Troubleshooting

Retention Time Changes

Changes in retention time can be a key to tracking down LC problems.

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LC Troubleshooting Editor

The retention time of a peak in a liquid chromatography (LC) separation can be a useful diagnostic tool to identify problems with a separation. If all variables are kept constant, the retention time also will be constant. However, it is impossible to have perfect control of every variable, so a small variation in retention is normal. Variations in the ±0.02–0.05 min range are normal, and for some methods, perhaps ±0.1 min. Larger changes in retention time from run to run generally are indicative of a problem that must be addressed for reliable method operation.

The characteristics of changes in retention can help to identify the problem source. Is the variation random? Do retention times drift? When changes occur, are the retention times always larger? Is the magnitude of change different in different parts of the chromatogram? Much can be learned from how the retention varies.

Longer Retention

Perhaps the most common change observed is the case in which all retention times are larger than expected. A subset of this condition is seen when peaks in the first part of the run are normal, but later peaks exhibit longer retention. The change can occur in a stepwise fashion, in which all retention times earlier than the change are normal, but retention times for peaks in subsequent runs are retained longer. The cause of these problems usually is related to a reduction in the flow rate of the mobile phase. An increase in flow rate is highly unlikely unless a change in flow-rate setting is made, but a decrease in flow can result from several possible causes.

Bubbles: An air bubble that passes through the pump can create a momentary drop in the flow rate. This will increase the retention time of all peaks that are eluted after the bubble. If the bubble is a single event, all peaks should shift approximately the same, but if the bubble problem continues, retention times will get larger with time. Of course, the passage of bubbles through the pump also will cause a reduction in system pressure, but this might not be noticed in unattended runs. Fortunately, bubble problems generally can be eliminated by thoroughly degassing the mobile phase. Some LC systems will work reliably with a single batchwise mobile phase degassing each day, whereas others will require continuous degassing. The in-line vacuum degassers common on many of the newer LC systems are a good preventive maintenance measure to minimize bubble problems.

Check valves: After bubble problems, the second most common cause of irregular retention times is faulty check valves. When clean and operating normally, pump check valves can be very reliable. However, it takes only a microscopic bit of debris to cause a check valve to leak. A leaky check valve, like a bubble, can cause a reduction in flow rate either on a continuing or intermittent basis. Check-valve problems rarely correct themselves, so some remediation will be required. Cleaning or replacement is recommended.

Often check valves can be cleaned in an ultrasonic cleaner, although this might not be suitable for all designs. Simply remove the check valve from the pump head and place it in a beaker of methanol and sonicate for a few minutes in an ultrasonic cleaner. If you have not done this before, be aware that some check valves are designed in such a way that they will come apart if they are tipped over. The array of balls, seats, washers, and other small parts can be daunting if you don't know how to reassemble them. If the check valve is likely to come apart, place each check valve in a separate beaker so the parts do not get mixed up. If reassembly is required, use forceps and avoid fingers, tissues, or gloves that might reintroduce oil or lint, thus counteracting the cleaning process.

If a check-valve problem is determined to be the cause of erratic flow, be sure to correct the root cause, which is usually some source of particulate matter. Particulate matter can come from the mobile phase, damaged pump seals, or precipitation of buffer salts in the system. Mobile-phase filtration, use of an inlet line frit in the

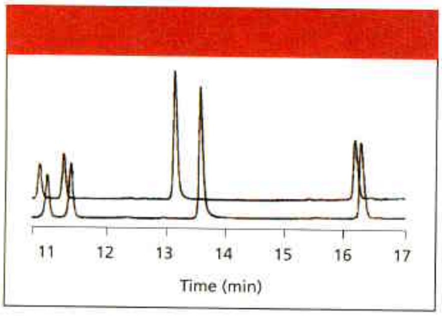

Figure 1: Two consecutive reversed-phase gradient runs showing larger retention errors for peaks near the gradient midpoint. See text for details.

mobile-phase reservoir, and regular replacement of pump seals will help to minimize recurrence of check-valve contamination.

Pump seals: Pump seal failure is a third common cause of reduced mobile-phase flow rate. Pump seals in today's LC pumps are quite durable, and with improved pump design, seals can last for a year or more. However, the seals eventually will wear out. Worn pump seals will not hold pressure as well as new ones, so they will begin to leak with age. Initially, the leaks can be small, but are sufficient to disrupt the flow rate. As wear increases, the mobile phase can drip from the drain hole at the rear of the pump head. Depending upon whether the pump is a one- or two-headed design and operated in the low-pressure or high-pressure mixing mode, the failure of a single seal can have a different influence on the flow-rate disruption. A worn seal also will begin to shed particulate matter as wear increases. These fragments of seal can foul the outlet check valve or work their way downstream to block the column inlet frit or some other part of the system. Some pumps have filters mounted on the outlet of the pump to catch seal debris; pump pressure will increase as these filters become blocked.

How long should a seal last? As with so many other LC parts, the answer is "it depends." I have seen systems in which the pump seals had to be replaced weekly and others in which the seals lasted for more than a year. Mobile phases that contain buffers or salts usually result in shorter seal life than those without these additives. The liquid seal between the pump seal and the piston is never perfect, and the film of mobile phase on the pump piston lubricates the seal. However, when the pump is shut off, that film of mobile phase will evaporate, leaving behind an abrasive buffer residue. This residue can abrade the seal before it dissolves in fresh mobile phase the next time the pump is started. This is one good reason to flush the LC system with nonbuffered mobile phase prior to shutdown. For high-salt mobile phases (for example, more than 50 mM buffer or salt), use of the pump's seal wash feature can help to extend seal life.

If good records are kept, you might be able to determine the normal seal life for your system. Then you can institute a preventive maintenance schedule for seal replacement (I recommend replacing the seal at approximately 75% of its useful lifetime so that failure will be avoided). In the absence of any other compelling reasons, replace the pump seals annually.

Readers should be aware that all pump seals are not created equal. Some pump seals work better in aqueous solvents and others in all organic mobile phases. In a recent seminar I gave, an attendee pointed out that rapid seal wear occurred when tetrahydrofuran was used with the seals designed for use with methanol or acetonitrile. Check with the pump operator's manual or call the manufacturer's technical support group to determine if there is a difference between the seals that are available for your pump.

Leaks: Leaks are still another source of retention time variation. A leak anywhere in the system can decrease the flow rate and thus increase the retention time of peaks. Leaks usually are spotted easily by a puddle of mobile phase. Some LC systems have leak detectors that will shut off the system if a leak is detected. Leaks often can be corrected by tightening an offending connecting fitting. If plastic fittings and tubing are used, it is a good idea to stop the pump flow, loosen the nut, reseat the tubing in the fitting body, then retighten the nut before proceeding. The tube end sometimes can slip in the nut if it is tightened with the flow on, resulting in a hidden dead volume in the system.

Retention Drift

Flow-rate problems caused by bubbles, check-valve failure, pump-seal wear, or leaks always result in larger retention times. In contrast, changes in the column temperature, mobile-phase composition, or column aging can increase or decrease retention times.

Flow rate: When on-line blending of mobile phase is used, any of the flow-rate-related failures mentioned earlier also can cause mobile-phase composition errors. Such problems can be corrected using the techniques discussed earlier.

Temperature: Retention time drift over a series of samples often is due to a change in column temperature if the column temperature is not controlled. Retention in isocratic reversed-phase separations can change 1–3% per 1 °C change in column temperature. The laboratory temperature can change several degrees throughout the course of the day in some facilities, resulting in a drift in retention times. Additionally, a change in peak spacing can occur with a change in temperature, so I strongly advise the use of a column oven with every LC system.

Mobile phase: A gradual change in the mobile-phase composition can result in changes in retention time. For example, selective evaporation of a volatile component of the mobile phase would cause a one-way drift of retention. A new batch of mobile phase would be expected to correct the problem. I have rarely observed this problem, so I believe it is not a significant concern, at least for reversed-phase separations. If selective evaporation is a problem with your method, make smaller batches of mobile phase and cover the reservoir to minimize evaporation (be sure to leave a vent so that a vacuum is not formed as mobile phase is pumped out).

A more common mobile-phase-related problem is the selection of a mobile phase that is not operated in a stable region. For ionic samples, the pH of the mobile phase should be controlled with a buffer. Remember that a buffer is effective within ±1 pH unit of its pK_a, so select a buffer that is effective in the desired pH region. Use of a buffer outside of the buffering range can result in more variable retention times as the pH of the mobile-phase changes with a change in laboratory temperature or some other external variable. In a similar manner, retention times of sample components will be most constant if the mobile-phase pH is at least 1.5 pH units away from the pK_a of the sample. Methods that operate near the pK_a of sample components generally will have more variable retention times. And be sure to use a high enough buffer concentration to buffer the sample. Generally, a buffer concentration more than 10 mM (in the total mobile phase, not just the buffer component) should be sufficient for adequate buffering in analytical methods.

Column aging: Column aging also can

result in retention changes, but such changes typically are over hundreds of samples and weeks of use. Such changes might not be noticed as readily as the short-term retention changes discussed here. Retention caused by column aging might or might not be of significance in the performance of the method. Replacement of the column should correct this problem.

Variation Within a Run

Flow-rate or mobile-phase changes, as discussed earlier, are the most likely causes of retention, but other instrument problems can be the problem source. A recent "LC Troubleshooting" column (1) stressed the importance of checking proportioning-valve performance for low-pressure mixing systems. Several years ago, I observed a rather dramatic example of retention time change during a gradient run that resulted from poor proportioning performance (2). As shown in the chromatograms of Figure 1, retention-time variation in the middle of the run was greater than at either end. In two consecutive runs for this sample, the peaks that were eluted near 11 and 16 min differed by 0.10–0.12 min. This is larger than one would like but was not nearly as bad as the variation for the peak eluted near 13 min, which differed 0.43 min between two consecutive runs. Obviously, this was unacceptable. A gradient step test was run (see reference 1 for this procedure). The performance across most of the gradient range was acceptable, for example, ±0.1–0.2% inaccuracy for a 5% step size. However, the step from 45% to 50% measured 8.4%, a 3.4% error. This low-pressure mixing system used a different proportioning algorithm for the 0–50% and 50–100% portions of the gradient. There was a severe calibration error at the changeover point that was confounded by the elution of the peak of interest at this point in the gradient. Recalibration of the instrument corrected the problem.

Conclusions

Retention time changes within or between chromatographic runs can be the result of a number of possible root causes. Some retention-time variation is normal, but when changes greater than expected are observed, the source of the problem must be identified and corrected. Some problems can be the result of physical failure of system components such as pump seals or check valves. Errors in mobile-phase composition, either from instrument malfunction or an injudicious selection of pH, can be more difficult to isolate. The causes discussed in this month's "LC Troubleshooting" are by no means the only sources of retention-time variation, but they identify some of the major causes.

References

(1) J.J. Gilroy and J.W. Dolan, *LCGC* **22**(10), 982–988 (2004).

(2) J.W. Dolan, *LCGC* **14**(4), 294–299 (1996).

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