LC TROUBLESHOOTING

Column and Mobile Phase Problems

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Just because most separations are performed on reversed-phase columns doesn't mean that other types of columns don't have problems that need to be addressed.

Most analytical liquid chromatographic (LC) separations are performed in the reversed-phase mode on a C8 or C18 bonded phase, so most of the emphasis in this column is placed on troubleshooting these techniques. This month, however, we look at readers' problems associated with methods used for anion, cyano, and silica columns. In addition, we answer a question regarding baseline drift that occurred under conditions commonly applied to the separation of proteins and peptides.

ION CHROMATOGRAPHY

Q: I am using an ion chromatographic system for the analysis of chloride, nitrite, nitrate, and sulfate anions. The 25-cm silica-based anion column is used with a mobile phase of 4 mM potassium hydrogen phthalate at pH 4.5 and a conductivity detector.

My samples are aqueous, but they also contain mineral oil and emulsifiers. To protect the column, I use a C18 guard cartridge, but this does not seem to provide sufficient protection. I observe rapid deterioration of the analytical column, with the nitrate and nitrite peaks shifting toward the chloride peak, as shown in Figures 1a and 1b. Column regeneration procedures and guard column replacement do not restore column performance.

I suspect that the mineral oil is not being trapped by the guard column and thus poisons the analytical column. I've tried using solid-phase extraction tubes, but they all release small quantities of chloride. Do you have advice to improve the performance of this assay?

JWD: The shifting retention times that you observe (including the movement of the sulfate peak to later times) are characteristic of some, and perhaps all, anion columns as they age. I suspect that you may be experiencing premature column aging caused by the mobile phase conditions rather than by the sample composition. The lifetime of these columns is related to the total time that they are exposed to buffer and is accelerated when the buffer pH is increased. Such column deterioration, to my knowledge, cannot be restored. The working pH of 4.5 can accelerate aging. For example, you would expect the column to last much longer if you could operate at pH 3.9. Unfortunately, operating at pH 3.9 would not allow you to analyze nitrite in this assay. If you store the column in mobile phase, you could be inadvertently aging the column, even though it is not being put to its intended use. You could slow aging by storing the column in 50:50 (v/v) methanol/water rather than in buffer. (Be sure to flush the buffer from the column with water before adding methanol to minimize potential buffer precipitation problems.)

If I were performing this assay, I would do everything possible to remove the mineral oil from the sample before it is injected. I suspect that the C18 guard column quickly becomes saturated with mineral oil and therefore doesn't provide as much protection as you desire. I would try solvent–solvent extraction as a clean-up technique. The mineral oil should partition into the organic phase and the anions should remain in the aqueous phase.

CYANO COLUMN PROBLEMS

Q: I have a problem with the deterioration of a cyano bonded-phase column. The mobile phase that I am using is 99:1 (v/v) 1.0 mM octanesulfonic acid, pH 3.5/acetonitrile. The separation is satisfactory with a new column, but a column void appears much more quickly than it should. I have tried a number of other conditions that have not improved the separation or column stability. I also have tried other manufacturers' columns, but they do not separate the sample components. The sample is a pharmaceutical preparation and is as clean a sample as one could wish for. I have tried using a guard column, but it increases the bandwidth to the point that I cannot obtain the required separation. Is there anything I can do?

JWD: With the particular method that you have chosen, you should expect to have shorter column lifetimes than you could expect with a C8 or C18 column operated in the reversed-phase mode. Cyano columns, in general, do not last as long as the other reversed-phase columns. I'm not sure why (it may be that the shorter bonded-phase chain does a poorer job of protecting the silica backbone), but most users agree that the cyano columns are not as robust. Ion-pairing reagents, such as the octanesulfonate you are using, are harder on columns than mobile phases that contain only organic solvent, water, and buffers. Furthermore, methods using buffers or mobile phase additives at concentrations less than ~10 mM are more susceptible to reproducibility problems than if the additive concentrations are ≥20 mM. All of these factors point to a method with a lot of potential for problems, and this is borne out by your experience. However, if your methods-development efforts have been rigorous, you will probably not be able to modify the mobile phase conditions and improve the assay.

There are two likely sources of the problem — the sample or the mobile phase. I suspect that the problem is with the mobile phase because of the relatively pure nature of your sample. You could confirm this diagnosis by running the system without injecting sample or by injecting mobile phase alone. Although this experiment may confirm that the mobile phase is the problem, it may not be worthwhile because you'll ruin another column and waste a lot of time that you could have used to run samples.

The approach that I would take is to do everything possible to protect the column. This would be an ideal situation for using a guard column. The guard column should provide chemical and physical protection of the analytical column. It is puzzling that the guard column has caused peak broadening in your system. A good guard column should not decrease the plate number of the analytical column, although it may not increase it either. I would check this carefully with a test compound, measuring the bandwidth with and without the guard column. Be sure that all the connections are correctly made so that the tubing seats properly in the fittings and that you are using 0.010-in. i.d. (or smaller) tubing of minimum length to connect the guard column to the analytical column.

If the guard column experiment is not successful, perhaps a precolumn will be useful. A precolumn (sometimes called a saturator column) is a guard column or old analytical column that is placed upstream from the injector. The precolumn's function is to pretreat the mobile phase so that it is not so damaging to the main column. In this way, the precolumn acts as a sacrificial column, protecting the main column from attack by the mobile phase. Be sure to use an in-line filter (0.5-μm porosity) between the precolumn and the injector to keep any partially dis-

solved packing from the precolumn from getting into the injector or main column frit and causing more problems. The use of a precolumn may provide enough protection to eliminate your problem or to at least extend the useful lifetime of the column.

As a last resort, you could try filling the column void to extend column life. In general, I feel that it is false economy to try to repair voided columns, but when all other avenues have failed, this technique may be useful. I recommend the method described in reference 1, in which the void is filled with a slurry of old packing material and then the column is reversed before placing it back in service. Column reversal serves two purposes. First, it places the relatively unstable new packing bed at the end of the column, where the pressure is lower, so that compaction of the filled void is less of a problem. Second, it places the well-packed region, which originally was at the end of the column, at the head, so that a stable column top will be available. If you use this technique, be sure to flush about 10 column volumes of mobile phase through the column before connecting it to the detector. This procedure will prevent any stray packing material from getting into the detector and causing further problems.

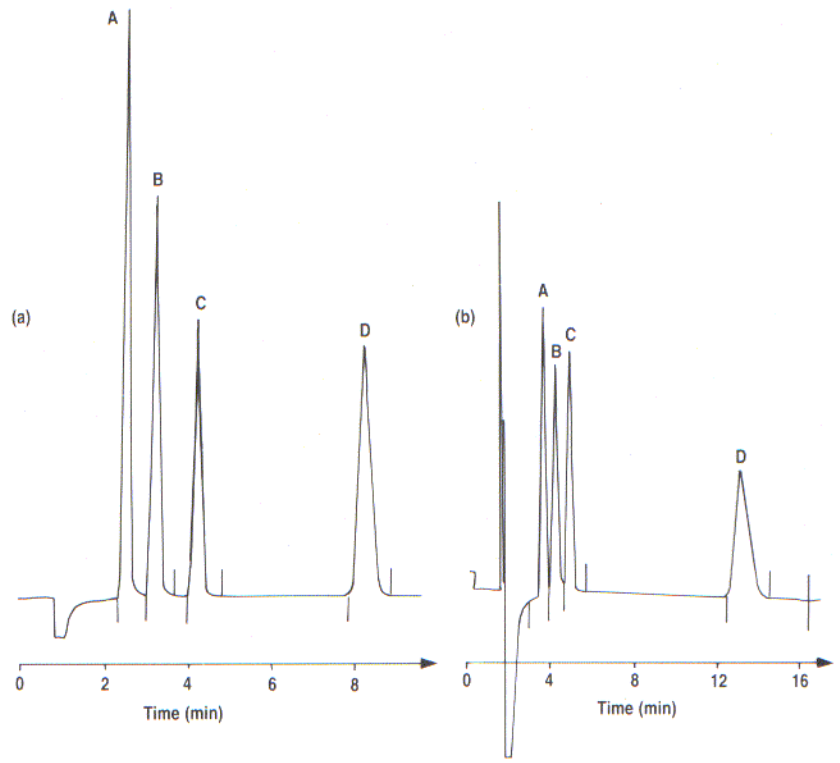

FIGURE 1: Sample chromatograms showing the analysis of several anions (a) using a new column and (b) using the same column after 50 injections. Peaks: A = chloride, B = nitrite, C = nitrate, D = sulfate.

BASELINE STABILITY

Q: I have a baseline stability problem with my LC system. I'm using a C18 column in a two-pump system with 0.1% trifluoroacetic acid in water as solvent A and acetonitrile as

solvent B. When I run a gradient, the baseline looks horrible, with lots of noise (both short-term and long-term) as well as an unacceptable amount of baseline rise. When I run just a water–acetonitrile gradient, everything is fine. I've substituted all the system components and prepared new solvents, but the problem persists. What can I do?

JWD: I suspect that you have a twofold problem — poor mixing and an absorbance mismatch with your solvents. I would start by adding trifluoroacetic acid to both the water and the acetonitrile. In the present setup, you are running a gradient in trifluoroacetic acid as well as one in solvent strength. This means that your column never gets equilibrated with trifluoroacetic acid, so baseline upsets are not surprising. We have found that we can eliminate most of the baseline drift if we match the absorbance of the A and B solvents by adding 0.115% trifluoroacetic acid to solvent A and 0.1% to solvent B to compensate for the difference in absorbance between water and acetonitrile at low wavelengths. This change may be sufficient to correct all of your problems. If you still see a wavy baseline, try premixing some acetonitrile into the water phase. For example, if you normally run 5% to 80% B, add 5% acetonitrile to the A reservoir, then start your gradient at "0%" B (actually 5%). This will help to overcome the initial mixing problem when pure organic solvent and pure water are mixed.

COLUMN LOADING

Q: I'm running a normal-phase method to separate my sample on a silica column. When I make the first injection, the peak is strongly retained and barely comes off the column. By the fifth or sixth injection, the retention time stabilizes to a reasonable value, but the peak begins to look broad and "lumpy." Why does the retention behave like this? I suspect that my compound of interest is degrading on the column, causing the poor peak shape. How can I verify this?

JWD: First, the retention-time shifts that you see are a result of deactivating the column. Your first few injections result in a masking of the strong retention sites for your sample. Once these sites are deactivated, the retention stabilizes. You could probably accomplish this deactivation by adding a similar compound to the mobile phase to provide a steady flow of the site-masking compound through the column. Some workers load their columns at the beginning of each day by making several large injections in a row without waiting for the sample to elute between injections. This procedure may speed the loading phenomenon that you observe.

Breakdown of your compound on the column is a possibility, but column overload may be the culprit instead. First, try reducing the injection volume by a factor of 10 to reduce the sample mass. The resultant chromatogram should look better (after making the appropriate adjustment in detector attenuation) if overload is the problem. If sample breakdown is the problem, no improvement should be observed.

You can verify sample breakdown by changing the conditions to encourage or discourage the chemical reaction causing the breakdown. Increase the residence time of the sample on the column by making an injection, stopping the flow for several minutes, and then starting the flow again. If breakdown is a problem, the "product" peaks should increase under these conditions. Alternatively, increase the flow rate to reduce the residence time, and the peak for the original sample should be sharper. If after doing these experiments you determine that sample breakdown is the problem, I suggest changing to a less reactive column, such as a normal bonded-phase column (for example, cyano) or a C1 phase (which can act like a deactivated silica) to see whether improvements occur.

REFERENCE

(1) J. Vendrell and F.X. Aviles, *J. Chromatogr.* **356,** 420 (1986).

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Lafayette, California, USA, and is a member of the Editorial Advisory Board of LC•GC. *Correspondence concerning this column can be sent to "LC Troubleshooting,"* LC•GC, *P.O. Box 10460, Eugene, OR 97440, USA.*