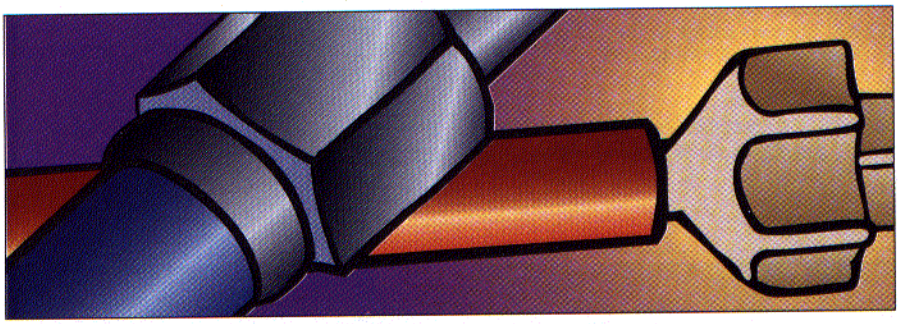
LC Troubleshooting



Starting Out Right, Part I — Selecting the Tools

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This first part of a multipart series tells you how to put your best foot forward and maximize your chances for a successful separation.

Liquid chromatography (LC) problems tend to fall into one of two categories. The first type of problem is associated with instrumentation. Problems with check valves, mixers, detector lamps, and other hardware can be irritating and tedious to isolate, but the solution usually is simple after the source has been identified. Many instrument problems can be prevented through disciplined preventive maintenance. The second type of problem is associated with the separation itself. Poor peak shape, inadequate separation, and drifting retention are some examples of separation problems. Unfortunately, the symptoms of separation problems can be obvious, but identifying the problem source can be difficult and correcting it can seem impossible.

One of the key factors in minimizing separation problems is to start out with a good method. By using a good method, it is much easier to maintain operation within specifications and to correct problems when they arise. For the next few months, "LC Troubleshooting" will focus on the components that make up a good separation and discuss how to put them into action. The staff at my company has been teaching chromatographers these skills in short courses for more than 25 years. Over time, a fairly simple approach to method development emerged — we use this approach in our courses and contract laboratory — and I will use it as the core of this series.

OPEN YOUR CUPBOARD

If you were about to cook a gourmet dinner, you wouldn't just walk into the kitchen and start cooking. First you'd consult a recipe, check your supplies, and go shopping to obtain the ingredients. You'd make sure the pots and pans were clean and the knives were sharp. Sure, you may need to cut some corners and make some substitutions, but you would use the right ingredients and follow the recipe to get the best results. The same approach holds true for developing an LC method. You need specific tools and reagents to obtain the best results. If you start developing a method without proper planning, you may get an adequate separation but the likelihood of a robust method that is tolerant of small changes is less than if you had used a systematic approach.

COLUMN SELECTION

One of the most important choices in method development is column selection. Chromatographers can choose from hundreds of columns, so where do you start? One tool I use to keep tabs on trends in column usage is Ron Majors' annual Pittsburgh Conference review in his "Column Watch" columns in the March and April issues of *LCGC* each year. Since 1984 (1), Ron has shared information about new columns, the changes in column technology, and column usage habits.

For example, most separations performed today use reversed-phase columns, and C8 and C18 phases are the most popular of these. Chromatography, however, is not just a popularity contest. The reason why these two phases are so popular is that they obtain the desired separation in most cases. Although some standard sample types don't point to these columns as the first choice (see Table I), the C8 or C18 columns usually offer the best stationary phase with which to start. There's not enough difference between the C8 and C18 phase for me to strongly recommend one over the other — I prefer C8 for most applications, but I'll leave that choice up to you.

Most samples encountered by average chromatographers will be amenable to reversed-phase separation; however, some compound types likely will require another LC method for successful separation. The sample and method combinations listed in Table I are

TABLE I: Alternative Sample and Method Combinations

Sample Characteristics	Preferred HPLC Method or Column
High molecular weight	Special columns usually required; size-exclusion and ion-exchange high performance liquid chromatography (HPLC) often preferred
Optical isomers (enantiomers) present	Special chiral columns required
Other isomers (stereoisomers or position isomers) present	Normal-phase often best, especially with unmodified silica
Mixtures of inorganic salts	Ion chromatography
Carbohydrates	Amino bonded-phase columns with reversed-phase conditions; ion-exchange resins
Biological samples	Special conditions often required for life-science samples; may not require different approach

suggested starting points. For more information on these samples, see reference 2 for a general treatment of method development.

The production of low-metal, high-purity silica is perhaps the most important practical column development in the last 15 years. Detailed descriptions of the silica surface are available elsewhere (2,3). These publications describe the traditional chromatographic silica as a heterogeneous, acidic surface. After attaching the bonded phase to the surface, the resulting columns could be characterized by tailing peaks for basic analytes and often lower-than-desired column-to-column reproducibility.

Recent advances in silica production have resulted in a nearly metal-free silica with a very homogeneous distribution of low-acidity surface silanol groups. The practical result of these developments is columns that are more stable, more reproducible, and produce much better peak shapes. This improved silica often is called *Type B* silica. Because of the benefits of these phases, most manufacturers prominently distinguish these with names touting their superiority such as Inertsil (GL Sciences Inc., Tokyo, Japan), BDS (base-deactivated silica, Hypersil Inc., Needham Heights, Massachusetts), Symmetry (Waters Corp., Milford, Massachusetts), and YMC-Basic (YMC, Inc., Wilmington, North Carolina). Most column manufacturers make a Type B silica column, so check with your favorite column supplier for the one you should use. Because of the superiority of these phases, I strongly recommend that you set aside your older, Type A phases and start every new method development project with a Type B phase.

COLUMN SIZE

Another important choice is the column dimensions and packing particle diameter (d_p). The most popular particle size is 5-µm d_p, but 3.0- and 3.5-µm particles also are widely used. Any of these particles are suitable for starters, but most chromatographers still prefer the 5-µm particles because of their long usage history. Smaller particles produce columns with higher plate numbers as well as higher back pressure. The 3.0-µm particles are packed with smaller frits that are prone to fouling, so some manufacturers produce 3.5-µm particles that enable the use of traditional 2.0-µm frits, which are less susceptible to fouling.

I prefer the 150 mm × 4.6 mm column size because it generates a sufficiently high plate number (N) with 5-µm particles to obtain adequate separations with most samples. An alternative configuration is the 75 mm × 4.6 mm, 3.5-µm d_p column, which provides separations similar to those of the 150-mm long, 5-µm d_p column but in half the time. An added benefit of either of these column configurations is that the flow rate can be set at 1.5–2.0 mL/min with reasonable back pressures (for example, less than 2000 psi). Higher flow rates mean shorter run times, and as the saying goes, "time is money." Columns as long as 250 mm generate a few more plates, but the benefit is marginal (N grows only with the square root of the length increase) and the penalties are longer run times and higher back pressures.

Some workers suggest 30- or 50-mm column lengths for initial screening, but these columns often do not generate a sufficiently high plate number for a reasonable separation. Another option is narrow-bore (2-mm i.d.) or microbore (≤1-mm i.d.) columns. These columns use less solvent, but during the method development stage they place unnecessary demands on the LC system, such as requiring smaller sample volumes and minimizing extracolumn plumbing and detector cell sizes. My preference for using any of these short or narrow diameter columns is to wait until the fine-tuning stage at the end of method development.

In other words, I'll stick with my 150 mm × 4.6 mm, 5-µm d_p column as the workhorse for method development. My alternate is the 75 mm × 4.6 mm, 3.5-µm d_p column. Typically I recommend running these columns with a 1.5-mL/min flow rate.

ORGANIC SOLVENT SELECTION

Another choice that can have a bearing on the success of the separation is the organic solvent. Reversed-phase separations provide three choices: methanol, acetonitrile, and tetrahydrofuran. Each solvent has unique selectivity advantages, but chromatographers seldom can predict which solvent will be the best choice on this basis. So you must choose the starting solvent based on other considerations.

Most of the work in my laboratory requires analyzing pharmaceutical compounds. Many of these samples have very poor UV absorbance, so analysts often find themselves working with detector settings of 220 nm or less. Because of its high background absorbance, tetrahydrofuran is not very useful below about 240 nm. Although low concentrations of methanol can be used at low wavelengths, gradients with methanol usually drift off scale at wavelengths shorter than 220 nm.

You want a solvent that is nonreactive with samples and the atmosphere. Tetrahydrofuran can degrade and form peroxides, so workers must take special care if they use it. Some workers have found that adding water to tetrahydrofuran greatly diminishes this problem (4). Tetrahydrofuran also is much slower to equilibrate with the column and LC system than is acetonitrile or methanol — it may take twice the solvent volume for initial equilibration with tetrahydrofuran. And, of course, tetrahydrofuran's unpleasant odor is another negative factor.

A final beneficial property of acetonitrile and methanol is their relatively low back pressure at flow rates of 1–2 mL/min when compared with tetrahydrofuran.

When I consider the various desirable solvent properties — low viscosity, low UV absorbance, low reactivity, and convenience — my first choice for an organic solvent is acetonitrile. However, I would not argue with anyone who preferred methanol as a starting solvent.

AQUEOUS PHASE SELECTION

If the samples are neutral compounds, you may be able to use water as the aqueous phase. However, ionic compounds generally are present in pharmaceutical analysis and many other applications. Ionic compounds require analysts to maintain pH control to obtain reproducible methods. You will get the best results if the compounds are fully protonated or fully ionized. Thus, the pH of the mobile phase should be at least 1.5 units above or below the pK_a if possible. For organic acids, working at pHs lower than pH 3 generally will be satisfactory. However, bases will require buffers with pHs higher than pH 8, which exceeds the recommended working pH of most silica-based columns. Silica columns with extended pH ranges are available now, but few have extensive experience with these materials at high pH, so I recommend organic buffers to reduce silica dissolution. These factors make me wary of routine use of high-pH mobile phases with silica columns.

Sample characteristics and column pH stability combine to suggest that a low pH is preferable to a high pH for routine operation. Most C8 and C18 columns are stable down to a pH of approximately 2. Under these conditions, most acidic samples will be protonated, bases will be fully ionized, and the ionization of surface silanols will be minimized. For many routine methods, 25 mM phosphate buffer at pH 2.5 will be a satisfactory starting aqueous phase. If the method is destined for use with a mass spectrometer, you will need to use a volatile buffer. Although not as useful as true buffers, 0.1% trifluoroacetic acid or formic acid will provide satisfactory pH control for many LC–mass spectrometry applications.

OTHER FACTORS

You should consider a few other factors before embarking on developing a new LC method. Because retention changes by 1–3% for a 1 °C change in temperature, it is important to control the column temperature. Changes in temperature also can affect selectivity, which should give you additional incentive to control the temperature. I generally operate the column oven at a temperature just higher than room temperature (for example, at 35 °C) for ease of control and to reduce the solvent viscosity for lower-pressure operation.

Sometimes you'll need to use additional reagents such as ion-pairing reagents or triethylamine for special sample types. I recommend, however, that unless you know that you will need these additives, you should start with an additive-free mobile phase. Remember the KISS principle — Keep It Simple, Stupid — and don't complicate the mobile phase unless it is necessary.

TABLE II: Recommended Starting Conditions for Reversed-Phase LC Method Development

Separation Variable	Preferred Initial Choice
Column	
Dimensions	150 mm × 4.6 mm
Particle size	5 μm
Stationary phase	C8 or C18
Mobile phase	
Solvents A–B	Water–acetonitrile
% B solvent	Variable
Buffer	25 mM phosphate (pH 2.5) or 0.1% trifluoroacetic acid or formic acid
Additives such as ion-pair reagents and amines	As necessary
Flow rate	1–2 mL/min
Temperature	35 °C

Finally, I strongly recommend that you select a column that will be available for years to come. Chances are that your method will be used for several years, and the heart of reproducible separation is obtaining a column of similar chemistry year after year. Use a column vendor that you are confident can provide controlled column chemistry over the long haul.

IN OTHER WORDS

I cannot overemphasize the importance of choosing starting conditions carefully. The choices of column and mobile phase are ones that you — and likely others, too — will live with for years. Make those choices wisely. I've summarized my recommendations for starting conditions in Table II.

The 150 mm × 4.6 mm, 5-μm d_p Type B silica column is a workhorse, and when operated at low pH under controlled temperature it will be an excellent place to start method development with most samples.

Now that I've described the tools to use, next month I'll begin looking at the steps toward obtaining the best separation in the minimum amount of time and effort.

REFERENCES

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(3) U.D. Neue, *HPLC Columns. Theory, Technology, and Practice* (Wiley-VCH, New York, 1997).
(4) J. Zhao and P.W. Carr, *LCGC* **17**(4), 346–352 (1999).

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