LC TROUBLESHOOTING

Retention-Time Variation: A Case Study

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An awareness of the important variables affecting retention time will help us to develop methods that yield more consistent results.

This month's "LC Troubleshooting" will cover one reader's question about problems with retention-time reproducibility. A problem of the magnitude reported may cause great concern for some workers, whereas others will shrug their shoulders and continue with their work. If we are aware of the important variables that can affect retention times, we will be more likely to control the variables that are important in our particular separation.

THE PROBLEM

Q: I am observing retention times that vary from day to day in the assay for a chemical intermediate we prepare. The method involves a 25 cm × 4.6 mm C18 column operated at 2 mL/min, a mobile phase of 75:25 (v/v) acetonitrile/phosphate buffer, pH 6.9, and UV detection at 230 nm. We inject 25 µL of sample using an autosampler. I'm concerned about the product peak at ~12 min (peak 2, Table I), which changes by almost a minute between days in some cases. What could be going wrong here, and how can I correct the problem?

JWD: In Table I, I've compiled a summary of retention-time data for several peaks from the chromatograms that you submitted. Table II contains a brief summary of the effects of changes in different variables on the separation. We can use these data to help diagnose the problem. In Table II, we see four variables that are most likely to affect retention in a reversed-phase separation: flow rate, temperature, organic solvent, and mobile phase pH. Let's look at each of these to assess the likelihood of an important contribution by that variable.

SOME POSSIBILITIES

Flow rate: The flow rate is the easiest variable to check. If the pump is not delivering the prescribed flow, changes in retention can result. You can measure the flow rate by doing a timed collection of the column effluent in a graduated cylinder. In your case, you didn't notice the problem until the second day, so determining a flow rate change by direct measurement is impossible. However, you can use the chromatogram to assess flow rate changes. If the flow rate changed from day 1 to day 2, you would expect retention for all the bands to change in proportion to the flow rate change. Note, however, that the t_0 peak at the column void volume does not vary between days. Retention of the t_0 peak should be affected only by the flow rate and not by the other variables under consideration. A constant t_0 indicates that the flow rate is constant. You can also do a rough verification of the flow rate by estimating t_0 and comparing the estimate with the observed value. A typical void volume (V_m) of a 4.6-mm i.d. column is ~0.1 mL/cm. In your case, the 25-cm column should have a void volume of ~2.5 mL. Convert void volume to dead time ($t_0 = V_m$/flow) so that $t_0 \approx 1.25$ min = 2.5 mL/2.0 mL/min. The observed t_0 peak is at ~1.25 min, so the pump is probably delivering the proper flow rate.

Temperature: Changes in the column temperature will change the retention time of all peaks (except t_0) in the chromatogram. As shown in Table II, an increase of 1 °C will reduce the retention times by 1–2%. The retention-time shifts shown in Table I are ~8%, which could be accounted for by a difference of perhaps 5–10 °C between the two days. Changes of such magnitude are common in many labs, especially in the summer and win-

TABLE I: Summary of Retention Data

Day	Time	t_0	Retention Time (min) Peak 1	Peak 2	Peak 3
1	10 a.m.	1.25	2.17	12.12	17.84
	1 p.m.	1.26	2.19	12.17	17.82
	4 p.m.	1.24	2.18	12.15	17.85
2	9 a.m.	1.24	2.36	13.03	19.19
	11 a.m.	1.26	2.35	13.01	19.22
	1 p.m.	1.25	2.38	13.06	19.21

TABLE II: Effect of Change in Separation Conditions on Sample Retention

Variable	Method	Change in Variable	Average Change in t_R (%)
Flow rate	All	+1%	−1
Temperature	All except SEC	+1 °C	−(1–2)
Mobile phase composition			
Organic solvent	Reversed phase*	+1 %	−(5–10)
pH	Reversed phase	+0.01 unit	±(0–1)
Strong solvent	Normal phase	+1%	−(1–2)
Buffer, organic solvent	Size exclusion	+1%	0

* including ion-pair LC

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ter when air conditioning or heating is reduced at night to save energy. I have worked in labs in which the summer temperature was ~22 °C (72 °F) during the middle of the day but reached 27 °C (80 °F) or more by the time I left for home because the air conditioning had been shut off at about 4 p.m. Similarly, the laboratory would be hot early in the morning until the air conditioning took effect. Many labs experience similar temperature variations, but the changes tend to be cyclic. Chromatograms run under such conditions should vary within the day as the temperature changes, but in this case the data in Table I show that the retention times are quite constant within the day. For this reason, I suspect that the observed retention-time changes are not temperature-induced.

pH: Mobile phase pH can have a profound effect on retention time. A change of 0.1 pH units can change retention time by 10% (Table II). Few labs may routinely control mobile phase pH to within <0.05 units. When pH changes, however, the direction and magnitude of retention-time changes seldom are the same for all peaks. Because the pK_as of the various sample components will differ, the retention-time change caused by a given change in pH will vary for the different components. For example, the retention time of a neutral compound will not change with changes in pH, whereas retention times of ionized species might change in opposite directions, depending on the species' properties. Thus, although the retention of all sample bands might drift by the same magnitude in the same direction, it is highly unlikely. Consequently, a change in mobile phase pH is probably not the cause of your problem.

Organic solvent: The remaining variable to examine is the organic-solvent content of the mobile phase. As the data of Table II show, a 1% change in the organic-solvent concentration can change retention time by 5–10%. I suspect that a change in the mobile phase composition is the cause of your problem.

What could cause such changes? If the mobile phase is hand-mixed, the most obvious problem source is a formulation error. Be sure to carefully measure the volume of each mobile phase component before mixing, rather than measuring one component and "bringing to volume" with the other. For example, to make 1 L of your mobile phase, measure 750 mL of acetonitrile and 250 mL of buffer and pour them together, rather than placing 750 mL of acetonitrile in a graduated cylinder and bringing the volume to 1 L with buffer. If you use the latter technique, the mobile phase composition can vary by 1–2% or more depending on whether acetonitrile is added to a volume of buffer or vice versa. Careful measurements using graduated cylinders will be adequate for most applications, but the use of volumetric glassware will increase the precision and accuracy of the mobile phase composition.

If the mobile phase is mixed on-line using a low-pressure mixer, an air bubble trapped in the mixer or in a solenoid valve can affect the delivery of one solvent and thus change the mobile phase composition. Similar problems can occur if the inlet-line frit in one solvent reservoir is partially blocked, reducing the delivery of one solvent. When high-pressure mixing is used, a small bubble in one pump head, a malfunctioning check valve, or a leaky pump seal can reduce the delivery from one pump, resulting in a mobile phase compositional error.

A more insidious source of changes in mobile phase composition is the selective evaporation of one mobile phase component. If the mobile phase reservoir is left uncapped overnight, enough solvent could evaporate that a retention-time change would result. In reversed-phase systems, the organic component would evaporate first, resulting in a weaker mobile phase and thus longer retention times. This outcome is consistent with the observations summarized in Table I. The likelihood of compositional changes increases when helium sparging is used to degas the mobile phase. In a study of the effectiveness of helium at removing dissolved air from various liquid chromatographic (LC) solvents, relatively small volumes of helium were required to fully degas reversed-phase solvent systems (2). For example, only 1.3 L of helium is required to remove 99% of the dissolved oxygen from 1 L of acetonitrile; just 140 mL of helium will degas 1 L of water. Thus, effective degassing can occur by vigorously sparging the mobile phase for only 5 min. The mobile phase can be kept in a degassed state by maintaining a slow trickle of helium (for example, 10 mL/min) if the reservoir is capped with a vented cap; however, evaporation problems can occur if the sparger is left running for an extended time. For example, although an acetonitrile loss of only ~0.03% occurs when the solvent is minimally degassed by sparging with 1 L of helium (2), this low rate of loss can be important over a long period. If the helium is left flowing at 50 mL/min (as much as 30 L during a 16-h overnight period), a loss of 1% of the acetonitrile from the mobile phase can occur (30 L × 0.03%/L = ~1% loss). So inadvertently leaving the sparger on overnight could have a noticeable effect on retention.

STRATEGY FOR ISOLATION AND PREVENTION

Now that we've covered the most likely causes of the retention-time drift that you observe, let's see how to use these data to isolate the cause and prevent it from recurring. We eliminated a flow rate problem from the list of suspect conditions because the t_0 peak was constant from one day to the next.

We also decided that temperature changes were not a likely cause because the within-day retention-time variation was quite small. If you have a recording thermometer available, record the lab temperature for a few days and you should get an idea of the magnitude of typical temperature fluctuations. Because temperature generally fluctuates in a cycle throughout the day, you would expect to see similar cyclic changes in retention times. However, controlling the column temperature is a good idea if the laboratory temperature is subject to fluctuation.

For best operation, the column should be in a thermostated oven. We run our columns at 30–35 °C using a block heater to control the temperature (the manual injector also is enclosed in the column compartment). Running the column at elevated temperatures not only controls temperature-related retention-time drift but also reduces the system back pressure and increases the column plate number. If you don't have a column heater, you should insulate the column. Fit a piece of foam water-pipe insulation (available at a hardware store) or a piece of split vacuum hose around the column to keep drafts away. Insulating the tubing between the column and the detector also will help prevent temperature-related baseline drift. If an air conditioning or heating duct vents near the LC system, move the system or make a cardboard deflector to redirect the draft.

A change in mobile phase pH was not the problem because all the retention times changed in the same direction by about the same magnitude. You should, however, take care when buffering the mobile phase. Several pointers are valuable here: First, when a method is developed, check its sensitivity to pH changes by preparing the mobile phase at the desired pH (6.9 in the present case) and comparing its retention time with those of mobile phases prepared at ±0.1–0.2 pH units. When you know what to expect when the pH is changed, you'll have an indication of how carefully you need to control the pH. Second, be consistent about how the mobile phase is prepared. A thorough discussion of pH adjustment is contained in earlier "LC Troubleshooting" columns (3,4). The key factors are measuring the pH *before* organic solvent is added to the mobile phase, staying within the buffering range for the buffer type chosen, using a sufficiently high buffer concentration, and keeping the buffer concentration constant. In your case, pH 6.9 is within the pH 6.2–8.2 buffering range for phosphate. In general, as long as the buffer concentration is not lower than ~20 mM, everything should be OK. So with regard to this variable, your system is fine. The best way to prepare a constant (and known) buffer concentration is to prepare known concentrations of acid (for example, 20 mM H_3PO_4) and base (for example, 20 mM K_2HPO_4) and blend these to get the desired pH. Add the organic solvent after checking the pH. Other buffer preparation techniques may be used, but the key to good results is consistency in buffer preparation.

Thus, we concluded that the most likely cause of the retention-time shifts you observe was a change in the organic concentration. This theory should be easy to check by carefully preparing fresh mobile phase (and optionally using a second batch prepared with 1% more or less organic solvent) and observing the results. How to pinpoint your problem source will depend on the LC system configuration. If on-line mixing is used, check for bubbles or leaks that can affect the proportion of each mobile phase component delivered to the mixer. Verify that inlet-line restriction is not excessive because of blocked inlet-line filters (check this by removing a filter temporarily to see if conditions improve). If the mobile phase is prepared manually, take extra care so that batch-to-batch variations are minimized. When helium sparging is used for degassing, turn the sparger down to a trickle after initial degassing, and shut the sparger off overnight if the system is not in use.

For the best results, use a sparging system that keeps a slight head pressure on the reservoirs and adds helium only as mobile phase is pumped out (several of these systems are commercially available). Always cover the reservoirs to reduce evaporation and keep dust out of the mobile phase, but don't seal them tightly because a vapor lock can occur when solvent is pumped out of the reservoirs. I like to use a bottle cap with a 1-mm vent hole drilled in it, a piece of aluminum foil, or (for compatible solvents) Parafilm (American Can Company, Greenwich, Connecticut). Each of these techniques provides reasonable protection against evaporation and dust and allows for sufficient venting. Finally, prepare fresh mobile phase on a regular basis, such as every day or every other day. I prepare a buffer concentrate at 10 times the desired concentration (200 mM in the present case) to speed mobile phase preparation. Dilute the buffer, check the pH, and you should be ready to run.

AND FINALLY. . .

After all this discussion of the potential causes of retention-time drift, we should ask what impact the problem has on the usefulness of the data. Retention-time changes of 8–10% are greater than most of us would like on a day-to-day basis, but the data are probably usable. If standards and control samples are run with each batch of samples, peak identification should not be a problem, and quantitation should be possible. When the sample is simple and generates few potential interfering peaks, retention-time changes of this magnitude will cause little confusion. On the other hand, if the chromatogram is complex, containing closely eluting metabolites or interferences, the magnitude of variation you have observed may be unacceptable because of the increased likelihood of peak identification errors. In chromatography, as in other analytical techniques, there aren't always hard-and-fast rules — you'll have to use your judgment to decide which variables to control and how much variation is acceptable for the method used.

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