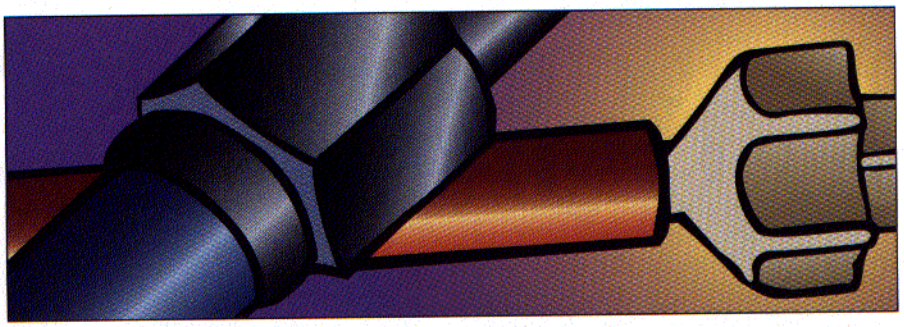
LC Troubleshooting



Retention Time Problems

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Retention time instability creates frustrating problems.

Analysts conducting qualitative analyses in liquid chromatography (LC) commonly use retention time for compound identification. If the retention time of a standard and an unknown coincide, analysts assume that the two represent the same compound. Depending on the objectives of the method, the identity of the analyte may require confirmation. Whenever the retention time of a chromatographic peak is not constant, the possibility exists for error in interpreting the data, and analysts will have less confidence in their data. This month's "LC Troubleshooting" addresses three readers' questions about method problems related to retention time changes in the chromatogram.

RETENTION TIME DRIFT

Q: I developed and use an LC method on a 15 cm × 4.6 mm C18 column at 50 °C. Mobile-phase solvent A is 60:40 acetonitrile–0.1% phosphoric acid, and solvent B is acetonitrile. I use a gradient of 0–50% B in 15 min with a return to starting conditions at 15.1 min and reequilibration for approximately 10 min before the next injection. I sparge the mobile phase with helium at 30–50 mL/min.

The retention times for the peaks of interest have increased over time. I suspect the increased retention times may be caused by a change in mobile-phase solvent A. Is this likely, and if so, how can I overcome the problem?

A: I think you identified the problem correctly. Although I have seen this problem only a few times over the years, a change in mobile-phase composition is possible when using helium sparging. As you presumed, a vigorous sparging stream can selectively evaporate the more volatile component of the mobile phase, thus changing its composition. In the present case, the acetonitrile in solvent A would be more volatile, and loss of acetonitrile would make solvent A weaker, thus lengthening retention times. A similar loss of acetonitrile from the B reservoir would occur, but because the reservoir contains only acetonitrile, it remains at 100% acetonitrile.

To solve the problem, you must use sparging conditions that do not evaporate too much acetonitrile from solvent A. If you use low-pressure mixing, you must degas the mobile phase on a continuous basis, or air bubbles will be a problem. If this is your situation, try sparging vigorously for 3–5 min and then turn the helium flow down to a minimum, so that only a few bubbles trickle from the sparging frit. This step may solve the problem. An alternative solution is to invest in a helium sparging system that uses pressurized reservoirs. These systems allow helium sparging in the normal manner, then users must close a valve to allow helium pressure (less than 5 psi) to build up in the reservoir. Additional helium bubbles through the solvent to replace the solvent that is pumped out of the reservoir. The headspace comprises helium saturated with mobile phase, so no air can diffuse back into the reservoir. These systems are robust, conserve helium, and eliminate mobile-phase evaporation problems.

If, on the other hand, your LC system uses high-pressure mixing, it may be unnecessary to sparge the mobile phase continuously. In my laboratory, analysts use high-pressure mixing LC systems and operate successfully by sparging the mobile phase off-line in the morning before a new batch is placed on the system. The degree of degassing obtained in this fashion is sufficient to avoid outgassing problems for an entire day's runs. As with many other problem solutions, you should test this scenario with your system to check that it corrects the problem.

Another alternative to helium sparging is on-line vacuum degassing. This technique, illustrated in Figure 1, passes the mobile-phase solvents through a thin-walled tubing coil in a vacuum. The tubing is permeable to air but not to liquid, so the gas in the solvent passes through the tubing to the vacuum and is pumped to waste, and the degassed solvent continues toward the pump. Users of these systems find them to be acceptable alternatives to helium degassing in many cases.

For the case described above, I assumed that the retention drift problem would be corrected when a new batch of mobile phase was prepared. If replacing the mobile phase fails to correct the drift, however, the problem may be related to a change in the column chemistry. Some columns are prepared using a process called *endcapping*, in which a trimethylsilyl

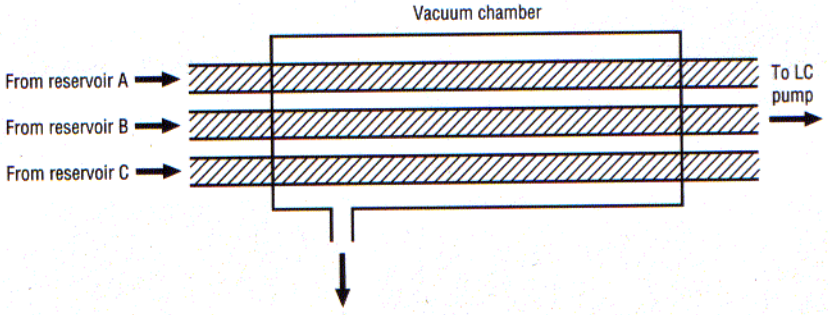

FIGURE 1: On-line vacuum degassing of mobile phase. In practice, each reservoir line is in a separate vacuum chamber. (Reprinted with permission from LC Resources Inc., Walnut Creek, California.)

function is bonded to the silica after the C18 phase is added. This endcapping covers some of the residual silanols left after the primary bonding is complete and provides better peak shape in some cases. Endcapping is useful at intermediate pH values (for example, >pH 6), but the Si–O–Si bond is unstable at low pH values (1). Thus, under the acidic conditions of your mobile phase, the endcapping may wash off the column over time and make more of the polar silanol groups available for retention. If your sample compounds are basic, you may observe increased retention. A nonendcapped column would correct the problem easily.

MORE RETENTION DRIFT

Q: I am having problems with random retention time shifts with my method, as the data of Table I show. My method uses a 25 cm × 4.6 mm amino column and a mobile phase of 60:40 acetonitrile–20 mM phosphate buffer (pH 3.5). This method is used to analyze ascorbic acid in foods using external standardization. Do you have any suggestions about what is causing the retention drift and how to correct it?

A: Three possible problem sources come to mind. Any one of these situations or some combination could be the source of your trouble.

First, amino columns are notorious for their instability. This problem probably results from a combination of the basic amino group, the acidic silanols on the stationary phase, and the short chain length of the bonded phase. For C_n-bonded phases, the stability is greater when n is greater (1). For this reason, you shouldn't be surprised to find that the stability and lifetime of an amino column is much less than that obtained from a C8 or C18 bonded-phase column. Because of this instability, analysts rarely use amino columns for anything but carbohydrate analyses, in which they provide separations unobtainable with other columns. I'm not sure why you are using the amino column with ascorbic acid, which should be amenable to a C8 or C18 column.

Second, retention relates directly to temperature. The rule of thumb is that you will observe a 1% change in retention for every 1 °C change in temperature for reversed-phase separations. You are not thermostating your column, so a change in laboratory temperature can cause retention times to vary. Even if your laboratory temperature is relatively stable, a heating–air conditioning vent blowing on your system can cause significant local temperature fluctuations. Depending on the season and the construction of the air handling system, different retention drift patterns can occur. Some systems can be especially problematic during the fall and spring seasons when the system changes from heating to air conditioning and back again. Under these conditions it may be possible to cool but not heat or heat but not cool the laboratory, even though the outside and inside temperatures demand different performance on different days. The data of Table I could result from temperature changes. The easy fix for this problem is to run all your LC columns in a temperature-controlled environment. In my laboratory, we routinely set column temperatures at 35 °C. This setting is high enough above room temperature to maintain a constant column temperature.

TABLE I: Retention Times

		30 April		1 May	
Vial Number	Sample	Injection Time	Retention Time	Injection Time	Retention Time
101	Standard	11:21 A.M.	5.429	9:24 A.M.	7.060
102	Standard	11:33 A.M.	5.522	9:36 A.M.	7.041
103	Standard	11:44 A.M.	5.697	9:47 A.M.	7.026
104	Standard	11:56 A.M.	5.815	9:59 A.M.	7.005
105	Sample	12:07 P.M.	5.887	10:10 A.M.	6.980
106	Sample	12:19 P.M.	5.943	10:22 A.M.	6.947
107	Sample	12:30 P.M.	5.985	10:34 A.M.	6.908
108	Sample	12:42 P.M.	6.005	10:45 A.M.	6.866
109	Sample	12:53 P.M.	6.012	10:57 A.M.	6.803
110	Sample	1:05 P.M.	6.011	11:08 A.M.	6.743
111	Sample	1:16 P.M.	5.985	11:20 A.M.	6.662
112	Sample	1:28 P.M.	5.972	11:32 A.M.	6.545
101	Standard	1:56 P.M.	5.905	11:58 A.M.	6.275
102	Standard	2:07 P.M.	5.863	12:09 P.M.	6.141
103	Standard	2:19 P.M.	5.818	12:21 P.M.	6.003
104	Standard	2:30 P.M.	5.767	12:32 P.M.	5.862
105	Sample	2:42 P.M.	5.715	12:44 P.M.	5.716
106	Sample	2:53 P.M.	5.671	12:56 P.M.	5.573
107	Sample	3:05 P.M.	5.627	1:07 P.M.	5.432
108	Sample	3:17 P.M.	5.582	1:19 P.M.	5.334
109	Sample	3:28 P.M.	5.531	1:30 P.M.	5.329
110	Sample	3:40 P.M.	5.478	1:42 P.M.	5.328
111	Sample	3:51 P.M.	5.420	1:54 P.M.	5.326
112	Sample	4:03 P.M.	5.363	2:05 P.M.	5.330

A third possible problem source is the mobile-phase pH. Changes in pH can affect retention greatly, especially when analyzing acids or bases. The rule of thumb is that a buffer is effective at ±1 pH unit from the pK_a. Phosphate has a pK_a of 2.1; therefore your buffering capacity is marginal at pH 3.5. An alternative buffer is acetate with a pK_a of 4.8, but it is useful only as low as roughly pH 3.8. Thus, a no-man's-land exists between approximately pH 3.1 and 3.8 where the pH is poorly controlled by either buffer. However, a mixture of the two seems to work fairly well in this pH range. In my laboratory, we have found retention and drift problems when analyzing benzoic acids at pH 3.5 with phosphate, so I'm not surprised to see a similar problem with your method. If it were my analysis, I would lower the pH to 3.0 to make the system more stable. Also, remember that the temperature can shift the pH curve for the analytes, so the possibility exists for additional problems with the present method.

So my recommendations are thermostat the column, lower the pH to 3.0, and if these fixes are not totally satisfactory, shift to a C8 or C18 column.

POOR RETENTION

Q: I have been having difficulty analyzing herbicide residues in soil using a method provided by the herbicide manufacturer. The method uses a 15 cm × 4.6 mm C8 column at ambient temperature. The mobile phase is 80:19:1:0.3 water–acetonitrile–formic acid–triethylamine at a flow rate of 0.75 mL/min. I inject 20 µL of sample and use UV-absorbance detection at 240 nm. The method appears to work very well in the handout supplied with the method, but it does not work in my hands.

My sample peak at 3 min goes sharply negative and up immediately to form a positive peak. Because there is no baseline separation between the negative and positive peak, I cannot integrate the negative side of the peak. We are unable to quantify the amount of herbicide present. I tried to use mobile phase without triethylamine but saw no improvement. How do I solve this problem?

A: I suspect that the primary problem is related to insufficient retention. For good chromatography, analysts need retention times that generate retention factors (k) of 1–20 — or better yet $2 < k < 10$. Recall that we calculate retention factor as

$$k = (t_R - t_0)/t_0 \qquad [1]$$

where t_R and t_0 are the retention time and the column dead time, respectively. The column dead volume (V_m) can be estimated from the

column dimensions for 4.6-mm i.d. columns as

$$V_m \approx 0.1\,L \qquad [2]$$

where L is the column length in centimeters. Finally, the dead time is obtained by

$$t_0 = V_m/F \qquad [3]$$

where F is the flow rate in milliliters per minute. So in your case, the column volume is approximately 1.5 mL, and the flow rate is 0.75 mL/min, so the dead time is approximately 2.0 min. Therefore, the retention factor is approximately 0.5, which is less than desired. When retention is this small, interference with early eluted compounds and the baseline upset at t_0 is common, as you observed.

You need to increase the retention time substantially to improve the method. You can take advantage of the *Rule of Three,* which states that the retention factor will increase approximately threefold for a 10% decrease in mobile-phase organic solvent content. So, in your case, I would reduce the organic solvent content of the mobile phase by approximately 10% as a first attempt to improve the method. Modify the mobile phase so that it contains 90% water and 9% acetonitrile. This step should increase the retention factor to approximately 1.5, which converts to a retention time of approximately 5 min. I suspect that the negative deflection is part of the baseline upset that often is present at t_0, and the retention increase will move your sample peak away from this disturbance, allowing adequate quantification.

CONCLUSION

The retention time is affected by several variables in any LC method. The mobile-phase composition (both strength and chemistry), stationary phase (type, brand, and condition), column temperature, and mobile-phase flow rate are the primary factors that control retention time. A change in any of these factors can change the retention time, so it is important to set up your LC system to minimize the likelihood of change. In each of the cases examined above, better control of one or more of these variables likely will improve the retention time reproducibility.

REFERENCE

(1) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley and Sons, New York, 2nd ed., 1997), p. 196.

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