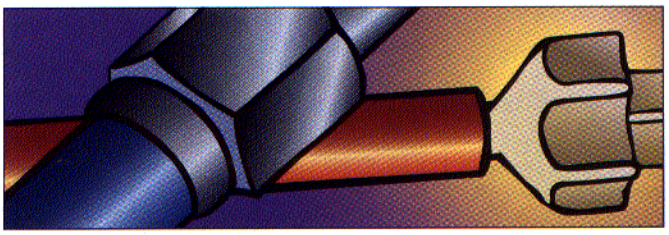
LC Troubleshooting



Solvent Changeover and Column Equilibration

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The intuitively simple process of switching mobile phases may be more complex than it first appears.

Changing from one solvent to another or equilibrating a column between runs seems like such a simple matter that it hardly merits any attention. It is true that both of these liquid chromatography (LC) tasks are fairly simple, but you must avoid some pitfalls to get reproducible results. As more laboratories rely upon chromatography simulation software to speed method development, stable column conditions are essential. The saying "garbage in, garbage out" certainly holds true when trying to predict LC separations based on unreliable input runs, so it is especially important that columns are equilibrated fully before gathering data for these applications. This month's "LC Troubleshooting" column covers some basic rules to obey if you want to get the best results out of your LC system.

For many workers, column equilibration consists of washing the column for a while without regard to important variables that can influence the equilibration process. Let's look at some of these variables and the roles they play.

HOW BIG IS THE COLUMN?

Although the time allowed for solvent changeover or equilibration is important, you should think of all solvent change processes in terms of *column volume*. Time is important, but the number of column volumes of solvent that pass through the column is the key parameter in column washout or reequilibration. The column volume, V_m (for the volume of mobile phase), can be calculated accurately, but an estimate is sufficient for our purposes.

The column merely is a piece of expensive pipe, and the volume is calculated as the familiar $\pi r^2 L$ (where r is the radius and L is the length). But this calculation provides the volume of the empty column — we need to account for the packing material in the column. Generally, the packing is a porous silica that occupies approximately 40% of the column volume. That is, when all the pores and spaces between the particles are accounted for, approximately 60% of the column is filled with solvent. This percentage varies somewhat between packings of different porosities, but 60% is a good practical estimate of column porosity for totally porous particles.

With these data, we can calculate the solvent volume in the column. As an example let's use a standard 15 cm × 0.46 cm column, so L = 15 cm and r = 0.23 cm. Our calculations give the column volume as $V_m = \pi \times (0.23\ cm)^2 \times 15\ cm \times 0.6 =$ 1.5 mL. This amount conveniently equals 10 times the column length, giving us the common rule for estimating column volume:

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

where the volume is in milliliters and the length is in centimeters. This estimate is useful for 0.46-cm i.d. columns, but you must make adjustments to estimate other column diameters. For other column diameters, the estimate is modified to

$$V_m \approx 0.5d^2L \qquad [2]$$

where d is the column diameter in centimeters. The derivation of equation 2 from equation 1 is straightforward.

EQUILIBRATION VOLUME

A common rule of thumb states that you must use 10 column volumes of solvent to equilibrate a column. For many cases this rule holds, and it is a good starting place. Using this rule makes it easy to determine how long to equilibrate the column, following equation 3:

$$t_E \approx 10V_m/F \qquad [3]$$

where t_E is the equilibration time in minutes, V_m is the column volume in milliliters, and F is the mobile-phase flow rate in milliliters per minute. So if you used a flow rate of 1.5 mL/min with a 15-cm column, equilibration would take roughly 10 min. This equilibration time is a good starting place, but only one method is effective to determine if the column is equilibrated: empirical experiments. Inject a standard two times: if the retention times are constant (within experimental error), the column is equilibrated; if not, more equilibration time is necessary. Some workers watch for stabilizing baselines or pressure as an indicator of equilibration, but this misleading practice will give false security and cause problems eventually.

SOLVENT TYPE IS IMPORTANT

The 10-column-volume rule works well for many common mobile phases such as mixtures of methanol and acetonitrile with water or buffers. Other solvents such as tetrahydrofuran can take more than twice as long to equilibrate. Viscous solvents such as isopropanol also equilibrate slowly. Equilibration times depend on the physical nature of the solvents (for example, viscosity) and the chemical interactions between the solvents and the column. Because equilibrium is related to diffusion, higher temperatures generally speed equilibration. This fact supports the argument for using a column oven and operating the column at temperatures slightly higher than ambient temperature (for example, 35–40 °C).

ION PAIRING — A SPECIAL CASE

As the mobile phase becomes more complex, column equilibration becomes more demanding. Systems that use ion-pairing mobile phases are some of the most difficult to equilibrate.

In addition to the solvent equilibration, the ion-pairing reagent also must equilibrate with the stationary phase, and this process is slow. The ion-pairing equilibration is very temperature sensitive, as are ion-pairing separations, so it is unwise to operate any ion-pairing system without column temperature control. Even so, you may need to use 40 column volumes or more of solvent to equilibrate such mobile-phase systems. For a 25-cm column you could use 1 L or more of mobile phase and at least 1 h of your time for equilibration.

A further complication of ion-pairing mobile phases is that the ion-pairing reagent may take even longer to wash out of the column

than it took to equilibrate. For this reason, it is best to dedicate a column to ion-pairing applications and never use it for standard reversed-phase applications after it has been used for ion-pairing applications.

An expedient way to help remove ion-pairing mobile phases is flushing with a mobile phase of 50:50 (v/v) methanol–100 mM potassium phosphate (pH 7) buffer. The combination of high salt, intermediate pH level, and methanol will help reduce the time it takes to wash the ion-pairing reagent from the column. Even with this technique, however, the column should be dedicated to ion-pairing applications.

As with other mobile phases, the best way to determine the equilibration requirements for ion-pairing mobile phases is to make a series of injections and continue the equilibration process until the retention times are constant. For more details about ion-pairing applications see last month's "LC Troubleshooting" column (1).

THE DIFFERENCE IS . . . THE DIFFERENCE

As intuition would tell us, equilibration time is influenced heavily by the difference between the starting and ending conditions. For example, we would expect it to require longer to reequilibrate to starting conditions for a gradient of 5–80% acetonitrile than for a 30–50% acetonitrile gradient. The adjustment in equilibration time should be roughly linear for different conditions. That is, if the shift from 80% acetonitrile back to 5% takes 10 column volumes, the 50% to 30% change-over should take about 3 column volumes ([50 − 30] ÷ [80 − 5]).

What about running a reversed gradient as compared with a step from the final conditions back to initial conditions? As long as the initial conditions have at least 5% organic solvent in the mobile phase, you should expect no difference between the techniques.

If you are impatient, however, the total equilibration time will be shorter if you step from the final mobile phase back to the initial one (or run a very steep reversed gradient). For example, if the system requires 10 column volumes of mobile phase at initial conditions, it will take longer to ramp back to the initial conditions and wait for the column to equilibrate than to step back and allow 10 column volumes to pass through the column. The only time a reversed gradient would be beneficial is when you are using some specialty columns that change physical characteristics (such as shrinking or swelling) when the mobile phase is changed. These special requirements will be stated clearly in the column data sheet that was shipped with the column.

AVOID THE WATER

We commonly hear about workers who routinely flush their reversed-phase columns with water to remove buffer or water-soluble interferences. This practice is misleading and probably does not accomplish what you desire.

To understand what happens, we need to take a microscopic look at the stationary-phase surface. The C18 bonded phase can be visualized as long strands bonded to the silica. When the surface is fully wetted, as is the case when organic solvent is present, these strands are extended, much like seaweed in the ocean. This extension occurs because the organic solvent does a good job of wetting the surface (like dissolves like). When 100% water (or buffer) is used, however, the bonded phase collapses, much like the seaweed when the tide is out. This collapsed bonded phase can trap solvent or sample molecules, and they won't be removed by normal washing with water. It requires the addition of organic solvent to allow the bonded phase to extend so that the mobile phase has access to the stationary phase.

Another problem is that the resolvation of the bonded phase can be very slow, so it is best to avoid conditions that cause the bonded phase to collapse. Bonded phase collapse occurs somewhere near 2% organic solvent, so we recommend that you use a minimum of 5% organic solvent for normal applications. Select mobile phases containing less than 5% organic solvent only for special cases.

If your goal is to wash buffers, salts, or other water-soluble materials out of a reversed-phase column, the best approach is to use an unbuffered mobile phase. For example, if the normal mobile phase is 45% methanol–buffer, switch to 45% methanol–water as a wash solvent. You only need a short wash (5–10 column volumes) to remove most of the buffer. Then switch to 100% organic (methanol in the present example) to remove any strongly retained material. Do not change from buffered mobile phase directly to 100% strong solvent because buffer precipitation is very likely to occur, especially if acetonitrile is present.

CONCLUSIONS

So what are the practical conclusions we can draw from this rather detailed discussion of column equilibration?

- Column volume is the standard of measurement.

We saw that it is the volume of solvent that passes through the column that is important, not the time. This observation implies that we can reduce equilibration time by increasing the flow rate, which is practical and expedient as long as the system pressure is reasonable. Keep system pressures lower than 2500 psi to avoid undue stress on system components.

- Column volume can be estimated easily.

Equation 1 is suitable for estimating the column volume of 4.6-mm i.d. columns within approximately 10% — good enough for most applications. If you are using other column diameters such as narrow-bore or preparative columns, use equation 2 to estimate the column volume. Alternatively, you can inject an unretained compound such as uracil under strong elution conditions and determine the volume by the retention time. Reference 2 lists column dead volumes for a large number of commercially available columns.

- Equilibration volume depends on the mobile-phase composition.

The 10-column-volume rule for column equilibration is a good place to start, but it may be overkill for some applications and insufficient for others. A better solution is to determine equilibration requirements by injecting at least two standards and checking for constant retention times. When more-complex or higher viscosity mobile phases are used, equilibration volumes will increase. Mobile phases containing tetrahydrofuran or ion-pairing reagents are two common systems that require extra solvent volume.

- Equilibration volume depends on the magnitude of the solvent change.

Whenever a solvent change is made, smaller changes in conditions will require less equilibration volume than larger changes. Also, a change from one mobile-phase solvent or buffer to another will take longer than a simple change in solvent strength, such as when you reequilibrate after a gradient run.

- Water should be avoided.

Flushing columns with 100% water generally is ineffective and actually may increase equilibration times. Instead use mobile phases with no less than 5% organic solvent.

So changing solvents or reequilibrating columns involves no magic. Just use common sense — and be sure to verify equilibration requirements by injecting two or more standards to ensure that retention times have stabilized.

REFERENCES

(1) J.W. Dolan, *LC•GC* **14**(9), 768–775 (1996).

(2) R.J. Steffeck, S.L. Woo, R.J. Weigand, and J.M. Anderson, *LC•GC* **13**(9), 720–726 (1995).

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