figure 4. So you can see how this powerful tool can lead you quickly to fine-tuned conditions that give the best overall separation — it can save days of trial-and-error experiments.

Other Variables

The resolution map of Figure 3 is for a variation in the %B, and requires only two experiments to obtain the input data. Similar maps can be made for most of the other variables of Table I. Resolution as a function of column temperature requires just two input runs, whereas changing solvent type, ion pairing reagent concentration, or pH requires at least three runs to calibrate the retention model. The use of retention mapping in method development can greatly speed up the development process. It can help quickly identify the best conditions for the separation as well as danger regions to avoid.

Conclusions

Once we have adjusted the mobile phase strength so that $1 < k < 20$, to get the retention times in a region that is likely to give good chromatographic performance, we can move on to the adjustment of peak spacing. Because most samples contain analytes with a variety of functional groups, our samples usually fall into the category of "irregular" samples, as illustrated in Figure 2. When this is the case, adjusting the mobile phase percent organic can move peaks relative to each other so that we can hopefully find conditions where all the peaks are resolved from each other. The use of equation 1 allows us to generate resolution maps that will help to identify quickly the conditions for the best separation. Because the resolution map is constructed based upon real experiments, it can provide very accurate predictions of resolution. If the relationships between retention and mobile phase conditions are linear (or log-linear), two experimental runs are required. For more complex relationships, such as retention versus pH, more experimental runs might be required, but the resolution mapping concept works just as well for such variables. Any of the continuous variables of Table I (all except column type) are amenable to retention mapping.

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LC TROUBLESHOOTING

The Perfect Method, Part V: Changing Column Selectivity

How to avoid an expensive shot in the dark.

John W. Dolan
LC Troubleshooting Editor

For the last several months we've been working through the steps to develop a liquid chromatography (LC) method quickly and effectively. First we looked at setting separation goals (1) and selecting the starting conditions (2). Then we adjusted retention times (3) and mobile phase conditions (4) to get the retention and peak spacing to meet the goals we set. Changes in the mobile phase percent organic (%B), solvent type, pH, and temperature were easy variables to modify in an effort to fine-tune the separation, because these variables can be changed in a continuous manner. That is, the incremental change in the parameter from one run to the next can be made in any step size we desire, such as a change from 45% B to 46% B or 43% methanol, 35% acetonitrile, and 22% buffer to 44% methanol, 34% acetonitrile, and 22% buffer. One other variable that can be useful to change peak spacing is a change in the column type, for example, C18 to phenyl. Unfortunately, such column changes are in discrete steps — it is not possible to change from 44% phenyl and 56% C18 to 43% phenyl and 57% C18. And changing columns is expensive — typically $500 per column — so column changes have more budgetary impact than changes in pH or temperature.

This month's installment of "LC Troubleshooting" will focus on changing the column as a means to change the peak spacing in a chromatogram. We will consider two approaches — a traditional one of selecting the column by bonded phase type and a newer technique based upon the chromatographic properties of the column.

"Orthogonal" Columns

We often hear the term "orthogonal" to describe a column or separation change in the quest to obtain a better separation of two or more peaks. Strictly speaking, orthogonal conditions are those that produce a separation that is at right angles or perpendicular to the current one. As long as we are working with reversed-phase LC, hydrophobic interactions dominate the separation mechanism, so no matter what change we make, hydrophobic interactions are still the most important ones. As a result, there is no truly orthogonal separation condition in this context. Perhaps if we switched to a different retention mechanism, such as from reversed phase to ion exchange, we might get orthogonality, but some would argue that as long as we used LC as the analytical tool, we wouldn't achieve orthogonal results.

Our present goal is to get a significantly different separation than the one we currently have, and in this context, we'll refer to a set of conditions that achieves this goal as orthogonal. (Those of you who are purists had better stop reading at this point or take your blood pressure medicine!)

Contributions to Column Selectivity

There are three major contributions to achieving the desired selectivity, or peak spacing, in reversed-phase LC, the analyte chemistry, the mobile phase composition, and the column composition. For the most part, we're stuck with the analyte chemistry (with the major exception for ionic compounds when the mobile phase pH is changed), and we've already explored mobile phase changes. The column chemistry has two major contributions — the packing particles (usually silica) and the bonded phase. There was a time when we thought all silica was created equal and all bonded phases of the same description were the same. Thus, a C18 column was a C18 column . . . period. This gave rise to the L-1 classification in the *United States Pharmacopoeia*

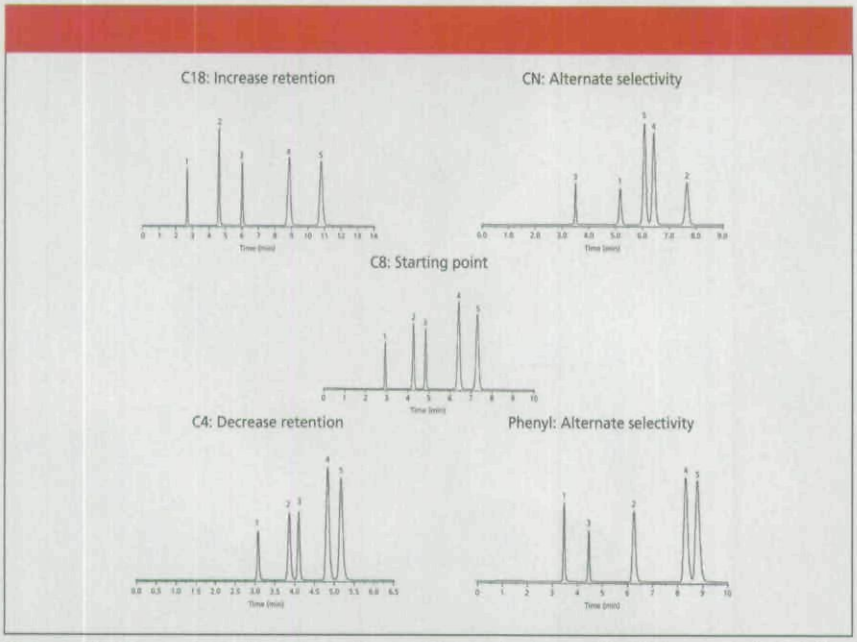

Figure 1: Separation of various drugs using columns with different selectivities. Columns: 250 mm × 4.6 mm, 5-µm d_p ACE; mobile phase: 80:20 (v/v) methanol–25 mM phosphate buffer (pH 6.0); flow rate: 1.0 mL/min. Peaks: 1 = norephedrine, 2 = nortriptyline, 3 = toluene, 4 = imipramine, 5 = amitriptyline. Courtesy of Advanced Chromatography Technologies (Aberdeen, UK).

(USP), grouping all C18 columns in one category. Now, unless you are very new at LC or very naive, you realize that not all C18 columns are created equal.

The Traditional Approach

Even though we know that all C18 columns are not the same, it seems logical that a change in the bonded phase type will be more likely to change peak spacing than a change to another C18 column. One common approach to try to change selectivity by a change in column is to change from a C18 column to a phenyl or cyano column. The logic is that both the phenyl and cyano columns have π–π interactions that are not present with the C18 phase and the cyano also has dipole–dipole contributions. To limit the variables, often columns are selected from the same family of phases from the same manufacturer. This means that the same silica particles are used, so the differences seen are the result of the bonded phase changes.

An example of selectivity changes with bonded phase changes is shown in Figure 1 for a sample of several drugs. These columns are all from the same manufacturer and are operated under identical conditions (see figure caption). The reference conditions use a C8 bonded phase, which gives a retention time for the last peak of approximately 7.5 min. The primary effect of changing to a C18 or C4 phase is that retention is increased or decreased, respectively. There are some minor changes in peak spacing, but all three columns use a hydrocarbon bonded phase, so little change in the bonded phase chemistry and, thus, peak spacing is expected or observed.

A change to the phenyl or cyano column makes a significant change in selectivity for this set of aromatic compounds, for which π–π interactions are expected to be significant. The phenyl column reverses peaks 2 and 3, whereas with the cyano column only peak 4 is in the same order as it was with the C8 column. I think we can agree that the cyano column gives an orthogonal separation for this sample; the phenyl column has some, but not as much orthogonal nature. I would expect the results to be much less dramatic for a sample that did not have a significant aromatic or dipole component.

Over the last ten years, bonded phases containing an embedded polar group (EPG) have become popular. These often incorporate a nitrogen-containing functional group, such as an amide or carbamate, near the base of the C8 or C18 bonded phase chain. The EPG phase can impart a significant change in selectivity over the comparable C8 or C18 phase, so it represents a viable alternative to the phenyl or cyano phases for a change in selectivity. EPG columns also have the advantage of being capable of operation in 100% aqueous mobile phases without phase dewetting (which we called "phase collapse" in the past), so they have added flexibility. For many workers today, the EPG column is the column of choice for alternate selectivity when a C18 column does not give the desired separation.

An Alternate Technique

The use of a cyano, phenyl, or EPG column to give different selectivity from a C8 or C18 column has stood the test of time, but is not a guarantee of orthogonality. Just as some pairs of different C18 columns give similar separations and other pairs exhibit changes in selectivity, some of the alternate non-C18 columns might give similar separations to the starting C18 column. It would be nice to have increased confidence that the chosen column would indeed make significant changes in the separation. In the last few years, several groups have been working on ways to quantify the differences and similarities between LC columns and translate this information into practical tools. The results from one of these studies has generated a database of more than 300 commercial reversed-phase columns, which allows the user to select columns that are similar or ones that are different from a chosen reference column. (See reference 5 for a recent review of this approach, the "hydrophobic-subtraction model.")

The database is available in beta-test version on the USP website (6) and is expected to be available in a released version in the future. The screen capture shown in Figure 2 depicts the database configured to look for columns of different selectivity ("View Different" button in upper right for Figure 2). You select your current column from the drop-down menu at the upper right. In this case, I've chosen the ACE 5 C8 column, the same one used as the starting place in Figure 1. Next, select the appropriate check boxes if the sample has acids or bases present and enter the pH of the mobile phase. After these selections have been made, the database searches for columns that are maximally different from the reference column and displays the 10 columns most different. The measure of the difference is the

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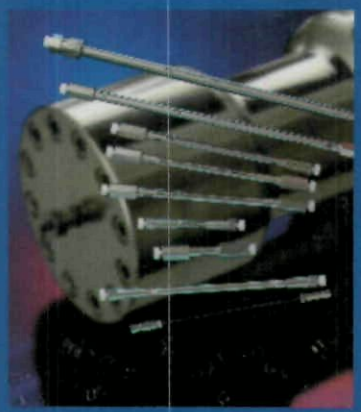

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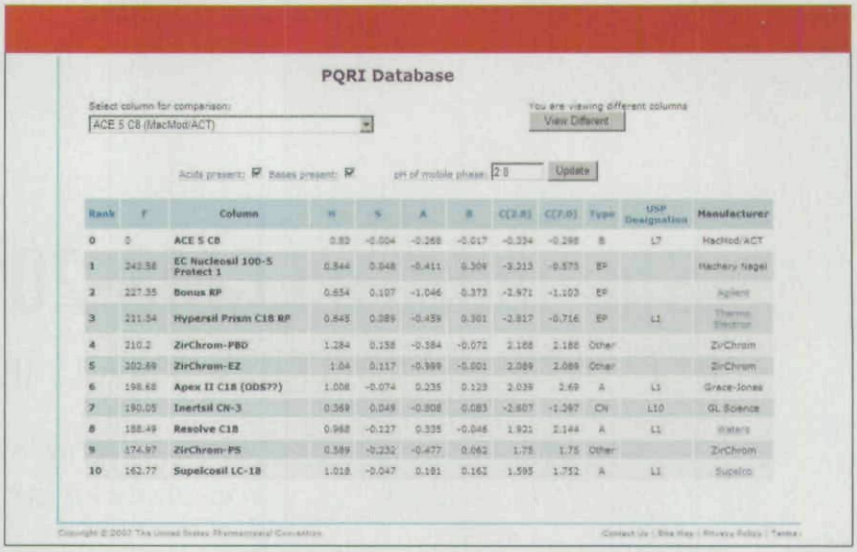


Rank	F	Column	H	S	A	B	C(2.8)	C(7.0)	Type	USP Designation	Manufacturer
0	0	ACE 5 C8	0.83	-0.004	-0.268	-0.017	-0.334	-0.298	B	L7	MacMod/ACT
1	243.58	EC Nucleosil 100-5 Protect 1	0.844	0.048	-0.411	0.309	-3.313	-0.575	EP		Machery-Nagel
2	227.35	Bonus RP	0.654	0.107	-1.046	0.373	-2.971	-1.103	EP		Agilent
3	211.54	Hypersil Prism C18 RP	0.845	0.089	-0.459	0.301	-2.817	-0.716	EP	L1	Thermo Electron
4	210.2	ZirChrom-PBD	1.284	0.158	-0.384	-0.072	2.188	2.188	Other		ZirChrom
5	202.69	ZirChrom-EZ	1.04	0.117	-0.999	-0.001	2.089	2.089	Other		ZirChrom
6	198.68	Apex II C18 (ODS??)	1.008	-0.074	0.235	0.123	2.039	2.69	A	L1	Grace-Jones
7	190.05	Inertsil CN-3	0.369	0.049	-0.808	0.083	-2.807	-1.267	CN	L10	GL Science
8	188.49	Resolve C18	0.968	-0.127	0.335	-0.046	1.921	2.144	A	L1	Waters
9	174.97	ZirChrom-PS	0.589	-0.232	-0.477	0.062	1.75	1.75	Other		ZirChrom
10	162.77	Supelcosil LC-18	1.018	-0.047	0.181	0.162	1.595	1.752	A	L1	Supelco

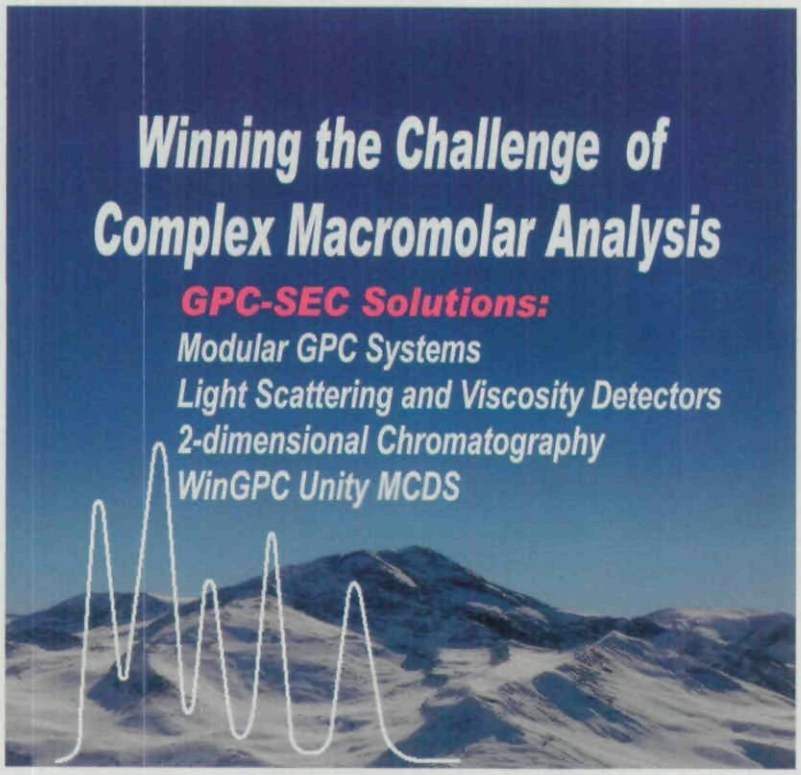
Figure 2: Column-comparison database of reference 5. See text for details.

F_s fitting value (abbreviated F in the database). If the F_s value is greater than approximately 65, the column has a high likelihood of giving different selectivity. You can see that the displayed columns all have F_s values greater than 162 — significantly different. The next few columns of the database display measured characteristics of the columns: H, hydrophobicity; S (abbreviated S^* in the table), resistance of the bonded phase to penetration by bulky molecules; A, and B, the ability to hydrogen bond with nonionized acids and bases, respectively; and C, the cation-exchange characteristics of the column at pH 2.8 and 7.0 (see (5) and related references for a more detailed discussion of these parameters). The column type is listed, the USP "L" classification, and the manufacturer at the far right.

For the reference column we selected, a sample containing both acids and bases, and a mobile phase pH of 2.8, it can be seen that the list of different columns contains several different phase options. Three of the choices are EPG columns (abbreviated EP in the table) and one is a cyano (CN) column, which is consistent with the previous discussion of the use of these phases as alternatives to the C8 or C18 column. There are also several columns with zirconia particles (ZirChrom, Anoka, Minnesota) indicated to exhibit different selectivity. Notice that three of the columns are Type A C18 columns. This highlights the difference that is sometimes observed between the older Type A silica particles and the newer, high purity Type B particle columns. Because of reproducibility and peak tailing problems with Type A columns, I recommend against using such columns for a new separation. Notice that in all cases except the Type A columns, the best choice columns for different selectivity is found with a different bonded phase from a different manufacturer. This means that the change in both the packing particle chemistry and the bonded phase are playing a part in giving alternate selectivity. It should be noted that this technique of choosing an orthogonal columns is not a guarantee of an orthogonal separation for your sample, but there is a high probability that this is the result you will obtain.

An Extra Point of Leverage

A change in stationary phase type using one of the two techniques discussed previously is likely to give you a change of selectivity, but if you want to increase the chances of obtaining a significantly different separation, there is one additional change you can make. It is well known that a change in the organic solvent type, such as changing from methanol to acetonitrile, can be a powerful way to change selectivity in reversed-phase LC. If you combine this mobile phase change with a change in the stationary phase, you will further increase the chances of achieving an orthogonal separation (7). Thus, if we use the data of Figure 2, we might change from the ACE 5 C8 column in a pH 2.8 phosphate buffer–acetonitrile mobile phase to a Bonus RP (EPG) column with the same buffer, but methanol instead of acetonitrile. This combination would be likely (but not guaranteed) to give a sepa-



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ration with different selectivity. In the event that this change was not sufficient, a change in mobile phase pH could be used as additional way to change selectivity (7).

Summary

It usually is best from an economic and convenience standpoint to attempt to obtain the desired separation by changing the mobile phase strength, solvent type, temperature, and pH as discussed last month (4). If these changes are unsuccessful, a change in the column should be explored. A cyano, phenyl, or EPG column often will give a change in the separation from a starting C8 or C18 column. The database of Figure 2 can be used to improve the chances of selecting a column for a successful orthogonal separation. A change in the column plus a change in the mobile phase solvent type from methanol to acetonitrile or acetonitrile to methanol will give added power to change the separation selectivity.

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LC TROUBLESHOOTING

The Perfect Method, Part VI: Make It Faster

Trade extra resolution for speed.

John W. Dolan
LC Troubleshooting Editor

Who doesn't want more speed? Whether you are looking at a new motorcycle, examining your times for a 10K run, or developing a liquid chromatography (LC) method, faster usually is better. Face it, most of us who work as chromatographers get paid, either directly or indirectly, by the number of samples we run. A faster method allows us to run more samples or get the sample set done more quickly so we can move on to something else. In the previous installments of this series on efficient development of LC methods (1–5), we have concentrated on improving resolution by modifying the mobile phase, choosing a different stationary phase, or changing some other condition, such as column temperature. In this month's "LC Troubleshooting" installment, we're going to look at trading some of that resolution for a faster separation.

One More Time

Throughout this series on efficient LC method development we have been using equation 1 as a guide. Usually our goal (1) is to develop a method that gives baseline resolution, R_s, for all components of interest. If it is to be a method used under the oversight of one of the regulatory agencies, $R_s > 2.0$ is recommended. As a starting point (2), we chose a reversed-phase C8 or C18 column, because this chromatographic mode has a high probability of success with most samples. A 150 mm × 4.6 mm column packed with 5-µm diameter particles or a 100 mm × 4.6 mm, 3-µm d_p column was used, because these columns generate approximately 10,000 theoretical plates, N, which is sufficient to separate most sample mixtures. As a bonus, these column sizes can be run at 1.5–2.0 mL/min for a reasonable run time without much concern about excessive pressure.

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

As soon as we had our starting conditions, we worked our way through equation 1 in an effort to develop a separation with the necessary resolution. First we tried adjusting the retention factor, k, which is most easily controlled by changing the mobile phase strength (3). We started with a strong mobile phase, such as 90:10 acetonitrile–water (or buffer) or methanol–water, then worked in a step-wise fashion to weaker mobile phases (more aqueous phase) until k was in the $1 < k < 20$, or better $2 < k < 10$, region. Because a change in k also results in a change in selectivity, α, for many sample mixtures, adjustment of the mobile phase strength may be enough to obtain the required resolution. If mobile phase strength changes are not sufficient, we can add more power to the process by concentrating on α through adjustments in the chemistry of the mobile phase (4) by changing solvents from acetonitrile to methanol (or vice versa), or changing the pH, temperature, or mobile phase additives. Selectivity also can be changed with a change in the column packing type (5), although this option often is reserved for later in the development process, because of the

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Table I: Influence of particle size on resolution, plate number, and pressure*

Parameter:	R_s	N	Pressure (psi)
Proportional to:	$1/d_p^{1/2}$	$1/d_p$	$1/d_p^2$
10 µm	1.08	6950	105
5 µm	1.62	13,850	425
3.5 µm	1.92	19,500	865
3.0 µm	2.04	22,200	1180
1.7 µm	2.46	32,250	3660

* Calculated values, 150 mm × 4.6 mm column, 65% acetonitrile–water, 35 °C, 1.0 mL/min

expense of purchasing additional columns.

At this point in the process, we hopefully have the resolution we need through the adjustment of k and α using a column that generated a sufficient number of theoretical plates. If the resolution is satisfactory and the run time is acceptable, we should be ready to validate the method. If resolution is larger than is needed, we can trade some of that resolution for shorter run times. If resolution is smaller than is needed, we may be able to adjust N to gain a little resolution.

The Column Parameters

I like to refer to the factors that influence only N, without a change in selectivity, as "column parameters." These are flow rate, column length, and particle diameter. Of course, temperature affects the plate number, but it usually changes selectivity, too, so it cannot be changed independently of peak spacing. My philosophy is to initially choose a column that generates a sufficient plate number to be likely to separate most samples, then when I have the best resolution possible, I will adjust the column parameters to increase or decrease resolution to fit my target value. This often will result in a faster separation. Let's look at some examples.

Flow rate: First, let's consider the mobile phase flow rate. One popular way of illustrating the influence of the flow rate on column efficiency is to make a van Deemter or Knox plot, as shown in Figure 1. This is a graph of the plate height, H, versus the mobile phase linear velocity. The plate height is inversely proportional to the plate number ($N = L/H$, where L is column length), so smaller plate heights mean larger plate numbers, or more efficient columns. The linear velocity is proportional to the flow rate (same diameter columns assumed), and in the case of Figure 1, a linear velocity of 2 mm/s is approximately equal to a flow rate of 1.2 mL/min. Look first at the top plot of Figure 1, for a 5-µm d_p column. You can see that there is a minimum in the curve at approximately 1–1.2 mL/min — this means that the column performs best at this flow rate. As the flow rate is increased, the line rises, meaning that the plate number drops and resolution will get worse. So speeding up the run by increasing the flow rate for a 5-µm d_p column will result in lower column efficiency. From a practical standpoint with real samples under real conditions, we usually can change the flow rate by a factor of two and not notice a change in resolution, but larger changes in flow can visibly reduce resolution. Of course, pressure increases in direct proportion to an increase in flow rate. If your initial method has excess resolution and you don't mind running at a higher pressure, an increase in the flow rate is the easiest way to shorten the run time.

Column length: Another way to speed up the method is to use a shorter column. If you started with a 150-mm-long column and have extra resolution, you may be able to move to a 100-mm column. The plate

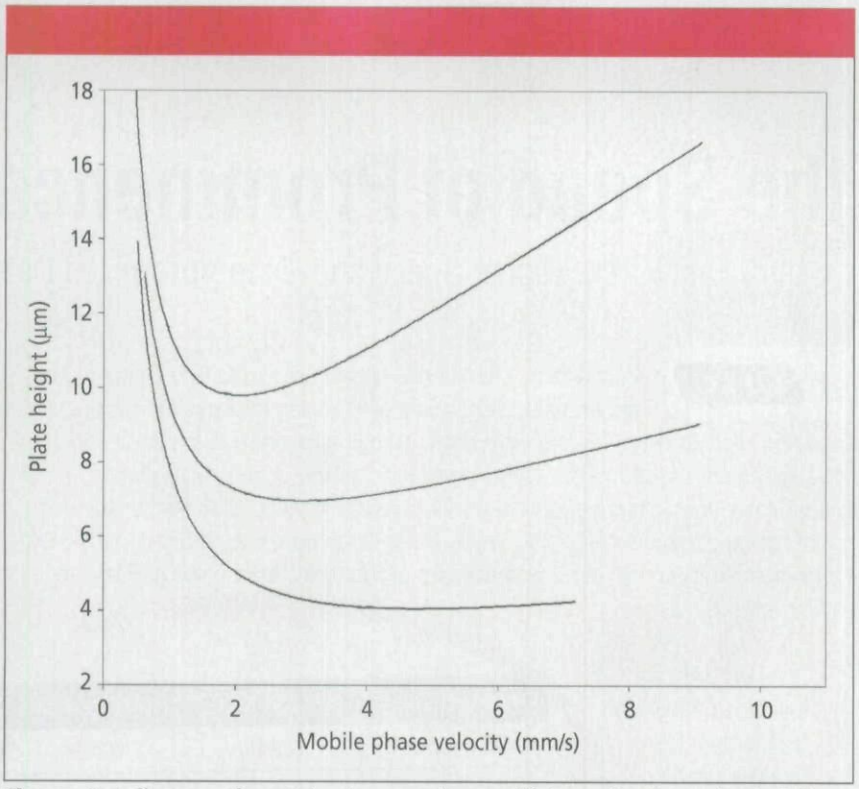


Figure 1: Influence of particle size on column efficiency for 5-µm (top), 3.5-µm (middle), and 1.8-µm (bottom) particles. 2 mm/s velocity ≈ 1.2 mL/min. See text for details.

number is proportional to the column length, but according to equation 1, resolution is proportional to the square root of the column length. This means that for the same flow rate, the run time (and plate number) drops by (150 – 100)/150 = 33%, but resolution drops only by ≈6%. And the shorter column means lower back pressure, so you may be able to increase the flow rate with the shorter column and gain even more time. A change in column length can be a very easy way to reduce the method run time, but if columns <100 mm long or <4.6 mm in diameter are used, be careful to minimize extracolumn volume or much of the theoretical gain may be lost to extracolumn band broadening.

Particle size: There has been a tremendous emphasis recently on the use of sub-2-µm d_p columns as a means to obtain faster separations. As the plots of Figure 1 show, the plate height is directly proportional to particle size. This means that a 1.7–1.8 µm particle column will generate approximately three times as many plates as a 5 µm one. This threefold increase in N translates into an increase of ≈1.7-fold in resolution. A second advantage of smaller particles is that plots as in Figure 1 stay nearly flat as the flow rate is increased. So, whereas a threefold increase in flow rate from 1.2 to 3.6 mL/min (2–6 mm/s) causes an increase in H (and corresponding reduction in N) by ≈25% for a 5-µm d_p column, there is no practical change in column efficiency for the 1.8-µm column with the same change in flow rate. A change from 5-µm particles to sub-2-µm particles gives an increase in N by approximately threefold. This increase can be traded for a shorter column, for example a 50-mm-long column instead of a 150-mm-long column, and give a threefold reduction in the run time, all other factors being held constant. The previous precautions about extracolumn band broadening hold in this situation, too. There is a penalty for smaller particles, however, and this is an increase in backpressure.

The Tradeoffs

As you can see from the above discussion, there are several opportunities to shorten run times by changing the column parameters. However, nothing comes for free, and the same holds true here – there always are tradeoffs with changes in column parameters. Column pressure changes in direct proportion to the flow rate, but for most routine separations, a two-fold change in flow rate will have little noticeable affect on resolution. Most workers run conventional LC systems in the 2000–3000 psi (130–200 bar) range, but most commercial LC systems are capable of operation up to 6000 psi (400 bar). You may have to tighten a few fittings to keep them from leaking, but otherwise, the equipment should function

Table II: Examples of column parameter changes

L (mm)	d_p (µm)	N	R_S	Pressure (psi)	t_R (min)
150	5	12,000	2.0	2000	15
100	3	13,300	2.1	3700	10
50	1.7	11,750	2.0	5750	5
150	5	12,000	1.7	2000	15
150	3	20,000	2.2	5550	15
75	1.7	17,650	2.1	8650	7.5

satisfactorily at higher pressures. The change in resolution and pressure can be calculated for a given change in column length, so as long as you stay within the equipment limits you should be fine.

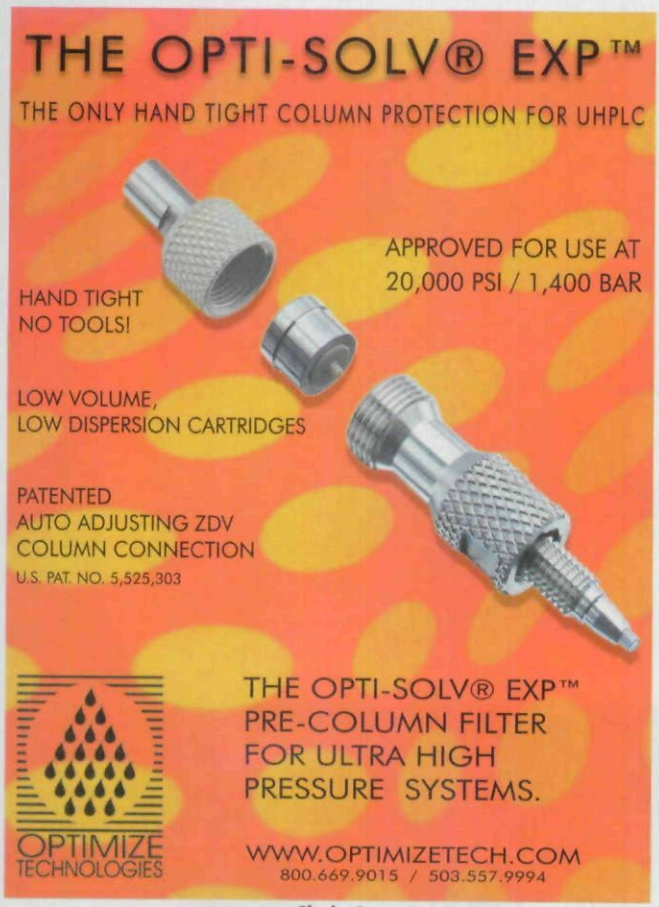
The impact of a change in column parameters becomes a bit more complex when particle size is changed. As is illustrated by the example in Table I, the plate number changes in direct proportion to the particle size, but the resolution changes only with the square root of the particle size change (equation 1). However, the penalty is in pressure — pressure increases with the square of the particle size reduction. So, for example, a twofold reduction in particle size improves resolution by only 40%, whereas the pressure goes up by a factor of four. If we want to take advantage of sub-2-μm particles at higher flow rates, as in Figure 1, it is very easy to exceed the pressure limits of conventional LC equipment. Several manufacturers now offer LC systems capable of pressures > 6000 psi to allow such operating conditions.

The practical use of a change in column parameters is shown with the data of Table II. In the first example, use of a 150-mm column packed with 5-μm particles resulted in a method with a retention time, t_R, of the last peak of 15 min, a pressure of 2000 psi, and the resolution for the least-resolved peak pair of 2.0. We can try using smaller particles to speed up the separation. A 100-mm-long column packed with 3-μm particles and all other conditions the same will generate the same resolution in 10 min, but at the expense of pressure. The new pressure of 3700 psi is higher than most workers operate their systems, but is well within the system specifications. Use of a 50-mm column packed with 1.7-μm particles will shorten the run time to 5 min, but now we are bumping up against the upper pressure limit for a conventional LC system. It is interesting to note, that if we are willing to operate at this pressure, an increase in the flow rate by a factor of three for the 5-μm column (not shown) will give the same run time, although the resolution will be slightly degraded and much more solvent will be used than with the shorter, 1.7-μm column.

So far we've talked about situations in which the method had excess resolution. What about the case where there isn't quite enough resolution? This is shown in the second example of Table II. The starting separation on the 150-mm, 5-μm column had a resolution of 1.7, but 2.0 was desired. By changing to a 150-mm, 3-μm column, the goal could be achieved, but with pressures just under the operating limits of the system. The 75-mm column packed with 1.7-μm particles also will solve the problem and halve the run time, but it will require an LC system designed for higher pressure use.

Conclusions

We've seen that a change in the column parameters — flow rate, column length, and particle size — can be used to speed up a separation for which excess resolution is present. Column parameter changes also can be used to increase the resolution of a marginal separation. Changes in flow



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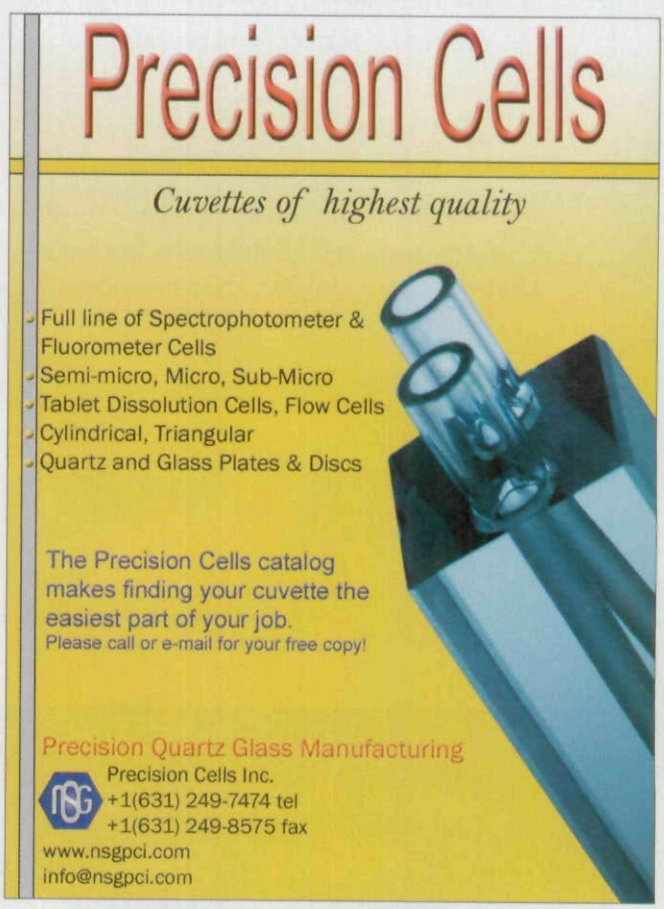

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rate will change retention and pressure in proportion to the change. Usually a change in flow rate by a factor of two will not cause a practical loss of column efficiency for most real samples when 5-μm particles are used. Smaller particles are less susceptible to flow rate changes. The results of changes in column length are easy to calculate - N, pressure, and retention time are directly proportional to the changes. Particle size changes can introduce more problems, as illustrated in Table I, because although N changes in proportion to the particle size change and R_s as the square root of the particle size, pressure changes with the square of the particle size change. Thus, particles with diameters < 3 μm may have limited use with conventional LC systems because of pressure limitations of the equipment.

Most of the results discussed here are based on theory. What you obtain with a method separating real samples in your laboratory is unlikely to gain the full benefit of the changes discussed. When any combination of particles < 5 μm, column diameters < 4.6 mm, and column lengths < 150 mm is used, extracolumn band broadening may further compromise the separation. If you are going to be using such conditions, take care to use short lengths of small diameter tubing (for example, 0.005-in. i.d.) to connect the autosampler to the column and column to detector and keep the injection volumes < 20 μL. In general, sub-2-μm particles will require an LC system designed for minimum volume from the injector through the detector, as well as the capability of pressures > 6000 psi.

Changes in column parameters can have a big impact on reducing run times if the initial method has excess resolution. They are not very powerful in trying to rescue a method with substandard resolution. For this reason, it often is useful to spend a little extra time during method development to obtain a method with more resolution than is necessary on the standard development column so that you can trade some of that excess resolution for shorter run times.

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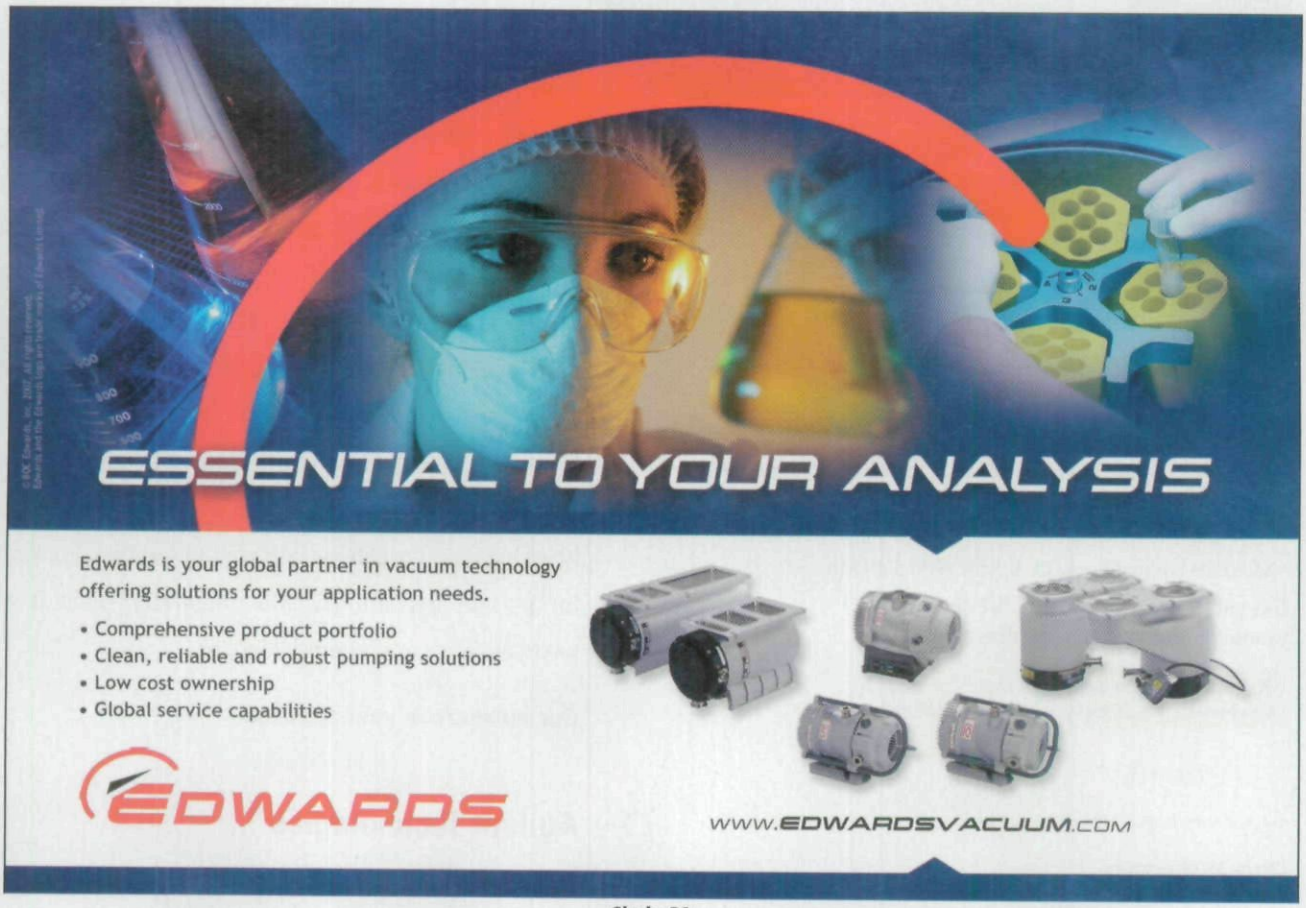

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