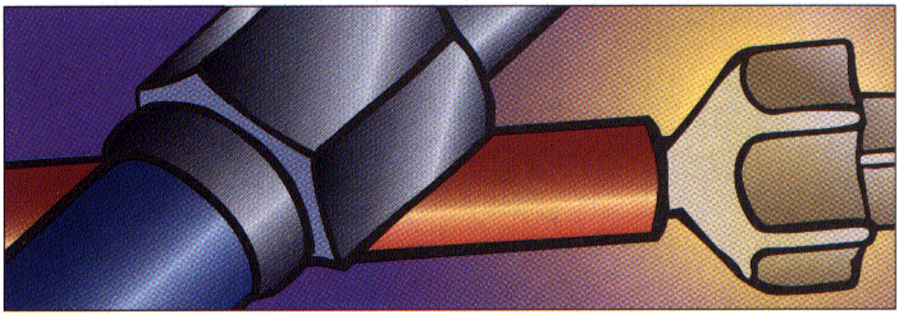
LC Troubleshooting



Column Temperature Effects in Gradient Elution

Robert G. Wolcott and John W. Dolan

There may be more to temperature control than meets the eye.

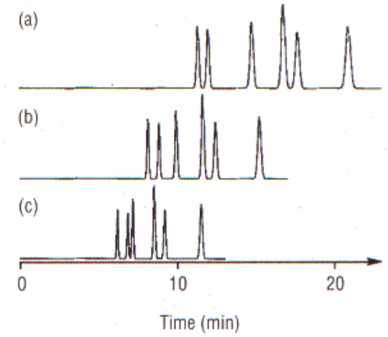


FIGURE 1: Simulated chromatograms based on isocratic LC separation of an adulterated vanilla sample at (a) 25 °C, (b) 35 °C, and (c) 45 °C. Column: 250 mm × 4.6 mm C8; mobile phase: 50:50 (v/v) acetonitrile–water; flow rate: 1 mL/min.

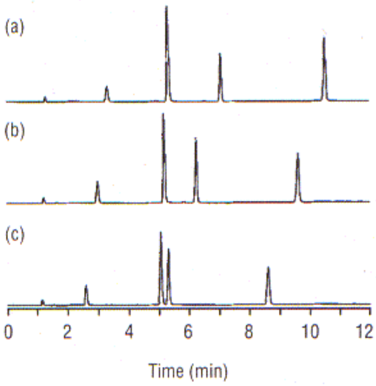


FIGURE 2: Gradient elution separation of substituted benzoic acids and anilines at (a) 38 °C, (b) 57 °C, and (c) 77 °C. Column: 150 mm × 4.6 mm, 5-μm d_p Zorbax SB-C18 (Mac Mod Analytical, Chadds Ford, Pennsylvania); mobile phase A: 50 mM potassium phosphate (pH 2.6); mobile phase B: acetonitrile; gradient: 5–65% B in 13 min.

Many, if not most, chromatographers operate liquid chromatography (LC) systems with the column at ambient temperature. If the laboratory temperature is constant, this situation will cause few problems. However, most of us work in environments with fluctuating temperatures. A further complication can arise if an LC method is developed in one laboratory and performed at another location. For example, the method that is developed in a laboratory in New Jersey and performed in a production facility in Puerto Rico may yield significantly different chromatograms because the temperature may differ by 10 °C or more. Although temperature affects all modes of chromatography, this month's "LC Troubleshooting" column discusses the impact of temperature on gradient elution LC.

ISOCRATIC RETENTION

Most chromatographers know that temperature influences retention for isocratic separations. Figure 1 shows results from runs performed at three temperatures. We see that all the peaks are eluted earlier as the temperature decreases. One common rule of thumb for isocratic separations is that retention drops by 1–3% for each degree Celsius increase in temperature (1). For the sample of Figure 1, the retention varies by approximately 2%/°C.

Even climate-controlled laboratories often have day–night thermostat settings, which may result in significant differences in ambient temperature during an overnight run. For example, our laboratory's thermostat is set to allow a rise of approximately 4 °C or a drop of approximately 7 °C during the nighttime cycle. Depending on the season, the nighttime laboratory temperature might be warmer or cooler than the normal workday temperature. These temperature changes could cause a peak to move out of an acceptable retention window and invalidate data gathered from the series of samples analyzed under those conditions.

Another potential source of local temperature variation depends on the location of the LC instrument within the laboratory. If the column is located in the path of a heating–cooling vent, the local temperature may vary by several degrees even though the overall laboratory temperature is quite steady. This potential for retention time variation with temperature changes is one reason we use column ovens with all our LC systems.

GRADIENT RETENTION

Temperature affects retention in gradient elution as well as in isocratic separation. Figure 2 shows chromatograms for a sample of substituted anilines and benzoic acids analyzed at three temperatures. The variation in retention for temperature changes is much less dramatic than for the data of Figure 1. In Figure 1, a 20 °C change in temperature changed the retention by a factor of approximately two, whereas in Figure 2, a 40 °C difference changes retention by approximately 20%. On average, the peaks in Figure 2 show approximately a 0.5%/°C change in retention. So it appears that gradient retention is less sensitive to temperature changes than isocratic retention (assuming that this example is representative of gradient separations in general). Even so, unless the column temperature is controlled in gradient elution, noticeable changes in retention may be common.

SELECTIVITY CHANGES

Inspection of the chromatograms in both Figures 1 and 2 reveals significant changes in selectivity when temperature varies. In Figure 1, the second peak is closest to the first peak at 25 °C, but as the temperature increases, it moves away from the first peak and closer to the third peak. Of the three conditions shown, the best separation of the first three peaks occurs at 35 °C. It is interesting to note that changes in selectivity are compound dependent. For example, the peak spacing of the last three peaks in Figure 1 changes very little for the temperatures shown.

In the gradient elution example of Figure 2, the selectivity changes as well. The relative spacing of peaks 1, 2, 4, and 5 does not appear to change significantly. However, peak 3 moves closer to peak 4 as the temperature increases.

The change in relative peak positions with changes in temperature is systematic. After a pattern is determined, it is very predictable. For example, if the temperature were increased to higher than 77 °C for the sample of Figure 2, peaks 3 and 4 would merge and at an even higher temperature, peak 3 would be eluted after peak 4. This change in selectivity with temperature is a fact that was unappreciated for many years. More recently, Snyder and colleagues have shown that analysts can use temperature as a powerful tool to control selectivity (2). Thus, the best selectivity (largest band spacing between peaks) is obtained at 35 °C for the sample of Figure 1 and at 38 °C for the sample of Figure 2 for the chromatograms shown. (The fact that both examples have similar temperature optima is coincidental; see reference 2 for other examples.)

CONTROLLING TEMPERATURE

From a practical standpoint, the data of Figures 1 and 2 should be sufficient to convince us that the temperature of the LC column needs to be controlled for consistent results. The use of column ovens is a preferred way to control column temperature. Two types of commercial column ovens are available: block heaters and air-bath ovens. Each design has strong and weak points, and often users are restricted to whichever design comes as part of a particular LC system. With block heaters, the column is clamped against a metal heating block or wrapped with a heating tape. Air-bath ovens are much like gas chromatography ovens, in which the column is suspended in a static or circulating air bath. A third design, immersion of the column in a liquid, such as a water bath, also is effective and easy to build from laboratory equipment, but generally this design is less convenient than the other oven designs.

One common rule of thumb for isocratic separations is that retention drops by 1–3% for each 1 °C increase in temperature.

If a column oven is unavailable, an inexpensive alternative is to insulate the column to minimize temperature fluctuations. For example, the column could be operated inside a foam sleeve or the column's shipping box. This technique works best when the laboratory temperature is fairly constant. Methods that rely on column insulation must allow for some variation in retention. Insulation also helps protect the column from local temperature fluctuations resulting from heating–cooling vents.

TEMPERATURE EQUILIBRATION

Chromatographers familiar with gradient elution know that reequilibration of the column following a gradient run is critical for reproducible separations. A rule of thumb is that 10–15 column volumes of mobile phase must pass through the column to achieve equilibration when a full-range gradient is used. For example, if an analyst uses a 5–100% B solvent gradient with a 150 mm × 4.6 mm column (~1.5 mL volume), column reequilibration will take approximately 10 min at 1.5 mL/min when the mobile phase changes from 100% B solvent back to 5% B before the next run. If the mobile phase is improperly equilibrated before the gradient, the retention time reproducibility will be poor.

Just as a column improperly equilibrated with the mobile phase provides poor retention-time reproducibility, a poorly temperature-equilibrated column will yield less than optimal results. This problem is particularly obvious when observing peak widths. In addition to changes in retention and selectivity, the peak width also changes when temperature changes. Increased temperatures generally result in higher column plate numbers (N) and, thus, narrower peaks. Because peak area is conserved, narrower peaks will be taller, so a temperature increase should yield better limits of detection. (The chromatograms in the figures shown in this article are not plotted on the same *y*-axis scale, so peak-height differences have no significance here.)

It is obvious that insufficient flushing of a column with equilibration solvent can leave it in a chemically unequilibrated state. But how can we have thermal equilibration problems if the column is operated in a constant-temperature environment? The answer lies in the supply of mobile phase to the column. If the column is operated at ambient temperature, the solvent reservoirs, pumps, and autosampler also are at ambient temperature, so the temperature throughout the system should be constant. However, if the column is heated and the system components and solvents are at ambient temperature, the inlet of the column will be cooler than the outlet. This temperature gradient along the column will be detrimental to peak shape.

Figure 3 shows chromatograms obtained with the column temperature set to 38 °C with a block heater and the incoming solvent at room temperature (~22 °C). In the lower trace, distortion of the last peak is obvious. It generally is impractical to heat the reservoirs, pumps, and autosampler to the same temperature as the column, so a solvent preheater is the best choice. In the present case, we used a 1-m piece of 0.25-mm i.d. stainless steel tubing as a preheater. The tubing was arranged in a flat coil approximately 10 cm in diameter and clamped against the heating block in the column oven. We plumbed the flow path so that the preheater was located between the autosampler and the column. Thus, the cool solvent from the autosampler passed through the preheater coil and increased in temperature before it reached the column. The upper chromatogram in Figure 3 shows the improved peak shapes we obtained by adding the preheater. Some commercial column ovens include preheaters, so check your column oven to see if it has one.

Peak distortion resulting from poor temperature equilibration increases as the temperature difference between the incoming mobile phase and the column increases. This problem is shown quite dramatically by comparing the lower traces of Figures 3–5. At 77 °C the later

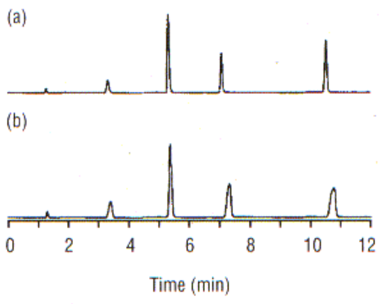


FIGURE 3: Separation of the substituted benzoic acid and aniline sample from Figure 2 at 38 °C (a) with and (b) without a preheater. Other conditions were the same as in Figure 2. See text for details.

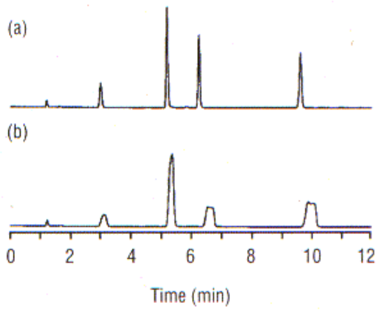


FIGURE 4: Separation of the substituted benzoic acid and aniline sample from Figure 2 at 57 °C (a) with and (b) without a preheater. Other conditions were the same as in Figure 2. See text for details.

peaks are so distorted that one might think that two components were present or that the column had a void in it. The poor peak shape collapses to a series of well-behaved peaks when the preheater coil is added in each case. Although we can't see it here because of differences in scaling, the peak areas for a given sample component in all the runs of Figures 3–5 are constant.

Why do we see such peak distortion? Peak broadening is easy to explain. The front edge of the band is at a higher temperature than the trailing edge, so it travels faster. When the front edge travels more quickly than the trailing edge, a broader peak will result. This phenomenon is the opposite of the band compression that is normal with gradients in which the leading edge is in a weaker solvent and travels slower than the trailing edge, which experiences a stronger solvent. The reason for the peak distortion is not as obvious, although the use of a preheater to correct peak distortion is a well-known solution (for example, see reference 3). In normal gradient behavior, each peak experiences the same environment and travels through the column at approximately the same velocity, resulting in similar peak widths characteristic of gradient elution. However, we were surprised that the peak distortion problems were more pronounced for later-eluted peaks than early peaks, as shown in Figure 4. Perhaps a reader has a simple explanation for this phenomenon.

Gradient retention is less sensitive to temperature changes than isocratic retention.

CONCLUSIONS

We have seen that column temperature can play an important role in gradient elution LC. First, we showed that increasing column temperature results in shorter retention times. We also saw that temperature can affect selectivity. Finally, we saw that poor temperature equilibration can result in severe peak distortion. These observations support the importance of operating the LC column in a temperature-controlled environment if you want reproducible and reliable separations.

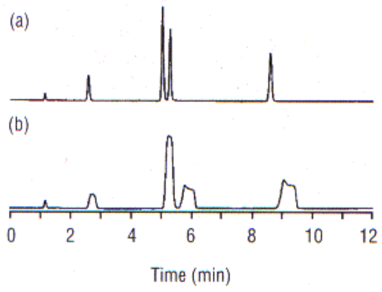


FIGURE 5: Separation of the substituted benzoic acid and aniline sample from Figure 2 at 77 °C (a) with and (b) without a preheater. Other conditions were the same as in Figure 2. See text for details.

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