LC Troubleshooting

The Importance of Temperature

Column temperature can be a powerful tool to control liquid chromatography separations, but it can create problems, as well.

Column temperature is an important variable to control in liquid chromatography (LC) separations. Many LC methods specify ambient temperature, which means that the column temperature need not be controlled. For simple separations, those conditions can be adequate, but if a separation is at all demanding, relying upon ambient temperature can be a problem waiting to happen. I have worked in several laboratories where the temperature varied by 5 °C or more during the course of a day — and often more during a 24-h period. In many production facilities, the temperature can vary much more than 5 °C as the seasons change, so production-control LC methods can be especially problematic.

This month's "LC Troubleshooting" focuses on several aspects of column temperature. Temperature can affect retention, selectivity, and peak shape as well as column pressure and other less important variables.

Retention Time

In gas chromatography separations, temperature is a primary variable used to control the separation, and it acts in a similar capacity as mobile-phase strength in LC. Most workers are aware that the column temperature can affect the retention time of sample components in an LC separation, too. Figure 1 shows how column temperature can affect the retention time of a set of seven test compounds. When the column temperature was changed from 54 °C to 75 °C, the retention time of the last peak dropped from approximately 13.5 min to roughly 10 min, a 26% decrease. This general observation of smaller retention times for increased temperatures can be summa-

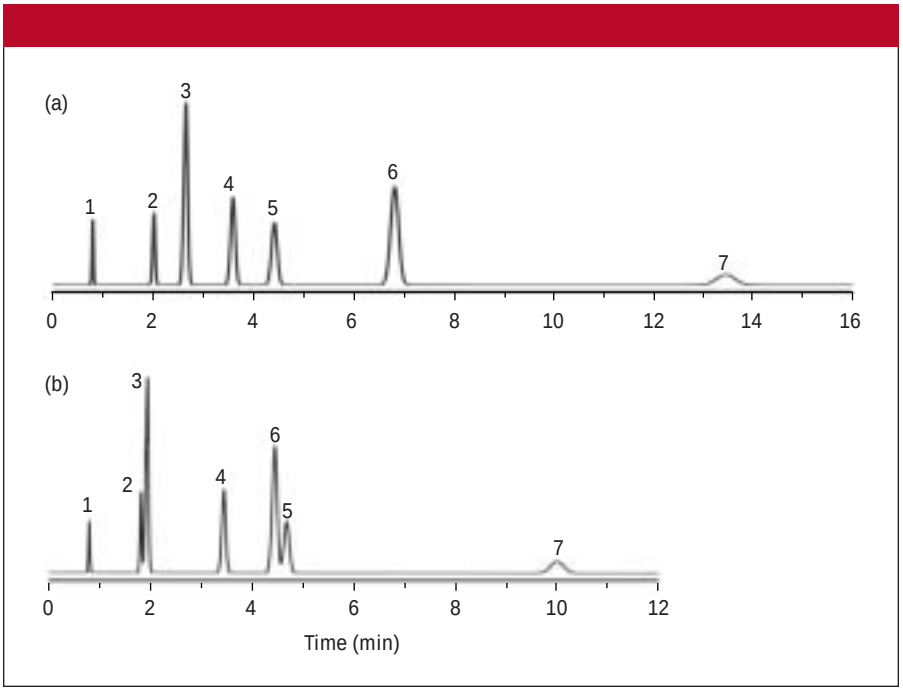

Figure 1: Separations obtained at (a) 54 °C and (b) 75 °C column temperatures. Column: 150 mm × 4.6 mm, 5 μm d_p C18; mobile phase: 90:10 (v/v) water–acetonitrile. Peaks: 1 = uracil, 2 = nitroethane, 3 = phthalic acid, 4 = 3,5-dimethylaniline, 5 = 4-chlorobutane, 6 = 3-cyanobenzoic acid, 7 = 1-nitrobutane.

John W. Dolan
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rized in a general rule that states that retention will change by 1–3% per 1 °C. For the present example, the change is approximately 1%/°C for the last peak.

Certainly, you won't commonly see a 21 °C change in temperature, as in Figure 1. What is the practical effect of temperature upon retention? Consider a method with a 15-min retention time in a laboratory with a 25 °C temperature. What happens when the temperature drops to 21 °C overnight? For a 4 °C change in temperature and a 2%/°C change rate, the new retention time would be approximately 16.2 min. If a data system had been programmed with a ±1.0-min retention time window for the peak, the peak would have drifted out of the window and would have been missed. If your method uses ambient column temperature, and you have any variation in the laboratory temperature, be sure to set a broad enough window on your data system for proper peak recognition.

Selectivity

In addition to the retention-time changes, Figure 1 also illustrates a change in selectivity. The most dramatic change is the reversal of peaks 5 and 6 when the column temperature is increased from 54 °C to 75 °C. A significant loss in separation also occurs between peaks 2 and 3 when the temperature is increased. Unfortunately, I have no simple rule for changes in selectivity when the temperature changes. Some peak pairs can be unaffected, and others can reverse. These sometimes dramatic changes in selectivity with temperature adjustments can be a powerful tool to control a separation, so temperature commonly is used in my laboratory to help obtain separations of complex samples. However, the reverse is true as well — if temperature is uncontrolled, you can lose a carefully developed separation.

Peak Shape

The changes in retention and selectivity discussed above should be great enough to convince you that column temperature should be controlled. Temperature control can be more difficult than it sounds. Consider the effect of temperature upon peak shape. Figure 2a illustrates the passage of a peak through a column in a fully equilibrated system. The peak enters as a narrow band, gradually gets broader as it moves through the column, and is eluted with a Gaussian-shaped peak in the ideal situation. This peak is what you're used to seeing with columns operated at ambient temperatures or in well-controlled systems.

Next consider the situation shown in Figure 2b. In this case, the column oven is set to a fixed temperature (70 °C), but the analyst made no effort to preheat the solvent before it entered the column. The cooler, incoming solvent cooled the inlet end of the column. Heat from the column walls heated the solvent as it passed through the column, but the effect was a temperature gradient from the column walls (warm) to the interior of the column (colder).

When a sample is injected, the molecules closest to the wall travel more quickly through a column than those in the cooler center of the column. This effect results in a band profile such as the one shown in Figure 2b. The net result is a broader peak at the column outlet than it would have been if the temperature was uniform throughout the column. The peak shape can become distorted significantly if the thermal transfer in the column is uneven.

Combined Effects

So, temperature can influence retention time, selectivity, and peak shape. Next, I'll look more at these three characteristics with elevated-temperature separations. In this next example, my co-workers and I placed a column in an air-bath column heater in which hot air was blown over the column to provide heating.

We used a custom-built mobile-phase heater (Rheodyne, Rohnert Park, California) that allowed us to control the temperature of the mobile phase entering the column. In addition, we used in-line thermocouples placed at various points in the system to measure the mobile-phase temperature. The example of Figure 3a shows a well-behaved separation in which the temperature throughout the column was 56 °C. This temperature was achieved by setting the oven temperature to 56 °C and preheating the mobile phase to 56 °C before it entered the column. The peaks are narrow and well shaped.

Many analysts place columns in column ovens, but make no special provisions for preheating the mobile phase. This practice can have undesirable results, as Figure 3b shows. In this case, the incoming solvent was heated to 39 °C by its passage through the transfer tubing within the oven. The situation of Figure 2b exists here, where cooler mobile phase enters a warmer column. The chromatogram deteriorates in three ways: First, the average temperature of the column is lower than the set temperature, so the retention times of all the peaks are greater than those in Figure 3a. Second, the selectivity has changed, much as it did in Figure 1. The evenly spaced peaks 3, 4, and 5 of Figure 3a have changed so peaks 3 and 4 are closer together than 4 and 5. Third, the nonequilibrium conditions similar to those in Figure 2b exist in Figure 3b. The distortion of the peaks is most dramatic for peaks 6 and 7.

You might ask what would happen if the average column temperature was increased so the cooler incoming solvent was balanced against a higher column temperature. Figure 3c shows this situation. In this case, my colleagues and I used no solvent preheating (other than passage through the transfer tubing), and we increased the column temperature until the retention time of the last peak was the same as that in

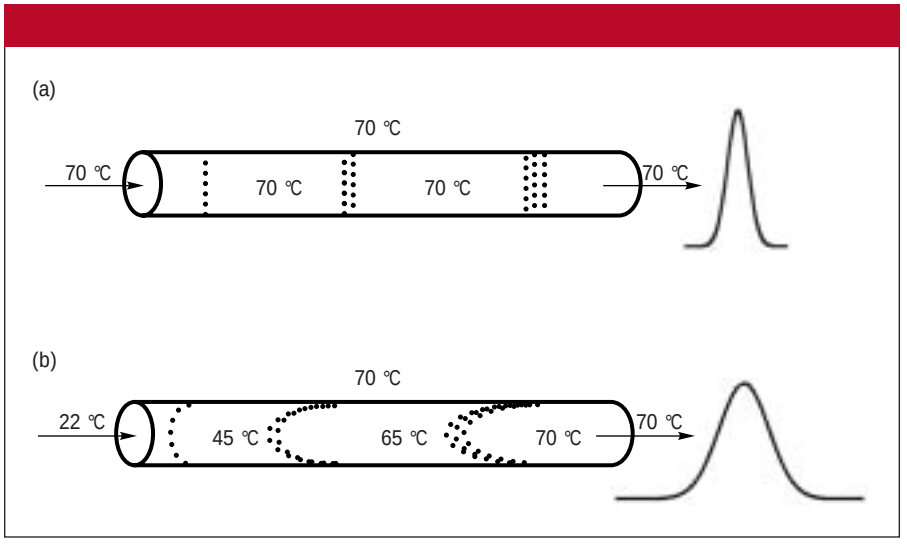

Figure 2: Illustrations showing the effect of thermal equilibration in an LC column. Shown are (a) a fully equilibrated column and (b) a column with cold incoming solvent.

Figure 3a. Under these conditions, the average column temperature was the same in Figures 3a and 3c, so the retention time for all the peaks was the same in the two runs. However, the temperature gradient along the column was even worse than in Figure 3b, so peak distortion was unimproved.

The runs of Figure 3 teach two very clear lessons: First, the *average* column temperature is most important in terms of retention time. Second, the *uniformity* of column temperature is most important in terms of peak shape. To obtain the best results, workers must control both of these factors.

Preheating the Mobile Phase

The above discussion makes it clear that the incoming mobile phase must be heated to avoid problems of peak distortion and retention-time changes. From a practical standpoint, if a solvent at a column inlet is within approximately 5 °C of the column temperature, the system will function quite well for most applications. With the air-bath oven, my co-workers and I were able to preheat the solvent by placing a coil of tubing in the heated air stream between the injector and the column. The efficiency of this preheater coil depends upon three factors: the difference in temperature between the column and the ambient conditions, the length of the preheater coil, and the flow rate.

Table I shows preheater coil lengths for various combinations of column temperature and flow rate. For example, if the room temperature is 25 °C and the column temperature is 55 °C ($\Delta T = 30$), a flow rate of 1 mL/min would require approximately 30 cm of preheater tubing. The tubing should be stainless steel with $^1/_{16}$-in. outer diameter and 0.005–0.007 in. inner diameter to minimize band spreading.

Be sure to place the preheater tubing *between* the injector and the column. It might be tempting to place the preheater in front of the injector to minimize extra-column bandbroadening, but the mass of the injection valve usually will strip any

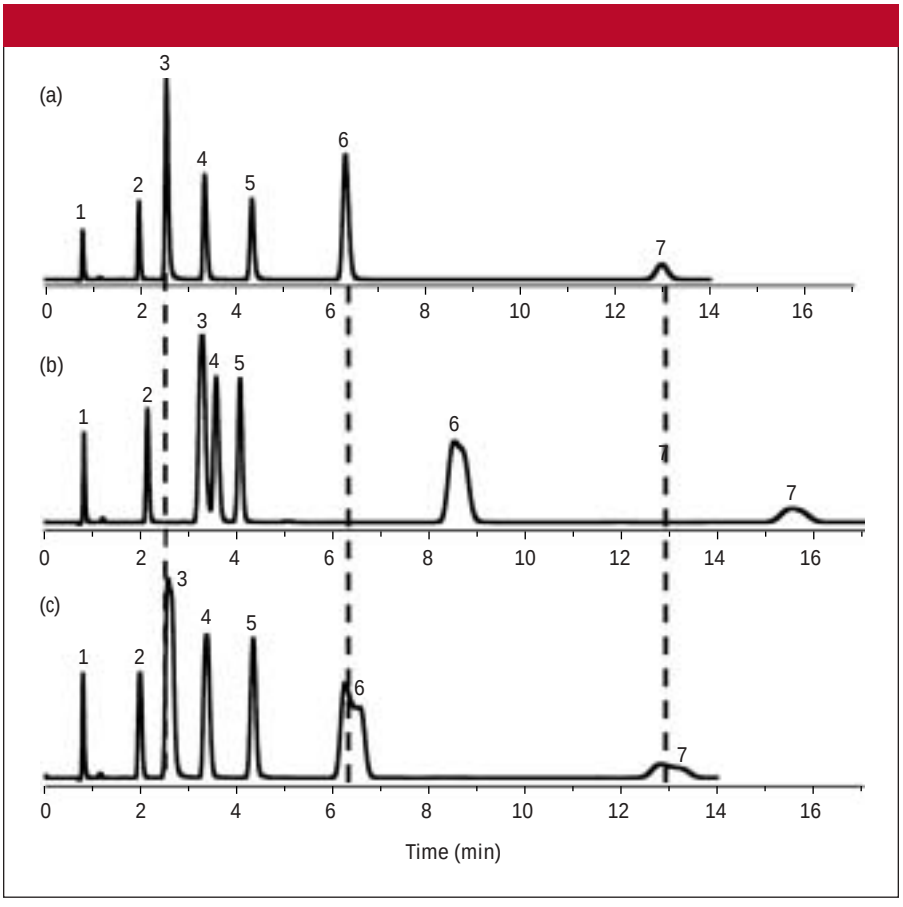

Figure 3: Separations showing the effect of incoming solvent and column oven temperature on retention and peak shape. (a) Inlet solvent 56 °C, column oven 56 °C; (b) inlet solvent 39 °C, column oven 56 °C; (c) inlet solvent 51 °C, column oven 77 °C. Other conditions were the same as in Figure 1. Peaks: 1 = uracil, 2 = nitroethane, 3 = phthalic acid, 4 = 3,5-dimethylaniline, 5 = 4-chlorobutane, 6 = 3-cyanobenzoic acid, 7 = 1-nitrobutane.

heat gain from the solvent and cool it to room temperature.

Table I also illustrates why chromatographers generally do not observe peak distortion when columns are heated to just above room temperature. For example, many workers operate their columns at 35 °C ($\Delta T \approx 10$) to provide better temperature control than they can achieve at ambient temperatures. In this situation, Table I indicates that 10 cm of preheater tubing would be needed. This length of tubing probably already would be used to connect the column under normal circumstances, so the preheater tubing is unintentionally installed. The problem escalates as the column oven temperature or the flow rate increases.

Other Ovens

The above discussion was based upon using an air-bath oven. Three other column oven configurations are common; the most common are block and Peltier heaters. Some workers use water baths for column heaters. Although water baths are very efficient, they are messy and make leak detection difficult. When using a block or Peltier heater, you can shorten the preheater tube lengths listed in Table I to 20–50% of those lengths. For temperatures to roughly 100 °C and flow rates as high as 2 mL/min, 25–50 cm of preheater tubing should be sufficient.

When using a block heater oven, I like to make a flat coil of the preheater tubing and sandwich it between two multiple-fold layers of aluminum foil. Then I like to clamp this sandwich against the heating block to ensure good heat transfer from the block to the coil.

Peltier-heated ovens can require extra care. Many oven designs include one or more pieces of stainless steel tubing embedded in an aluminum block that forms the heat transfer source for the Peltier heater. These tubes usually are sufficient to use as preheaters. For best performance, be sure that the column makes good contact with this block as well. One brand of heater in my laboratory uses fuse clips to hold the column. This setup is handy, but the contact with the heating surface is minimal, so the primary source of column heat is through the heated solvent.

Table I: Recommended preheater tubing*

	Tube Length (cm)		
ΔT (°C)	1-mL/min Flow Rate	2-mL/min Flow Rate	4-mL/min Flow Rate
10	10	20	40
30	30	60	120
50	40	80	160
70	45	90	180

*Data given for use with an air bath; when using a block or Peltier heater, multiply by 0.2–0.5.

Conclusion

Column temperature can be a very important parameter for controlling retention and selectivity in LC separations. It is important to avoid using ambient conditions because they are subject to changes beyond a chromatographer's control. Instead, always use a column oven to control temperature. A good alternative to ambient temperature is to set the column to a temperature sufficiently greater than ambient; for example, 30–35 °C, so you can achieve good temperature control. If temperatures much higher than this range are used, it is important to provide sufficient mobile-phase preheating so a temperature gradient does not occur along the column. For a more detailed discussion of temperature control, consult reference 1.

Reference

(1) R.G. Wolcott, J.W. Dolan, L.R. Snyder, S.R. Bakalyar, M.A. Arnold, and J.A. Nichols, *J. Chromatogr. A* **869,** 211–230 (2000).

John W. Dolan

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LCGC*'s editorial advisory board. Direct correspondence about this column to "LC Troubleshooting,"* LCGC*, 859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@LCResources.com.*

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