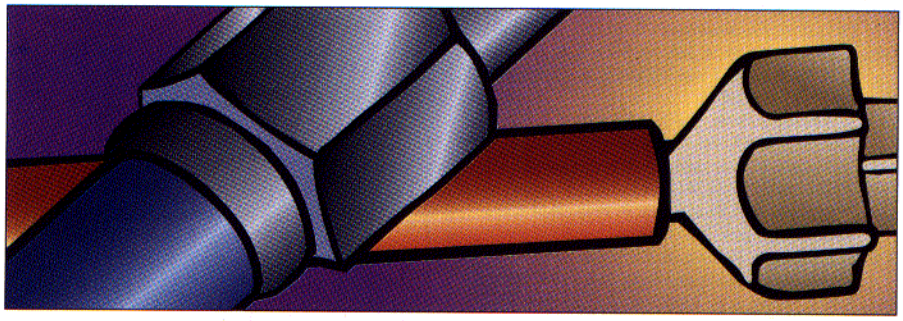
LC Troubleshooting



Method Development, Peak Distortion, and Interfering Peaks

John W. Dolan

The mailbag brings a variety of problems this month.

This month's "LC Troubleshooting" addresses a variety of problems submitted by readers. Although some of the questions are specific, their solutions can be applied for general use. For example, the first problem deals with a method for a specific compound — phthalic acid — yet the suggested approach is valid for any sample that contains acids.

ION PAIRING, ION EXCHANGE, OR . . .

Q: We developed a method for determining phthalic acid in food samples using a saponification procedure followed by analysis with a reversed-phase ion-pairing technique. After saponification, the matrix is very alkaline. Is the above liquid chromatography (LC) technique the best choice or would an anion-exchange chromatography technique be more suitable?

A: Ion pairing is an option for an acidic sample such as phthalic acid, and ion-exchange chromatography also should work, but I would start with a more traditional technique. I generally like to apply the *KISS Principle* — Keep It Simple, Stupid — to my methods. The simpler a method is, the less likely it will cause problems. For this reason, I would start with reversed-phase chromatography using a low-pH mobile phase. (I've added a few comments at the end of this section about how to proceed if you decide to use ion-pairing or ion-exchange chromatography.)

Start with a 15 or 25 cm × 4.6 mm, 5-µm d_p C8 or C18 column. I prefer to use one of the newer base-deactivated silicas as the column packing because they are less susceptible to tailing and are stable within a wider pH range than the older column types. To work in an ion-suppression mode, the mobile phase should be 1–1.5 pH units below the pK_a of the acid. Phthalic acid's first pK_a is 2.9, which would require working at pH 1.4–1.9. Generally, it is best to avoid working at pH levels lower than pH 2 unless you know the column is stable under those conditions. I would start with a pH 2.0, 25 mM phosphate buffer for the aqueous portion of the mobile phase and use acetonitrile or methanol as the organic solvent. Under these conditions, phthalic acid should be un-ionized sufficiently to provide a good peak shape and behave as a neutral compound. UV detection at 255 nm should be possible because of the molecule's aromatic nature.

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I prefer to use a scouting gradient to identify the proper mobile phase strength, as described in a previous "LC Troubleshooting" column (1) and in reference 2. Alternatively, use the stepwise approach for method development, starting with 90% organic solvent for the first run and stepping down in 10% increments until you obtain a reasonable retention time. You should adjust the pH of the sample before injection so that it is near the mobile phase's pH.

Ion suppression with a low-pH mobile phase offers many advantages over a high-pH mobile phase. At low pH, the ionization of the phthalic acid will be suppressed, so it should behave as a neutral molecule with predictable retention and acceptable peak shape. Low pH also suppresses the ionization of the silanol groups on the stationary phase, which helps to reduce peak tailing. All reversed-phase columns are stable in the $3 < pH < 7$ range, and the newer columns perform acceptably at $2 < pH < 8$ or an even wider range.

If you want to work at high pH, you should know that the base silica in the column packing will dissolve at pH levels higher than pH 8. Some columns are more stable than others, and endcapped columns are more stable at higher pH than their nonendcapped counterparts. Column stability is improved when you use organic buffers (for example, Tris or citrate) instead of inorganic buffers (such as phosphate) under alkaline conditions (3). An alternative is to use polymeric reversed-phase columns. These polymeric columns are insensitive to pH, but they tend to generate lower plate numbers and thus broader peaks than silica-based columns.

Ion-exchange chromatography, as you have suggested, is another possibility for separations at high pH. An ion-exchange phase attached to polymeric beads would have the pH stability you seek, but it is likely to suffer from lower plate numbers than the equivalent reversed-phase column.

PEAK DISTORTION

Q: While developing the above-mentioned method for phthalic acid, we found that the peak shape improved when we prepared the phthalic acid standards in water instead of methanol. What is the reason for this result? We used a mobile phase of 20:80 (v/v) methanol–5 mM phosphate buffer with 10 mM tetrapentylammonium bromide added for ion pairing.

A: This case is the result of injecting too large a volume of a solvent that is too strong. Figure 1 illustrates this problem. Figure 1a shows the distorted peak that results from injecting 30 µL of sample in acetonitrile into a

mobile phase of 18% acetonitrile in water. In Figure 1b, the same sample was prepared using mobile phase as the injection solvent, and it produced a well-behaved peak. When a sample is injected in a solvent that is different than the mobile phase, the sample solvent mixes with the mobile phase and becomes diluted. If the injection solvent is stronger than the mobile phase, the sample will behave momentarily as if it were in a stronger solvent and move more quickly through the column, which accounts for the shorter retention times of the peaks in Figure 1a. As the injection solvent mixes with mobile phase, some molecules will be mixed with the mobile phase before others, and their rate of travel through the column will change, thus distorting the band. Peak distortion is more of a problem for early eluted bands than later components, as Figure 1a shows. The key to minimizing injection solvent problems is either to inject a sample volume sufficiently small that this dilution takes place very quickly or to use a sample solvent that is no stronger than the mobile phase. Weaker solvents concentrate the sample on the column, and, in some cases, the peaks are narrower than they would be if injected in a stronger solvent.

The key to minimizing injection solvent problems is either to inject a sample volume sufficiently small that this dilution takes place very quickly or to use a sample solvent that is no stronger than the mobile phase.

So as a general rule, if a sample is in a solvent stronger than the mobile phase, the injection volume should be less than 25 µL. The volume will depend on how much difference exists between the injection solvent and the mobile phase. This difference is easy to check empirically — just inject larger and larger volumes until the peak shape of the early peaks becomes distorted and then back off by a factor of two or so. In the reader's case, the sample in methanol is too strong for the mobile phase, so he observed distorted, broad peaks. When he used water instead, the sample solvent was weaker than the mobile phase, and the peak shape improved. With ion-pairing chromatography, it always is advisable to use the mobile phase as the injection solvent to minimize the occurrence of baseline artifacts.

INTERFERING PEAKS

Q: How can I prevent a solvent-front peak from masking the peaks of interest in my reversed-phase LC separation?

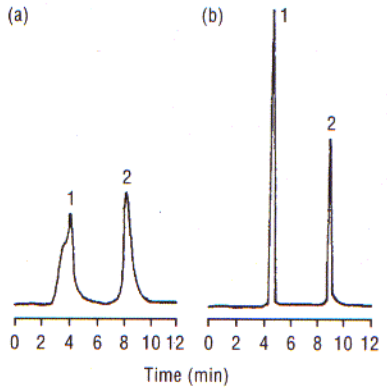

FIGURE 1: Reversed-phase separation with an 18% acetonitrile–water mobile phase. Shown are chromatograms generated by injecting a 30-µL sample in (a) acetonitrile and in (b) mobile phase. (Reprinted with permission from reference 4.)

A: The best way to prevent interference from unretained material at the beginning of the separation is to increase the retention of the analytes of interest. Generally, the chromatography and the separation will be better if the retention factor (k) is greater than 1. You can estimate k by using the solvent front as a ruler. The first peak in the chromatogram usually is the solvent front or garbage peak. This peak is eluted at the column dead time (t_0), which represents the time for an unretained material to pass through the column. Peaks that come out close to the dead time have little opportunity to interact with the column and tend to be poorly separated from the junk at the solvent front and other compounds. To obtain k greater than 1, the compound of interest must have a retention time of more than twice the dead time ($k = [t_R - t_0]/t_0$). For example, the peak at 9.03 min in Figure 1b has k of approximately 1.

You can increase retention by using a weaker solvent. For reversed-phase LC, the weak solvent generally should be water or buffer. Changing the percentage of organic solvent by 10% will change retention approximately threefold. You can use this *Rule of Three* to estimate how much you will need to change the organic solvent to obtain the desired retention.

WHERE TO START?

Q: I see much advice about selecting starting conditions for reversed-phase LC separations, but I'm totally confused. Is C8 or C18 better? Do I want a 15- or 25-cm long column? Should I use acetonitrile or methanol as the organic solvent? Can you give me some guidelines for starting conditions?

A: Let's examine each of the variables separately. First, is C8 or C18 a better choice or some other phase, for that matter? For most applications, it makes little difference which

stationary phase you choose. C18 is somewhat more retentive than C8, so less polar solutes may benefit from the use of C8, whereas more polar ones will be more strongly retained on a C18 column. For the most part, the choice is arbitrary. A more important choice is in the nature of the packing material. The newer, high purity, base-deactivated silicas (type B) are good for developing new methods. In my experience, these phases almost always provide better peak shape and less tailing than the older materials. I'm a firm believer that you get what you pay for with LC columns — a $200 column is unlikely to give you the same level of performance as a $400 column. Of course exceptions exist, but for the most part, when you spend thousands of dollars on method development and tens of thousands on analysis, does it make much sense to cut corners on the column?

Column length is another, somewhat arbitrary, choice. Most practical separations will require 8000–10,000 plates, and 15-cm long columns packed with 5-µm particles or 7.5–10 cm long columns containing 3-µm particles can generate these plate numbers for real samples. So from a plate number standpoint, 15- or 25-cm long columns are suitable. I favor 15 cm × 4.6 mm, 5-µm d_p columns because they can be used at 2-mL/min flow rates with back pressures of less than 2500 psi. The longer 25-cm columns will require lower flow rates for the same back pressure. The longer column generates roughly threefold longer run times due to the combination of column length and flow rate for a gain of approximately 30% in resolution. Using one of the new 7.5 cm × 4.6 mm, 3.5-µm d_p columns is an alternative. These columns generate plate numbers similar to the 15-cm long column in roughly half the time. You must take care to avoid extracolumn band broadening with shorter, small-particle columns. You also can use narrow-bore columns (1–2 mm i.d.), but they also are quite susceptible to band broadening caused by extracolumn factors.

Finally, the choice of organic solvent also is somewhat arbitrary. The solvent should be fully miscible with water, nonreactive with the analytes and column, of low viscosity, and suitable for the chosen detector. The three most popular solvents are acetonitrile, methanol, and tetrahydrofuran. Tetrahydrofuran generally is the worst choice — it is unpleasant to work with, chemically unstable (forming peroxides over time), and slow to equilibrate. Methanol is reasonably nontoxic and is a good choice for use at detection wavelengths higher than 220 nm. However, my first choice is acetonitrile, because my laboratory develops many methods that require low-wavelength detection.

To summarize, my preferred starting conditions are a 15 cm × 4.6 mm, 5-µm d_p C8 or C18 column, a 2-mL/min flow rate, and acetonitrile–water or acetonitrile–buffer as the mobile phase. My laboratory also has had good luck with the 7.5 cm × 4.6 mm, 3.5-µm d_p columns. Either of these columns provide a good starting point, because they provide a sufficient plate number for most separations, flexibility for low-wavelength detection, and fast runs. Having said this, I think it is very defensible to start with any combination of C8 or C18 columns in 15- or 25-cm lengths and to use acetonitrile or methanol — it's your choice.

CONCLUSION

Selecting the starting conditions for a new method involves choices of column, mobile phase, and injection solvent. Although many combinations of these factors may work, the use of a low-pH mobile phase, a reversed-phase column, and an injection solvent similar to the mobile phase will give the highest probability of initial success.

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