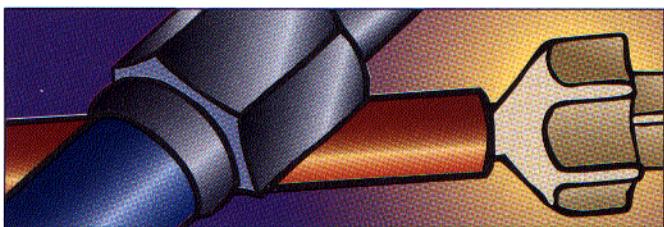


LC Troubleshooting



What is the True Temperature?

Peng-Ling Zhu and John W. Dolan

The column temperature you select may not be the temperature you get.

Our laboratory has been involved in a research project studying how the simultaneous use of gradient steepness (or isocratic solvent strength) and column temperature can provide an added dimension of selectivity (1-5). This work showed that for most sample types, the combined use of temperature and solvent strength variations results in significant changes in selectivity for liquid chromatography (LC) separations. From a practical standpoint, changes that can be made from the instrument console (gradient slope or isocratic percentage of solvent B and column temperature) often can provide sufficient selectivity to solve an LC separation problem. Using this method, you avoid the inconvenience of changing solvent and column types, and it is easier to automate.

During our studies, we observed results that were quite puzzling at first. Two LC systems with the same settings for all variables had retention-time differences of more than 10%. We began a systematic troubleshooting procedure to track down the source of this unexpected result.

Our findings will relate to any situation when temperature control is used with LC columns. Reference 6 discusses several additional potential problems that may occur when using elevated temperatures for LC separations.

THE SETUP

We used two LC systems in this study. System 1 comprised two model 10AD high-pressure mixing pumps (Shimadzu Scientific Instruments, Columbia, Maryland), a Mistral air-bath column oven (Spark Holland, Emmen, The Netherlands), and a model SPD10A UV-absorbance detector (Shimadzu) operated at 215 nm. A Rheodyne 7125 manual injector (Cotati, California) was mounted in the column oven. System 2 nominally was the same system, but it was equipped with a model CH-150 block-heater column oven (Eldex Laboratories, Napa, California) instead of an air-bath column oven.

We used 25 cm × 4.6 mm, 5- μ m d_p Zorbax SB-C18 columns (Mac-Mod Analytical, Chadds Ford, Pennsylvania) for all experiments. The gradient program was 20-30% 0.1% trifluoroacetic acid (in water)-acetonitrile in 15 min with a flow rate set at 1.5 mL/min.

The sample was a proprietary peptide mixture with an on-column sample size of less than 5 μ g. This sample type was appropriate because the resolution of the minor peaks changed dramatically when we changed the chromatographic conditions. This sample allowed us to compare chromatograms not only in terms of retention but also in terms of resolution.

INITIAL SUSPECTS

When we first observed the retention differences, the column oven was set at 55 °C for each system, but the retention time for system 2 was approximately 1.5 min shorter than for system 1. (Throughout this column, we refer to the retention time [t_R] of the main peak only.) Small system-to-system retention differences are typical using shallow gradients, but differences that large are unusual.

Whenever you observe retention differences between LC systems while using gradients, you should suspect the dwell volume as the first possible source of the problem. Commercial LC systems have dwell volumes ranging from less than 0.5 mL to more than 5 mL; this range alone can make differences of several minutes in retention time. In our study, however, the two systems had the same dwell volume, so dwell volume could not cause the observed retention differences.

Because the dwell volume was not the problem, the mobile phase became the most likely variable, and we examined it first. We mixed a new batch of mobile phase and tried it in each system, but we obtained the same results as before. Even when we used the same mobile-phase reservoirs for both systems, the problem persisted. Therefore we concluded that the mobile phase was not causing the problem.

The next item of potential variation was the column. Although most manufacturers produce columns that are highly reproducible from column to column, some separations are more sensitive than others to column chem-

istry changes. To eliminate the column as a variable, we used the same column in both systems. Again, we observed the same results — the retention differences persisted.

Flow-rate differences can cause retention-time differences, so we checked the pump flow rate next. Careful measurement of the flow rate of all four pumps indicated a difference of less than 1% in flow rate between any of the pumps, so we also eliminated this variable.

The remaining variable was the column oven, which we examined next. We moved the air-bath oven to system 2, and the retention-time difference moved with it. In other words, we discovered that the problem was related to the column oven.

COLUMN HEATING

Chromatographers commonly use one of three methods to heat their LC columns: air-bath, block-heater, and water-jacket ovens. Commercial column heaters use either the air-bath or block design.

Air-bath heaters are similar to gas chromatography column ovens; the air is heated and circulated with a fan. The column is suspended in the air current. The advantage of these ovens is that users can change the column temperature fairly quickly because there is little thermal mass to heat or cool. In addition, the entire column and connecting tubing in the oven are exposed to the same air temperature, so they should be the same temperature.

Block-heater ovens comprise a heated, grooved pair of blocks (usually aluminum) in which the column is clamped. The column is held in contact with the blocks and heat is transferred directly. This configuration changes temperature slowly because of the large thermal mass. Most designs use either different size or multiple blocks for different column sizes to give as much surface contact as possible. The column endfittings and connecting tubing usually do not contact the heater but are heated by convection

within the closed column compartment. A variation of the block heater is a unit with a flexible heating pad that is wrapped around the column.

A third method of column temperature control is a water jacket. In this design, the column is sealed inside a glass tube with end plugs and then heated water is circulated through the jacket. This configuration gives the column total exposure to the heat-transfer fluid, which is an advantage of the air bath, but the water provides a better heat-transfer medium than air. However, the inconvenience of assembly, tendency to leak, and general incompatibility with most commercial LC systems make the water-bath heater the least commonly used column heater.

BACK TO OUR PROBLEM

When we discovered that the retention differences were associated with the type of column heater used, we immediately suspected a calibration problem. At the time we had no thermocouple available so we used a laboratory thermometer, and we concluded that both heaters were generating the desired 55 °C temperature setting. When a thermocouple became available, we checked the temperature of a variety of column ovens in different laboratories participating in a group study. We found that most units deviated more than 0.5 °C from the set point — some were as much as 5 °C off, especially at higher temperatures (higher than 50 °C).

We discovered earlier that it was necessary to preheat the mobile phase to obtain the most consistent results and highest plate numbers. We placed a coil of 50–100 cm of 0.010-in. i.d. stainless steel tubing in the oven upstream from the autosampler. In this manner, the mobile phase from the pump passed through this heated coil before traveling to the injector valve and back to the column. For the balance of the experiments described here, we used LC system 2 and varied the column oven temperature, as described below. We used the same column and connecting tubing and left the column connected to the LC system throughout so that fittings were never changed, thereby eliminating possible er-

rors due to differences in plumbing connections.

We suspected that the effective column temperature was different in the two ovens, even though the settings were the same. We know that mobile-phase temperature directly affects viscosity, and we observed this principle as a change in system back pressure. Thus, higher temperatures yield lower back pressure when all other variables are held constant. We observed that the air-bath oven produced higher system back pressures than the block heater. At a 55 °C setting, the air bath and block heater produced back pressures of approximately 128 and 106 bar, respectively.

This difference in back pressure suggested that the column packing was cooler in the air-bath oven than in the block heater. This finding seemed logical because the heat capacity (the ability to transfer heat between the column and its external environment) of the air bath is lower than the block heater in direct contact with the column. This hypothesis should be simple to substantiate by repositioning the column in the block heater, so we tried the experiment.

ON OR OFF

Our first experiment compared the retention times for the system when the column was clamped to the block heater with those obtained when it was suspended in the column compartment but not in contact with the block. Figure 1a shows a chromatogram generated using the conventional configuration with the column

mounted on the block, and Figure 1b shows a chromatogram obtained using an off-the-block configuration. The difference in retention supports our supposition — we expected longer retention times when the temperature was lower. The back pressure

(Table I) also is lower for the block-mounted column, which provides additional support for our hypothesis.

To make sure that this problem was not one of insufficient preheating, we checked the influence of the preheating coil posi-

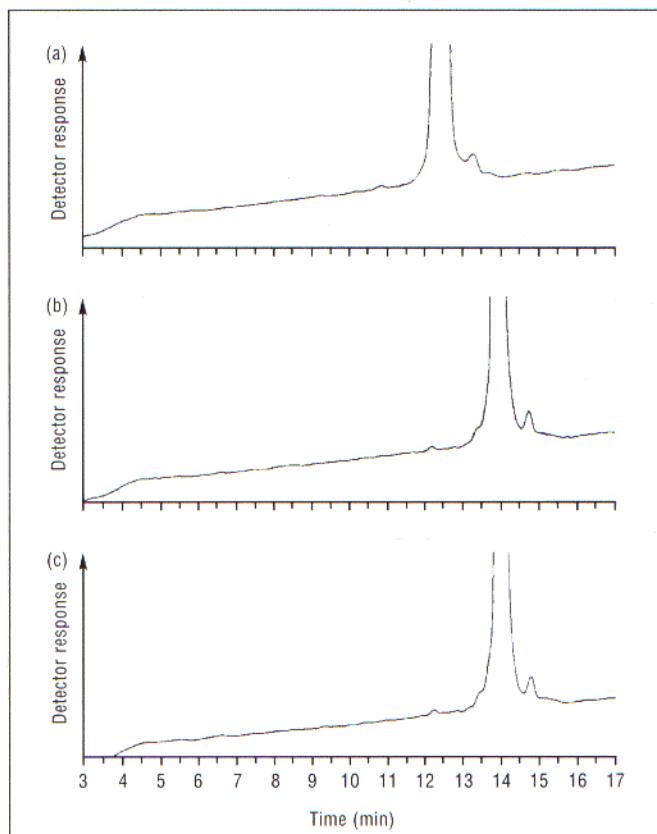


FIGURE 1: Chromatograms generated using a block-heater oven set at 55 °C and the oven door closed with (a) the column mounted on the heating block and the preheating coil mounted off the block, (b) the column and the coil off the block, and (c) the column off the block and the coil on the block.

TABLE I: Summary of Data from Figures 1–4*

Figure Number	Temperature Setting (°C)	Coil Position†	Column Position‡	Pressure (bar)	t_R (min)	Peak Width§ (min)
1a	55	Off	On	106–107	12.50	0.29
1b	55	Off	Off	128	13.98	0.21
1c	55	On	Off	128	14.01	0.21
2	42	Off	On	128	14.06	0.22
3	82	Off	Off	106	11.90	0.19
4a	55	None	On	—	18.68	0.33
4b	55	Off	On	—	19.39	0.24

* All data obtained using a block-heater column oven.

† Off = heating coil hanging free in oven; on = clamped to heating block.

‡ On = column clamped to heating block; off = hanging free in oven with the door closed.

§ Width at half-height for the major peak.

|| Somewhat different gradient conditions from other runs; the retention difference between Figures 4a and 4b cannot be attributed to temperature-related causes.

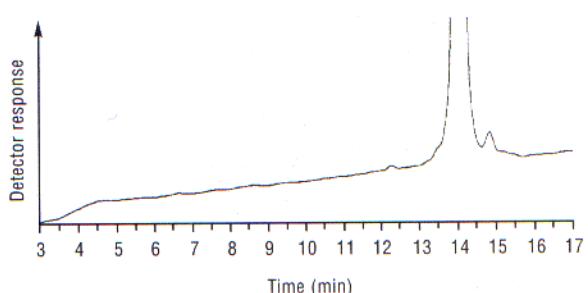


FIGURE 2: Chromatogram obtained with the column mounted on the heating block set at 42 °C.

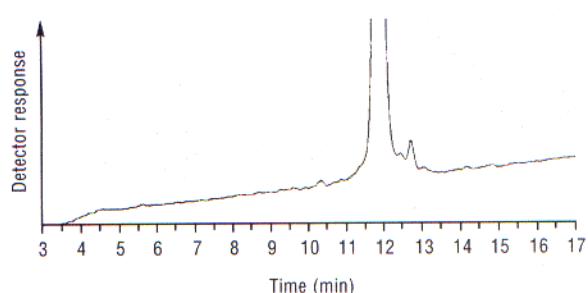


FIGURE 3: Chromatogram obtained with the block-heater oven set at 82 °C and the column suspended off the heater block.

tion. Figure 1c shows a chromatogram equivalent to Figure 1b, except that the preheating coil is clamped on the block heater for better heat transfer. The figure shows that this change is of no practical significance in retention or pressure.

EQUIVALENT TEMPERATURE

So far our findings suggested that the temperature inside the column indeed was different in the two oven systems. We observed a clear difference between the column contacting the block heater and the column in the same oven but without heater contact.

Next we decided to determine the effective temperature difference between the mounted and unmounted columns. As a reference, we used the conditions of Figure 1b (55 °C block temperature with an unmounted column). Then we remounted the column on the block and lowered the block temperature until the back pressure was the same as in Figure 1b (approximately 128 bar). We obtained this pressure at a temperature setting of 42 °C.

Figure 2 shows the chromatogram obtained under these conditions. As you can see, we obtained the same separation. This retention time was roughly the same as the time observed for the forced-air oven when the problem was discovered (results not shown).

As a final experiment, we determined what block temperature was necessary to yield a separation equivalent to that of the column mounted on a 55 °C block (as in Figure 1a). We suspended the column in the oven and heated the block until the pressure was approximately 106 bar. This pressure required a temperature setting of 82 °C. Under these conditions (see Figure 3), the retention time was close to the retention of Figure 1a. Although the retention match was imperfect, it illustrates that a much higher air temperature is needed to obtain retention times equivalent to the same column in contact with a lower-temperature heating block.

MOBILE-PHASE TEMPERATURE

It is important that the temperature of a column is constant from one end to the other because a

temperature gradient along the column is detrimental to peak shape. Early in our studies, we discovered that the peak shape at temperatures above approximately 50 °C was poor unless the injection valve was mounted in the oven. This configuration provided considerable thermal mass to preheat the mobile phase before it entered the column, thus reducing the temperature gradient along the column. Most LC systems, however, rely on autosamplers for routine use, so a system configured with a heated manual injector such as the one used here is impractical.

To provide similar performance between the heated manual injector and an autosampler without a heated injector, we added a preheating coil of tubing to the system. As mentioned earlier, this coil comprises a 50–100 cm length of 0.010-in. i.d. tubing placed in the column oven. The mobile phase is heated as it passes through the tubing and then some of the heat is transferred to the injection valve body of the autosampler or manual injector. By using this technique, we were able to obtain similar peak widths with manual injectors and autosamplers. However, a manual injector mounted in the heated oven always outperformed an autosampler. This finding suggests that an additional heating mechanism for the autoinjector valve would be beneficial to obtain the best performance at temperatures higher than 50 °C.

Figure 4 shows the effect of the mobile-phase preheating coil. In Figure 4a no preheating coil was used, and the peak width at half-height for the major peak is 0.33 min. Figure 4b shows a similar run but with the preheating coil mounted in the oven (55 °C); the width is 0.24 min. (The difference in retention times between the two runs can be attributed to day-to-day variation.) This difference in peak widths translates into a 1.8-fold difference in plate number, or more practically, a significant difference in resolution, as you can see by studying the minor peaks in the two chromatograms.

THE IMPLICATIONS

The results reported here indicate that the column temperature can play a major role in the ability to reproduce a separation from one

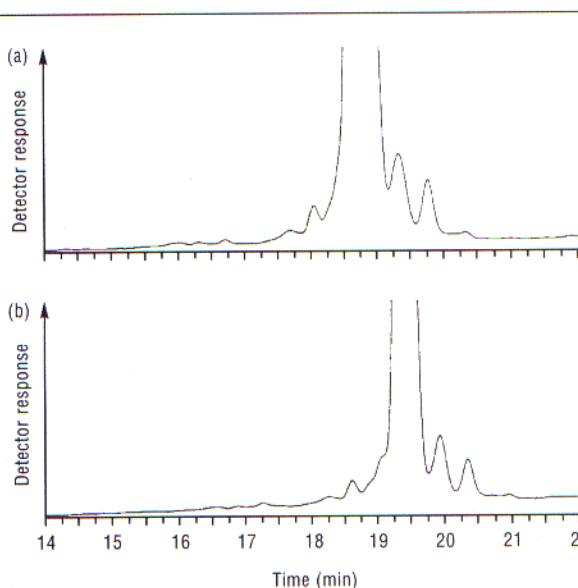


FIGURE 4: Chromatograms generated using (a) no preheating coil and (b) a preheating coil mounted in the oven off the heating block. The block-heater oven was set at 55 °C and the column was mounted on the block. The gradient conditions were slightly different than in Figures 1–3.

instrument to another. Commonly, chromatographers recommend thermostating columns to avoid problems resulting from differences in room temperature within individual days or between days. Our results also suggest that the manner in which column temperature is controlled can be important. For the examples cited here, the two heating techniques can produce column temperatures that differ by more than 10 °C for a nominal 55 °C setting.

Another variable is the evenness of the columns' heating. We observed that uneven heating can cancel out the gains in column plate number and selectivity generated by the use of higher column temperatures. This problem is suggested by comparing the peak width of the main peak and the resolution of the minor peaks in Figures 1a and 3 (also see Table I). Although the retention times and back pressure are approximately the same in the two examples, the separation is superior in Figure 3, which suggests that the temperature of the entire system is better controlled.

Our results lead us to conclude that different column heating techniques can yield dramatic retention-time differences. These differences can be corrected by adjusting either system to obtain an equivalent column temperature. Preheating the mobile phase and, if possible, the injection valve will improve system performance further. Although likely to be subject to error, the column back pressure is a metric that you can use to judge the equivalency of column temperature.

As our intuition tells us, higher column temperatures are more problematic than lower ones. In our experience, temperatures below approximately 50 °C are much less susceptible to problems than higher temperatures.

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