LC Troubleshooting

From the e-Mail Bag

John Dolan answers readers' questions related to columns and methods.

Usually "LC Troubleshooting" focuses on a particular theme each month. These topics often are drawn from questions sent to me from readers. This month, I've pulled a few of those questions from my e-mail box for consideration. If you like this random selection of topics, you'll want to become a participant in the lively on-line discussion of liquid chromatography (LC) troubleshooting problems on the Chromatography Forum web site (http://www.chromforum.com).

Column Chain Length and Life

Q: All of my experience is with methods that use C8 and C18 columns. I'm about to prepare a method that requires a cyano column. I've heard that these columns don't last very long. Is this true?

A: In our laboratory, my colleagues and I commonly use cyano columns and have encountered no particular problems with shortened column life. However, some evidence shows that any column with a shorter chain length can fail sooner than longer-chain-length columns under certain conditions (1).

Figure 1 shows an example of how bonded phases of various chain lengths deteriorate more quickly at low pH (1). In this case, a gradient was run with a pH 2 mobile phase at 50 °C. Under those conditions, the bond between the bonded phase and the silica surface was hydrolyzed. The retention of a nonpolar compound — 1-phenylheptane — was measured regularly throughout the test time. This neutral compound was retained only by the nonpolar bonded phase, not the exposed silica surface of the packing, so a change in the retention was a good measure of the bonded-phase loss. For example, a 20% change in retention should correlate to a 20% loss in bonded phase.

When expressed as a change in the retention factor (k), retention is normalized for different column types, so chromatographers can compare the stability of different

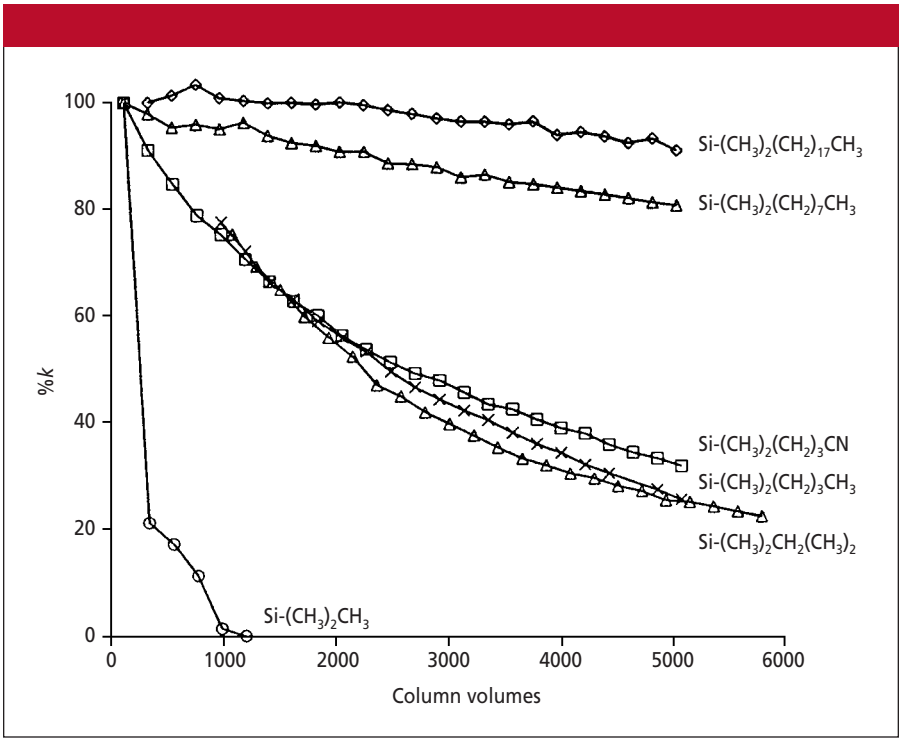

Figure 1: Effect of silane chain length on bonded-phase stability at pH 2. See text for details. (Reprinted from reference 1 with permission.)

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types of bonded phases. Time is measured in column volumes of mobile phase. For a 150 mm × 4.6 mm column, the column volume is approximately 1.5 mL, so 1000 column volumes represent about 1500 mL of mobile phase. Figure 1 makes it clear that column stability is related directly to bonded-phase chain length. The common C8 and C18 phases lose 5–10% of their retention in 6000 column volumes — roughly 9 L of mobile phase under aggressive conditions. Shorter-chain columns such as the propylcyano columns deteriorate much more quickly and lose more than 60% of their retention under the same conditions.

Do the data of Figure 1 mean that cyano columns are inherently less stable than C8 or C18 columns? In this test, yes. However, you should consider a few other factors. First, the work described above was reported in 1988, so these data probably were gathered using Type A silica columns rather than the newer Type B columns, which are more stable. Second, the test conditions are quite aggressive, so a higher pH (for example, pH 2.5–3) or lower temperature could reduce the rate of bond cleavage. Third, using a guard column should help protect an analytical column and mitigate the damage.

So although cyano columns potentially have shorter lives than their longer-chain cousins, many other factors are involved, making it hard to draw a blanket conclusion. I would set up the method and track the performance of the column over time to see what happens. Pay particular attention to changes in retention and peak shape as indicators of column deterioration. You can compensate for simple retention changes by adjusting the mobile phase, as long as the peak spacing is acceptable. A guard column also can help extend a column's useful life. Finally, it is important to remember that a column is a consumable item. I figure that as long as the cost of a column represents less than 5% of an assay's cost, it is of little consideration. So if a typical assay cost is $50 and a column costs $500, a column lifetime of 200 samples would hit that 5% threshold.

Tips for Successful Method Transfer

Q: I'm about to set up a method that is new to me. I always seem to have trouble getting the right results the first time with someone else's method. Can you look over this method and give me some pointers?

For mobile-phase preparation, I start by dissolving approximately 2.72 g of monobasic potassium phosphate in 1000 mL of deionized water. Next, I mix 550 mL of phosphate buffer, 400 mL of acetonitrile, 50 mL of methanol, and 1.5 mL of triethylamine. Then, I adjust the pH level to pH 7.0 ± 0.05 with dilute phosphoric acid and, finally, degas the mobile phase before use.

The method calls for a 250 mm × 4.6 mm, 5-µm d_p cyano column, a flow rate of 1.0 mL/min, and a detection wavelength of 214 nm. The injection volume is 10 µL, the temperature is 25 °C, and the run time is 30 min. The sample concentration should be 1500 µg/mL.

A: Before looking at your method, let me give you a note of encouragement: Method transfer can be a very difficult task, even for experts, because method descriptions often omit critical details.

Let's walk through the method step by step, starting with the mobile phase. Preparing the phosphate is straightforward, although I would use high performance liquid chromatography (HPLC)–grade water instead of deionized water. Deionized water works in many applications, but it has a lower level of purity than HPLC-grade water. My laboratory has a commercial HPLC water-preparation apparatus that has worked with only minor maintenance for more than 10 years, and it produces uniformly high-quality product at a low cost per liter.

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The blend of four components for the mobile phase suggests to me that the method was developed several years ago. One clue is the presence of triethylamine, which was used to suppress peak tailing on columns with a significant concentration of acidic silanol groups. Newer, Type B silica columns, which have overtaken the column market in the past few years, generally do not need triethylamine because they are manufactured with a low concentration of acidic silanols. Whenever possible, I abide by the KISS principle — Keep It Simple, Stupid — for mobile phases; in this case, the fewer the components, the fewer the things that can go wrong. I've found that by using some method development software such as DryLab (LC Resources, Walnut Creek, California), I usually can obtain a two-component mobile phase (organic solvent and buffer) to do the job. So you should measure the four components very carefully.

Adjusting the mobile-phase pH after adding organic components sends up a big red flag. pH meters are not designed to work well under these conditions, and the true pH can vary significantly from one batch to the next, especially if you try to adjust the pH to ±0.05 units. As a general rule, I usually expect pH control to be ±0.1 units using normal laboratory techniques when adjusting the aqueous portion of the mobile phase. I would convert the method to incorporate pH adjustment of the buffer component before adding organic solvent. This switch could change the apparent pH, however, so you might need to make several trials before you can achieve the same separation.

I approve of degassing the mobile phase, even if you use an on-line degasser. Degassing is high on my list of preventive maintenance techniques.

Now let's look at the chromatographic conditions. The column length gives me another hint that the method is an older one — workers today typically use a 150-mm column for most assays. A 250-mm column is suitable, but only if it provides a sufficiently better separation to justify the attendant increase in run time. The flow rate is fine — you'll have trouble running much higher than 1 mL/min and maintaining the pressure in a reasonable range. If I were reworking the method, I'd try a 150-mm column and a flow rate of 1.5 mL/min. If you can achieve satisfactory separation under these conditions, the run time will be approximately 40% of the specified 30 min. If you have a large number of samples to analyze, your investment in method modification time might well be paid back in analytical time savings.

The wavelength provides another hint that you're looking at an older method. A 214-nm wavelength dates from the days of using a zinc lamp in a fixed-wavelength detector. I would expect that if the same method were developed today, the devel-

oper would specify a 215-nm wavelength instead, and the method would have no observable difference in response.

An injection volume of 10 µL is satisfactory. However, when coupled with a 1500-µg/mL sample concentration, 15 µg of sample is being put on the column. A rough guide says that chromatographers can load as much as 10–20 µg of sample per gram of packing material. The 250-mm column will contain approximately 2.5 g of material, so more than 25–50 µg of sample would create an overload condition. Unless this assay indicates stability or examines trace components, 10 µL may be more sample than is necessary. Again, if I were modifying the method, I'd prefer to put roughly 10-fold less sample mass on the column.

The temperature is specified as 25 °C, which makes me suspicious. Nominal room temperature is in the 20–25 °C region, so this method was designed to run at room temperature. Unless you have a column oven with cooling capabilities, you will have a difficult time maintaining the column at this temperature. I find that 30–35 °C is as low as most column ovens can control the temperature reliably, unless they have a cooling function. You don't want to run an analysis without a column oven — remember that retention can vary by 1–3% per 1 °C.

This method has many potential problems. In total, these problems probably will result in what you fear — difficulty in transferring the method. On the other hand, you might be able to establish the method and have it perform reliably for years. Just because a method has suspicious characteristics doesn't guarantee that it will be problematic.

Why We Saturate Solvents with Water

Q: What is the purpose of saturating normal-phase solvents such as chloroform or methylene chloride with water?

A: The activity of bare-silica packings, common in normal-phase chromatography, is influenced strongly by the mobile phase's water content. Changes in activity often appear as changes in retention and peak shape. The best way to stabilize retention and peak shape in those cases is to include a polar additive in the mobile phase; the simplest way to achieve this is to saturate the mobile phase with water. For example, 0.15% water saturates methylene chloride. Because operation at true saturation is risky if phase separation occurs, which could happen with a change in room temperature, many workers prefer half-saturation of the mobile phase.

Half-saturation can be achieved by adding an excess of water to one aliquot of solvent to saturate it and then mixing equal volumes of this solvent with a dry solvent. Controlling the mobile phase's water content at concentrations of less than 0.1% can be tricky and subject to changes in the laboratory environment. I remember observing retention change in one separation when the humidity of the laboratory changed.

To avoid the difficulty of controlling the mobile phase's water content, you can use polar organic additives instead. For example, adding methanol or acetonitrile at a concentration of 1% will achieve the same effect as adding 0.1% water. The measurement is easier, and the resulting solvent mixture is less susceptible to changes in atmospheric humidity.

What's an Excipient?

Q: In many articles, I read references to excipients. What are they and how do they differ from foreign substances?

A: Excipients generally are materials that are intentionally combined with a drug substance (the active pharmaceutical ingredient) to make the final drug product. These materials include fillers such as talc, sugar, or cellulose; components that make the drug stable to oxidation or water uptake; coloring agents; and materials that help the drug product dissolve at a desired rate. Sometimes the only purpose of an excipient is to bulk up a small dose (for example, 2 mg) so that it can be taken conveniently. Basically, excipients are everything in the drug product that isn't the active ingredient.

Excipients differ from foreign substances, which are considered unintentional additives or contaminants. Excipients can present significant analytical challenges by clogging filters, interfering with the chromatographic separation, failing to dissolve, or making the solution too viscous to be drawn into a syringe. When chromatographers develop analytical methods, they generally test placebos containing everything except the active ingredient with and without the added drugs to make sure that the excipients don't alter the results.

More Input on High-pH Buffers

Last June, "LC Troubleshooting" contained a discussion of buffer effects and mentioned that chromatographers should avoid inorganic buffers such as phosphate at high pH levels, because they accelerate dissolution of silica column packing materials (2). Instead, the authors suggested Tris (tris[hydroxymethyl]aminomethane) and ammonium bicarbonate as alternatives. A reader suggested some additional buffers he had used successfully for high-pH work with the Zorbax Extend-C18 column (Agilent Technologies, Wilmington, Delaware) (3). The suggestions were

- pyrollidine (pK_a = 11.3),
- triethylamine (pK_a = 10.7),
- 1-methylpiperidine (pK_a = 10.3),
- glycine (pK_a = 9.8),
- borate (pK_a = 9.2),
- ammonium hydroxide (pK_a = 9.2), and
- diethylamine (pK_a = 10.5).

These buffers should be less aggressive than phosphate for any column at high pH, but to be safe, check your manufacturer's literature to determine the pH limitations of your column.

Keep Those Cards and Letters Coming

From this sampling of queries, you can see that I receive a wide variety of questions. Please feel free to send specific or general questions about LC troubleshooting problems to me by e-mail. Who knows — your question might even show up in print. You'll also receive well-thought-out responses to your questions at the Chromatography Forum web site.

References

(1) J.J. Kirkland, J.L. Glajch, and R.D. Farlee, *Anal. Chem.* **61,** 2 (1988).
(2) N.S. Wilson, R. Morrison, and J.W. Dolan, *LCGC* **19**(6), 590–594 (2001).
(3) T.J. Waeghe, personal communication, June 2001.

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For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.