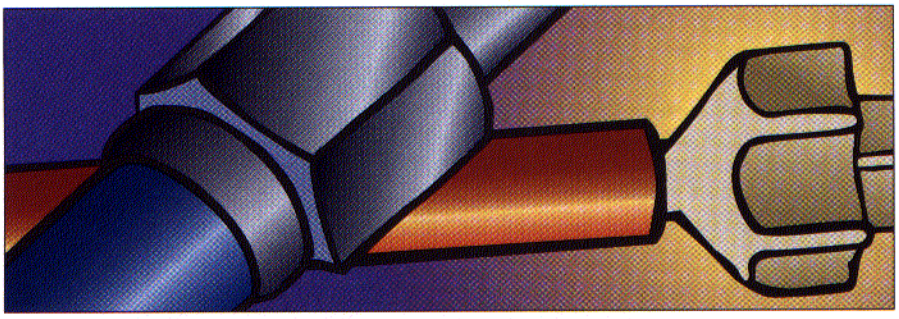
LC Troubleshooting



Lessons in Column Washing

Robert G. Wolcott and John W. Dolan

Like dissolves like . . . sometimes.

Most chromatographers wash liquid chromatography (LC) columns daily or when they need to change mobile phases to prepare a column for another application. Flushing is a common practice before columns are placed in short- or long-term storage. Strongly retained sample components can be removed from a column by washing with a strong solvent. The seemingly simple practice of washing a column, however, can create unintended results. This month's "LC Troubleshooting" column will discuss the column washing process and describe some pitfalls to avoid.

JUST WASH IT

The surface of a reversed-phase column's packing often is described as a brush phase. Figure 1a depicts the column medium's surface. The bonded phase (for example, C18 chains) is shown as bristle-like attachments to the silica stationary-phase support. When the surface is configured this way, the mobile-phase molecules (M) have ready access to the spaces between the bonded-phase chains. Because of the relatively open nature of the bonded phase, sample molecules can diffuse between the bonded-phase chains and undergo the interactions necessary for retention.

Sooner or later chromatographers must flush the column for one of the reasons listed above. If you are switching to another mobile phase or washing strongly retained materials from the column with a strong (high percent organic) solvent, the expected process takes place. Figure 1b illustrates this procedure. The new mobile phase or wash solvent (W) simply replaces the old mobile phase. If the solvent is stronger than the previous mobile phase, this process shifts the equilibrium of sample molecules toward the mobile phase, speeding their removal from the column.

OOPS

It is common knowledge that chromatographers should not try to wash a buffered mobile phase from the column with 100% organic solvent. For example, a mobile phase of 40:60 acetonitrile–25 mM phosphate buffer should not be exchanged directly for 100% acetonitrile because buffer precipitation can occur. This phenomenon is especially problematic with acetonitrile mobile phases because of the poor solubility of buffer salts in this solvent.

However, the use of too little organic solvent in the wash solvent also can cause problems. Following the rule taught in high school chemistry, "like dissolves like," it may be tempting to wash the buffer from the column with water. After all, buffer salts are highly soluble in water, so water should remove them more quickly than water–organic mixtures. Well, yes . . . and no. Water would be very effective at removing buffers and salts from the column if it weren't for the chemical nature of the column. Remember that the C18 bonded phase is hydrophobic — very nonpolar. So when a highly polar mobile phase like water is introduced, the bonded phase orients itself to minimize its exposure to the polar solvent.

Figure 1c illustrates the resulting bonded-phase collapse. The bonded phase collapses because the nonpolar phase achieves a lower energy condition more closely associated with other nonpolar molecules than with the bulk solvent. Now, rather than representing a brush, the stationary phase looks more like a shag carpet. This process is further complicated by the fact that the collapsed bonded phase traps molecules of the previous mobile phase as well as residual sample molecules and, of course, some wash solvent molecules. From a practical standpoint, these molecules are stuck in the bonded phase and not removed, even through extended water washes.

DOUBLE OOPS

This situation might not be so bad if everything returned to normal as soon as the col-

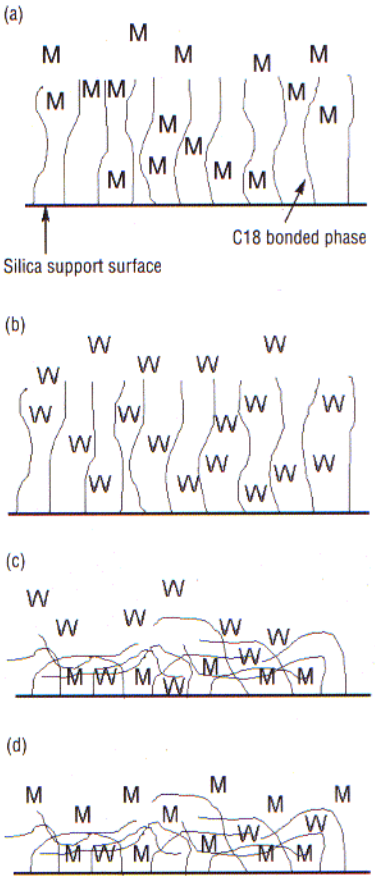


FIGURE 1: Diagrams showing bonded-phase orientations, including (a) extended or brush configuration when solvated with mobile phase (M), (b) brush configuration after successful replacement of mobile phase by wash solvent (W), (c) collapsed bonded phase after washing with too weak a solvent, and (d) collapsed bonded phase after unsuccessful resolvation with new mobile phase. See text for details.

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LC Troubleshooting Editor

umn was flushed with another mobile phase. A problem arises from the fact that after the bonded phase collapses, it requires a lot of energy to resolvate it. In other words, the bonded phase tends to stay collapsed even after the column is flushed with fresh mobile phase. Figure 1d illustrates this problem, making it clear that the appearance of the surface to a sample molecule would be much different than the surface shown in Figure 1a. The collapse and subsequent resolvation cause not only an apparent surface area change, which would result in shorter retention times, but also a chemical change because of the trapped molecules of the previous mobile phase and wash solvent.

Over time, continued washing with the new mobile phase will resolvate the bonded phase and normal bonded-phase behavior should return. Analysts would observe a gradual change in retention and selectivity as this resolvation occurred. However, restoration of normal conditions may require hundreds of column volumes of flushing.

FOR EXAMPLE

How does this phase collapse affect a separation? The separations of Figure 2 illustrate the practical impact of this phenomenon. In each case, a 150 mm × 4.6 mm, 5-µm d_p Zorbax Stablebond SB-C18 column (Mac-Mod Analytical, Chadds Ford, Pennsylvania) was operated at 75 °C with a mobile phase of 10:90 acetonitrile–50 mM phosphate buffer (pH 2.6) at a flow rate of 2 mL/min. Normally the separation looked like the chromatogram shown in Figure 2c, with the last peak eluted at approximately 9.4 min. The half-height width of the nitrobutane peak at 9.42 min was 0.196 min, resulting in a plate number (N) of approximately 12,800. Note that the second and third peaks, nitroethane and phthalic acid, are barely baseline separated.

The column was flushed with water and stored overnight at room temperature. The next day, we turned on the column oven again, flushed the column for 20 min with mobile phase, and then performed a series of injections of the sample. Figure 2b shows the sixth injection. Three dramatic differences are apparent when comparing Figures 2b and 2c. First, the retention time of the last peak is reduced approximately threefold following the water wash (9.42 min versus 2.93 min). Second, the early part of the chromatogram in Figure 2b shows a selectivity change and the poor peak resolution. Finally, the plate number before the last peak (half-height width of 0.079 min) has dropped to approximately 7600. An analyst might think that the column was not washed sufficiently to reequilibrate it, but more than 90 column volumes of mobile phase had passed through the column before the chromatogram of Figure 2b was obtained.

Further flushing might help. Figure 2a shows a chromatogram obtained after more extensive mobile-phase flushing, approximately 170 column volumes in all. We can see that the column is returning to normal, but it is not there yet. The retention time has improved, but it remains 30% lower than the expected value. The nitroethane–phthalic acid peak pair is baseline separated again. The plate number is approximately 11,500 — almost back to normal.

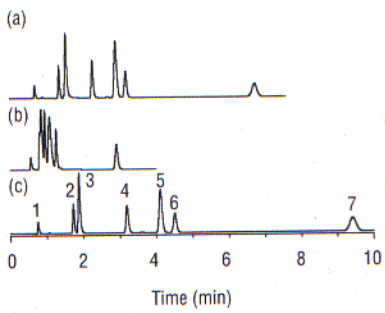

FIGURE 2: Chromatograms illustrating the practical impact of bonded-phase collapse. Shown are (a) a chromatogram generated after a water wash and 170 column volumes of additional mobile-phase flushing, (b) a chromatogram generated after a water wash and 90 column volumes of additional mobile-phase flushing, and (c) a normal chromatogram. See text for discussion. Peaks: 1 = uracil, 2 = nitroethane, 3 = phthalic acid, 4 = 4-chloroaniline, 5 = 3-cyanobenzoic acid, 6 = 3,5-dimethylaniline, 7 = 1-nitrobutane.

How much flushing is required to return the column to normal behavior? Impatience prevailed, so the mobile phase was replaced with 50:50 acetonitrile–water. After flushing with 40 mL of this mixture (approximately 25 column volumes at 75 °C), we reintroduced the 10% acetonitrile–buffer mobile phase. Within five runs, we obtained a normal-appearing chromatogram that matched Figure 2c.

This example shows the practical effect of the phase collapse illustrated in Figure 1. The chromatogram under normal conditions (Figure 2c) corresponds to the diagram of Figure 1a showing a properly equilibrated column. When the column was washed with water, the bonded phase collapsed (Figure 1c). Subsequent flushing with mobile phase removed the wash solvent, but the stationary phase remained in the collapsed configuration (Figure 1d), resulting in a change in retention and selectivity (Figures 2a and 2b). Extensive washing with mobile phase was insufficient to restore the bonded phase to the fully extended configuration — it required a much stronger (higher percentage organic) solvent to restore the column to normal performance (Figures 1a and 2c). This example used a column operated at 75 °C; a column operated at room temperature would take longer to resolvate the bonded phase.

GUIDELINES FOR COLUMN FLUSHING AND EQUILIBRATION

1. Avoid 100% water.
2. Initial flush with 5–10 column volumes of unbuffered mobile phase.
3. Secondary flush with 10–20 volumes of strong solvent (organic).
4. Store in strong solvent.
5. Equilibrate in 10–20 volumes of mobile phase.
6. Use constant retention to check for equilibration.

TAKE-HOME LESSON

What did we learn from this example that will help avoid future problems? Some guidelines are summarized in the accompanying sidebar. First, washing a reversed-phase column with water is not an effective way to flush the column. Bonded-phase collapse occurs for most columns at less than 2–3% organic solvent. As a practical rule, chromatographers should not use less than 5% organic solvent in the mobile phase unless a compelling reason exists. This guideline means that gradient elution runs should start at 5% B, not 0% B.

We know that buffer precipitation can occur if a buffered solvent is replaced directly by 100% organic solvent. For this reason, analysts should perform a short flush with unbuffered mobile phase. The easiest way to perform this task with on-line mixing is to replace the buffer reservoir with water and flush the column with the same ratio of organic solvent. For example, 10:90 acetonitrile–buffer would be replaced with 10:90 acetonitrile–water. The exact mixture is not important, but the direct replacement of buffer by water in the mobile phase is the safest approach. It takes only a few column volumes of organic solvent–water to flush the buffer from the system. (Analysts can estimate the column volume for 4.6-mm i.d. columns as 0.1× length in centimeters, so the volume of a 15-cm column is approximately 1.5 mL.)

After the buffer is removed from the system, washing with 100% organic solvent usually is the best choice. This procedure will quickly remove strongly retained materials and makes a satisfactory storage solvent as well. Generally, 10–20 column volumes of strong solvent are sufficient to flush a column. Knowledge of a sample's chemistry will help analysts decide on the best flushing conditions. For example, a change in mobile-phase pH may be more effective than 100% organic solvent at removing some ionic sample components.

Chromatographers can store reversed-phase columns in 100% organic solvent. For specialty columns, consult the manufacturer's literature for the best storage conditions. When the column is returned to use, typically 10–20 column volumes of mobile phase are required for reequilibration. Remember that the volume, not the equilibration time, is the critical factor. It may take more or less flushing to equilibrate the column, depending on the application and the difference in organic content between the storage solvent and the mobile phase. The column is fully equilibrated when successive injections yield the same results (retention, selectivity, and peak width).

AND NOW A WORD FROM OUR SPONSOR

Our discussion applies to the C8 and C18 silica-based reversed-phase columns that are the bread-and-butter columns for most chromatographers. Most analyses in reversed-phase mode are performed with mobile phases containing more than 5% organic solvent, so concerns about phase collapse are minimal for most applications. However, sometimes chromatographers need to use even weaker mobile phases to obtain sufficient retention. Conventional columns can be used with 2–3% organic solvent without experiencing phase collapse, but, for safety's sake, analysts should stay at 5% organic solvent or more. For applications requiring less than 5% organic solvent, consider one of the columns designed for low-organic solvent use. These columns are available from a number of manufacturers. Sometimes the column name includes "AQ" or "aqueous" to indicate that it can be used in the absence of organic solvent. Consult your favorite column supplier for more information.

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