LC TROUBLESHOOTING

Retention-Time Problems — Answers to Readers' Questions

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A run-to-run or day-to-day change in retention times can be one of the most aggravating problems in routine liquid chromatographic analysis.

Shifting retention times can cause a great deal of consternation in the lab when making routine liquid chromatographic (LC) runs. The symptoms of this problem can vary from abrupt changes in retention to gradually drifting peaks. Sometimes a change in peak shape or system pressure will occur, but at other times all of the system characteristics seem normal except for the retention times. This month's "LC Troubleshooting" addresses a number of retention-related problems by responding to several readers' questions.

RETENTION SHIFTS AFTER DEGASSING

Q: We commonly observe changes in peak retention if we accidentally leave the vacuum on for 10–15 min during solvent filtering–degassing. Frequently, this problem occurs during preparation of 30–60% acetonitrile in water. Can you comment on the cause of this problem and how to correct it?

JWD: This problem stems from a change in the composition of the mobile phase caused by the selective evaporation of one or more of its components. Many workers rely on vacuum filtration to degas their solvents, and in most cases it ensures reliable pump operation. When sufficient vacuum is applied to any solution for long enough, however, the more volatile components can boil off. Vacuum filtration accelerates the evaporation because solvents passing between the filter and the bulk solution are in a finely dispersed mist, whose very high surface area encourages evaporation. For reversed-phase separations, a 1% change in organic strength can cause a 5–10% change in retention time (1), so the retention changes you observe are not surprising.

The obvious solution to this problem is to shut off the vacuum as soon as all of the solvent has passed through the filter. Another strategy is to use helium sparging to degas your solvents. Be aware, however, that excessive helium sparging also can result in selective solvent volatilization with corresponding retention-time changes. As a third alternative, you can filter–degas or helium sparge the pure solvents before mixing them. When you degas each solvent separately, losses during degassing are unimportant because they do not change the composition of the pure solvent.

SAMPLE-DILUENT EFFECTS

Q: I have noted a common phenomenon when using three LC systems (electrochemical, UV, and fluorescence detection) with several compounds. When pure samples of compounds are reconstituted in pure organic solvent (acetonitrile or methanol), the peaks have a poor shape and a prominent forward shoulder. In all cases, if some water (~30–60%) is added to the reconstituted solution, the peaks are sharp and symmetrical and the shoulder disappears. Injections of pure solvent alone show no peaks, so I'm sure my solvent is not contaminated.

JWD: The occurence of broadened and fronting peaks with samples reconstituted in 100% organic solvent is a common problem. Injecting the sample in too much of a strong solvent can cause these peak distortions. A compound dissolved in a strong solvent travels through the column more quickly than one dissolved in a weak solvent. When it is injected into the strong solvent, the compound travels quickly until the strong solvent is diluted with mobile phase. Depending on the injection size and the injection solvent's relative strength (compared with that of the mobile phase), the injection of a strong solvent can have dramatic effects. Because mixing does not occur instantaneously or in one location in the column, part of the sample band can become diluted while another part remains concentrated. This uneven mixing enables different parts of the band to travel at different rates for a short time, which can cause peak distortion and often fronting. In some cases, it can also result in retention-time shifts.

The solution to the problem, as you have already discovered, is to dilute the sample. A rule of thumb is to keep the injection volume below 20 µL (preferably 10 µL) or to make the injection solvent no stronger than the mobile phase. In some situations (for example, ion-pair chromatography), you must match the strength of injection solvent with that of the mobile phase to prevent equilibrium problems. But generally, you can use mobile phase or weaker (corresponding to more water in reversed-phase systems) solvents successfully for injection.

In fact, sensitivity can be improved by diluting the sample and injecting a larger volume. For instance, you may be able to inject only 10 µL of sample in pure organic solvent, but if you dilute the sample to 50% of the mobile-phase composition (for example, 25% acetonitrile for injection, 50% as mobile phase), you may be able to inject 100 µL or more. Called on-column concentration, this technique is widely used to increase sensitivity for environmental and other trace samples. The action is just the opposite of the one you have observed. When a very weak solvent is used, the sample collects at the head of the column until a sufficiently strong solvent (the mobile phase) comes along to wash the sample down the column. Thus, some workers "inject" several liters of water-diluted sample on the column and then elute it in the normal fashion, which concentrates the sample into the equivalent of 10–100 µL.

COLUMN-TO-COLUMN REPRODUCIBILITY

Q: For one assay we run, columns last about three months. When a new column is installed, the retention times often shift. Most of the time, the magnitude of the shift is not a problem, but sometimes the peaks in one pair move too close together. To overcome this problem, we spend a day or so adjusting the mobile phase so that the peaks have acceptable resolution. The columns we use are made by the same manufacturer and are supposedly identical. Is there any way to avoid this problem?

JWD: Unfortunately, as you have discovered, the retention characteristics of nominally identical columns may show minor or major variations. Although some manufacturers seem to be better than others at making reproducible products, no vendor is completely immune to this problem. In fact, I've seen minor retention differences in columns that were packed on the same day by the same person and that contained the same packing material and had consecutive serial numbers.

A number of variables affect the retention changes you observe; you have control over some of them but not others. The major variables are mobile-phase composition, column chemistry, mobile-phase flow rate, and col-

umn temperature. Because your problem correlates with the change from one column to another, it is unlikely that either the flow rate or the temperature is the problem source.

Flow-rate changes should not correspond to a column change unless some system settings have been changed or a leak has occurred. In any event, no change in separation selectivity should be observed with a change in flow rate.

Temperature variations can cause a change in selectivity (peak spacing) and a retention-time drift (1–2% for every 1 °C change), but they should not result in abrupt, stepwise retention-time changes when a new column is installed unless the new column has not been allowed to equilibrate with the laboratory or column-heater temperature. To be on the safe side, all LC columns should be thermostated or at least insulated to avoid column-temperature changes during operation.

Changes in mobile-phase composition can affect both retention and selectivity. It is unlikely, however, that mobile-phase formulation is responsible for the changes you observe because the same type of variation would occur occasionally with an existing column when a new batch of mobile phase was used. As we'll see below, the components used in the mobile phase may be a key to solving your problem.

Changes in the column chemistry are the most likely source of the retention problems you observe. No matter how carefully manufacturers prepare columns, differences between nominally identical columns exist. For the most part, these differences are insignificant, but certain analyses (such as yours) may be overly sensitive to these minor changes. In fact, given the number of variables involved in column preparation, it is surprising that we don't have more problems. These variables include the manufacture of the base silica particles, the preparation of the bonding reagents, the bonding process, and the packing of the stationary phase into the column. Each step involves numerous secondary reagents, washing steps, purification, and so forth. The reagents may come from different suppliers, and each may contribute an impurity that was not present in previous column batches. That manufacturers can reduce column preparation to cookbook procedures that are reproducible for most applications testifies to the general ruggedness of LC separations.

The most critical factor determining selectivity differences between nominally identical columns is the amount of silica surface left uncovered in the bonding process. When the bonded phase is prepared, it is sterically impossible to cover more than about half of the silica surface. The remainder of the surface is covered with free silanol groups. These silanol groups retain basic compounds very strongly, which can be a problem because many analytes have amine functions. Small changes in the number of free silanols can result in band tailing and changes in retention. Manufacturers have addressed this problem by endcapping stationary phases (adding a C_1 function after the standard bonding reaction is complete) and by creating various polymeric and sterically protected phases that restrict analyte access to the silanol surface. These techniques successfully reduce silanol problems and improve column-to-column reproducibility, but even so, unbonded silanol groups remain — as does the potential for problems.

The simplest way to overcome the column-to-column reproducibility problem is to use identical columns. Columns packed with the same batch of packing material are closest to being identical. If your columns last three months each, buy four columns at a time and make sure that they come from the same batch. Using columns with the same packing will greatly decrease your chance of encountering the retention variations caused by switching from one column to the next. A year later, when you buy your next batch of four columns, you might have to adjust the mobile-phase composition, but a once-a-year adjustment shouldn't be too much work.

Another alternative is to switch to a type of column that is less likely to produce minor retention differences from one column to the next, such as a sterically hindered bonded-phase column. Using a column containing a polymeric packing instead of silica particles might also work because no silanols are present, but it would require method redevelopment and revalidation. Polymeric packings were once envisioned as the solution to all secondary retention problems, but the free silanols are a very important player in the separation process. We don't necessarily want to get rid of them — we just want to control them.

A final and perhaps more universal solution to your retention problems would be to modify the present method so it is not overly sensitive to changes in the column. Many workers routinely add triethylamine to the mobile phase (for example, at 25 mM) to "swamp out" silanol effects. Generally, the triethylamine improves peak shape, causes some changes in relative peak spacing, and makes methods less susceptible to small changes in the free-silanol content of the column. An alternative is to use ion-pair chromatography or another separation mode that may be more stable for your assay. And remember, any additional resolution that you can build into your method will mean that more-significant changes can take place before the column is no longer usable for your separation.

REFERENCE

(1) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (Humana, Clifton, New Jersey, 1989).

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