LC Troubleshooting

How Much Is Enough?

Better a little extra than not enough.

This month's "LC Troubleshooting" addresses two liquid chromatography (LC) practices. First, a reader asks about column regeneration after a reversed-phase gradient has been run. Another reader wants to know which degassing technique to use. In both situations, no hard-and-fast rules apply. Instead, analysts should use experimental evidence to determine the best operating conditions.

Gradient Equilibration

Q: I need an experimental method to determine the correct mobile-phase volume and the time needed to sufficiently reequilibrate a column after running a reversed-phase gradient. I know the general rule that approximately 10 column volumes of mobile phase is necessary for reequilibration, but I must demonstrate and validate that the reequilibration conditions are sufficient.

The method I am using calls for a 10-min, 20–80% acetonitrile–buffer gradient. Then, I use another 10-min gradient to reequilibrate from the final conditions back to the initial conditions, followed by 8 min of isocratic hold. The 250 mm × 4.6 mm column is operated with a 1-mL/min flow rate. The system dwell volume is 0.75 mL.

In my opinion, linearly returning to the starting conditions in 10 min wastes a lot of time, so I shortened this period to 3 min. I did not see any difference in the separation. Can you suggest some guidelines to help me validate that the reequilibration is sufficient?

A: You are quite right that the reequilibration conditions described in your method might not be producing the desired benefit. The rule that you quoted is intended to cover a full-range gradient (for example, 5–100% organic solvent) and refers to the amount of volume to reequilibrate at the initial conditions. Let's see how your method compares with an ideal case.

First, you must determine the column volume. For 4.6-mm i.d. columns, the column volume can be estimated as

$$V_m \approx 0.1L \quad [1]$$

where V_m is the column volume in milliliters and L is the column length in centimeters. So your column, which is 250 mm (or 25 cm), has a volume of approximately 2.5 mL. This amount suggests that 25 mL of mobile phase would be required for reequilibration. At 1 mL/min, reequilibration would require 25 min — longer than your method suggests. However, I have not considered how the gradient range and dwell volume fit into the process.

You can adjust the equilibration time in proportion to the gradient range. The range for your method, 20–80% acetonitrile, is a range of 60%, so 0.6 × 10 column volumes is approximately 6 column volumes or 15 mL — still more than your method requires.

Finally, you must take the system dwell volume into account because any reequilibration solvent is delayed by the time it takes to wash through this volume. (The dwell volume is the volume from the point at which the solvents are mixed until the point at which they reach the head of the column.) Your system has a dwell volume of 0.75 mL. At 1 mL/min, that value is only 45 s of additional time. However, some LC systems have dwell volumes of 5 mL or more, so it is easy to see the importance dwell volume can play in reequilibration times for these systems.

We can put all these factors together with the following guideline:

$$V_E = V_D + (10V_m)(R) \quad [2]$$

where V_E is the equilibration volume, V_D is the dwell volume, and R is the gradient

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range (as a percentage). You can convert this value easily to the equilibration time by dividing by the flow rate in milliliters per minute. In your case,

$$V_E = 0.75 \text{ mL} + (10)(2.5 \text{ mL})(0.6) = 15.75 \text{ mL} \quad [3]$$

This number is close to the total regeneration time, including the negative gradient.

But let's take a closer look. The reequilibration depends upon the volume of solvent passing through the column at the initial conditions, which is only 8 mL in your case. The negative gradient is a throwback to the earlier days of chromatography when columns were less stable than they are today. Historically, some packing materials — especially polymer-based packings — tended to swell or shrink when the mobile-phase composition changed. Even the packing structure of silica-based packings shifted in some cases when the packings were exposed to sudden changes in solvent composition or flow rate. Today's columns are very stable to flow and solvent-composition changes, so reverse gradients rarely are required. Gel-permeation columns based upon soft gels still can be problematic, but you shouldn't worry when using silica-based packings. So, for your method, I would eliminate the reverse gradient and simply transfer a step from the final conditions back to the initial conditions. By adding the reverse gradient time (10 min) to the isocratic time (8 min), the equilibration time now exceeds the recommendation calculated above.

Now that you have a guideline, you need to determine how much time is needed to satisfactorily reequilibrate the column. I would start with 15 min (15 mL) of reequilibration, which is just a little less than the guideline of equation 2. Make three or four runs under these conditions and observe the variation in retention time of a peak early in the chromatogram. Remember that early peaks will be more influenced by poor equilibration than later ones. Determine if the variation in retention time is acceptable (generally, less than 0.05 min). If the variation is acceptable and you would like a shorter total cycle time, then reduce the equilibration time, perhaps to 10 min, and repeat the experiment. If the variation was too large, then increase the equilibration time to 20 min and repeat the runs. Using this process, it should become quickly apparent how much reequilibration is necessary for acceptable retention time variation. I like to leave a few extra minutes in addition to the minimum, just for a safety margin.

I don't see any need to separately validate that the equilibration conditions are satisfactory. As you perform the method validation for a method, you can confirm that the retention time variation is acceptable. Of course, other factors can influence retention repeatability, but if the variation is satisfactory, the reequilibration conditions must be acceptable as well.

Finally, don't forget that reequilibration is a volume-related process, so if you can increase the flow rate, you'll shorten the time. In the case of a 250-mm column, you probably won't be able to use flow rates much greater than 1.5 mL/min because of pressure issues, but even this change would reduce the equilibration time by one-third.

Which Degassing Technique?

Q: I began using LC when helium sparging was popular and recognized as a very effective and efficient means of solvent degassing. Vacuum degassing performed while stirring or with applied sonication was, and still is, very popular. I have switched to in-line vacuum degassers, but the quality control laboratories I support rely upon vacuum degassing. With their reduced turnover volume and more-rugged spaghetti-like tubing, the newer low-volume in-line units have become my degassers of choice.

My question: How do the procedures compare as far as effectiveness? I have discovered that many experienced analysts do whatever feels right without any real knowledge of effectiveness. Vacuum with sonication feels right to me, because a large amount of gas is eliminated immediately, but how long should the vacuum be maintained? Is vacuum with stirring as good as vacuum with sonication? What about sonication alone? Prolonged vacuum will compromise solvent composition, resulting in too much of a good thing.

A: Solvent outgassing occurs when the solubility of a gas in a solvent mixture is exceeded. For example, when air-saturated water and methanol are mixed, the solubility of air in water–methanol blends is less than that in pure solvents. The result is bubble formation. If a slight vacuum is drawn on the mixture, as is the case when a low-pressure on-line mixing system is used, the problem is intensified. The goal of

mobile-phase degassing is to remove enough air that outgassing does not occur within the LC system. Further complicating matters is the observation that some pumping system designs are more tolerant to bubbles than others. I had pumps at two extremes in my laboratory at one time. One system did not require solvent degassing. In fact, I could start the pump with a dry inlet line, drop the inlet line in the reservoir, and the pump would prime itself. The other system required helium-sparged and pressurized reservoirs. If the helium tank ran out of pressure, the system would generate bubbles within a few minutes.

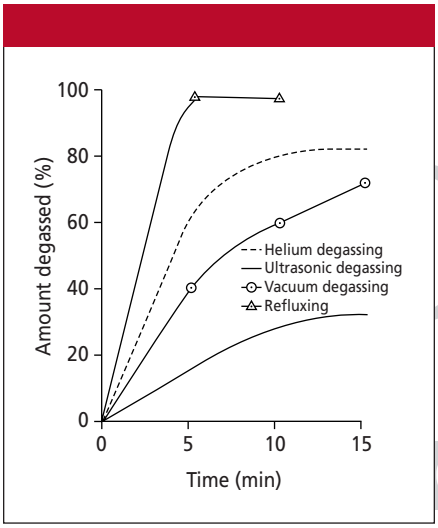

Figure 1: Comparison of degassing techniques in removal of oxygen from methanol. Reprinted from reference 2 with permission.

I haven't read much recent literature that compares degassing techniques, but the data supporting the original helium sparging patent (1) and an article comparing several techniques (2) are classics. Additional information can be found in reference 3. The data from Figure 1 address some of your questions. Although I don't recall the exact conditions being compared, Figure 1 shows that helium sparging is much more effective than vacuum degassing. Both techniques remove more than one-half of the dissolved gas, which should be sufficient to avoid outgassing problems (1). Note that sonication was less effective than either helium or vacuum treatment. In some cases, sonication can *increase* the amount of dissolved gas (4). Sonication with vacuum degassing is more effective than sonication alone (4), but I've never seen any experimental data that compare the combination of sonication and vacuum degassing with vacuum alone. However, as you observed, the practice is widespread, and many workers use the technique routinely. Of course, all of the gas can be removed by refluxing the solvent to boil off the gas (see Figure 1), but that isn't very practical and would certainly change the composition of mobile-phase mixtures.

Today's in-line vacuum degassers are in at least the third generation of development. These devices have a gas-permeable tube that carries gas-laden mobile phase through a vacuum chamber. Much like a Gore-Tex rain jacket keeps the rain out but allows air to pass through, the tubing allows gas to pass through into the partial vacuum and provides an effective means of solvent degassing. The earliest models had as much as 30 mL of tubing per solvent, which made for huge washout volumes when changing from one solvent to another. Later models had volumes of 10 mL or less. The most recent models have a different polymer in the tubing that allows washout volumes of less than 5 mL. Literature from the manufacturers shows that in-line degassers are as effective as helium

sparging in preventing bubble problems in LC systems.

Which system should you use? If you have an in-line degasser, by all means use it, but it could be hard to justify adding one to an existing system because they are expensive. In my laboratory, helium sparging is the degassing technique of choice because it is simple and effective. We simply sparge with a vigorous stream of helium for 2–3 min and then place the reservoir on the LC system. Our LC systems primarily are high-pressure mixing systems, and once-a-day sparging is sufficient for the equipment we use. If you filter all the solvents with a vacuum filter, vacuum degassing could be the easiest technique. Sometimes the filtration process removes sufficient gas for reliable system operation. If not, put a stopper in the vacuum flask and pump for another few minutes. If you set the flask in an ultrasonic cleaner during this process, it probably will be more efficient. Anything that creates nucleation sites for bubble formation will aid vacuum degassing, so I suspect that (gentle) use of a stir bar will be better than none.

As mentioned above, some LC systems are more tolerant of dissolved gas than others, so whereas vacuum degassing can be effective for one system, it might not work with another. Helium sparging is the standard to measure against, but most workers now prefer in-line degassers because of their convenience.

Conclusions

For some aspects of practical LC, hard-and-fast rules exist for determining settings or selecting conditions. In other cases, such as the two discussed above, analysts must determine the set point empirically. For gradient equilibration, the equilibration time should be increased until retention variation is within acceptable limits. Similarly, workers should use sufficient solvent degassing so that bubble problems are minimized. In both cases, the penalty for excessive treatment generally is just a few minutes added to the analysis time, whereas taking too many shortcuts can result in an unreliable method, or worse, a method that works one day and doesn't work the next. Add a safety margin to equilibration and degassing — you won't be sorry.

Acknowledgments

This month's column is the first column of my 20th year of writing for *LCGC.* Although my name appears as the author of the column, two others deserve much of the credit for the editorial quality of the column. If you look at the staff box at the front of the magazine, you'll see the names of Lisa McAdam and Stephen Brown. Year after year these two colleagues have provided the background support necessary for a successful publication. Lisa, as Managing Editor, takes the manuscript I submit, fixes my grammar and sentence structure errors, and makes sure the column is readable. Steve, the Group Technical Editor, checks the content of each column to make sure there aren't any technical mistakes. I have to be pretty clever to slip errors by these two, but now and then I succeed. Thanks, Lisa and Steve, for your continued support.

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