LC TROUBLESHOOTING

What's Happening to My Column?

Some methods just do strange things to your column.

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This month's installment of "LC Troubleshooting" focuses on two column-related problems reported by readers. The first deals with a column that takes several injections to "settle down" for each batch of samples. The second relates to short column life due to early fouling of the column. Although both of these problems are not encountered with most liquid chromatography (LC) methods, they both appear often enough that we all should be aware of them. It is only a matter of time before you meet one of these problem types with one of your methods.

Variable Peak Areas

Nothing seems unusual about the first problem. A gradient separation is used, running from 90:10 to 10:90 0.1% phosphoric acid–acetonitrile. The column is a well known C18 embedded polar phase, but also appeared on a C18 column from the same manufacturer, a C18 column from a second manufacturer, and a phenyl column from a third manufacturer. The sample is a small molecule drug dissolved in 75:25 methanol–water; 10 µL of a 30-µg/mL solution is injected. Detection is at 215 nm.

The problem relates to the peak areas of the first few injections. Peak areas typical of the first five injections are 3632, 6247, 6759, 6989, and 6980, in order. After about four or five injections, the peak area is constant, with a relative standard deviation (RSD) of <1% for subsequent injections. Furthermore, other analytes in the same runs show an RSD of <1% including the first five injections. In other words, the response of one analyte increases to a plateau over several injections, whereas other analytes have a stable response from the outset.

Priming injections: The requirement of making several injections before retention and area stabilize is more common for large molecules (proteins, peptides, and so forth) than for small molecules (<1000 Da), but I have seen plenty of cases in which this process is necessary with small molecule analytes. This process is variously referred to as "doping," "priming," "saturating," and other terms that describe the buildup of sample on the column before the results stabilize.

Let's take a look at the likely causes for this phenomenon. It is easy to think of a C18 column as a silica surface that is completely covered with C18 hydrocarbon chains. We think of this working strictly as a reversed-phase surface where more hydrophobic molecules are attracted more strongly than hydrophilic ones. This is indeed the mechanism that dominates reversed-phase retention, but it is not the only process going on. The silica particle surface is covered with -Si-OH groups that we refer to as silanols. The C18 phase is bonded to the surface, typically through a silyl ether bond (-Si-O-Si-), and because of the bulkiness of the C18 groups, only about half of the silanols can be reacted before there is too much crowding at the surface to allow more C18 groups to attach. The unreacted silanols are called residual silanols. Some manufacturers use a process called endcapping to attach a trimethyl-silyl group to some of the residual silanols,

but even then approximately half of the silanols remain unbonded. In the older, Type-A silica columns that dominated the column market before the early 1990s, these residual silanols tended to be acidic and often served as cation-exchange sites. These were a primary cause of the strong peak tailing that was common for basic compounds run on these columns. Tailing was due to the slower equilibration and easy overloadability of the silanols. One common practice to minimize tailing was to add 25 mM triethylamine to the mobile phase. Triethylamine interacted more strongly with the silanols than did most analytes, so this unwanted secondary reaction with sample components was minimized and tailing was reduced. Today's Type-B silica columns use much higher purity silica than the Type-A columns and proprietary treatments of the silica result in a much less acidic surface, with correspondingly fewer peak-tailing problems. In fact, the silanol activity of Type-B silica is sufficiently low that triethylamine is rarely used today. However, as every column manufacturer knows, if you pick a sufficiently basic compound and use a mobile phase pH of 6–8, you will see some peak tailing on even the highest purity columns available.

A further problem with the Type-A silica was the presence of significant concentrations of metals, such as iron and aluminum, which offered anion-exchange sites for acids and tended to increase the acidity of silanols. In some cases, tailing for acids was seen for these materials, although it was not as common as base-tailing. Type-B silica has very low concentrations of metals, so most of the metal-related problems are gone. The high surface area of the stainless steel frits at the column inlet and outlet provides additional sites of chemical interaction for some molecules.

So what! So, how does all this surface chemistry relate to the problem of increasing peak areas for one compound and not for others in the same sample? First, consider the well behaved analytes. These compounds likely are retained primarily by hydrophobic interactions with the C18 phase and have minor or weak interactions with the silanol groups. Thus, from the outset they behave in a consistent manner. It is likely that the problem analyte (which I suspect is somewhat basic in nature) interacts strongly with some site on the column, such as the silanols, metals, or some other undefined interaction. There will be a finite number of these strong adsorption sites on any column, and if the interaction is very slow, once the sites are saturated, there will be no net change in availability of the sites. This would explain the pattern observed. Many molecules of analyte in the first injection are "soaked up" by the active sites, making fewer available for the next injection, and so forth. After a few injections, the sites are sufficiently saturated that no net change takes place with each successive injection, so peak area stabilizes. If the column were to run for several hours without an injection or were left without mobile phase flow for several hours, the adsorbed analyte would equilibrate with the mobile phase and some of it would wash out when the system was restarted. This would expose some of the active sites so that the saturation process would be repeated the next time the column was used.

Thus, you can see that the equilibration process can be very rapid, as is the case with most compounds, with no change in peak area from the first injection. At the other extreme would be the injection of a compound that permanently adsorbed on the column and never came out, but once the active sites were saturated, the compound would flow through the column as is the case in frontal analysis. Fortunately, most compounds in reversed-phase separations fall in the first category and the problem compounds usually saturate the active sites within a few injections.

The solution: There are several ways to solve the problem. The easiest might be to do nothing and just add four or five priming injections of a high-level standard before running system suitability samples. In my experience, the process is related more to mass-on-column than to time, so either a large injection of a normal-concentration standard or a small injection of a high-concentration standard usually will speed up the process. For example, I expect that the reader has a stock solution of analyte at 30 or 300 µg/mL — making just one 10-µL injection of this high-concentration standard might be sufficient. Still another option is to make five injections, one right after another, without waiting for the gradient to run. Then run the gradient after the five injections. Any one of these practices is likely to solve the problem. (The reader tried injecting a large volume of sample and found that the column was stabilized by the second injection.) So the bottom line is that, although priming injections usually are not necessary, they are common enough to be well accepted in the LC community.

Early Column Demise

The second column problem has symptoms that are more or less the opposite of the first one. The response starts off in a satisfactory manner then disappears after about 50 injections. The analysis is performed on a Type-B C8 column at 40 °C with a gradient from water–methanol to water–tetrahydrofuran, both containing 0.05% trifluoroacetic acid. A guard column is used. The sample is a hindered amine that is in a polymer extract. The samples are dissolved in toluene and precipitate in methanol. All samples are filtered before injection. The analyte has no UV chromophore, so a chemiluminescent nitrogen detector is used. This detector responds only to nitrogen, which means that no nitrogen-containing compounds can be in the mobile phase. Thus, acetonitrile is not allowed as a mobile phase component.

After about 50 injections, the peak for the amine analyte disappears. This is somewhat dependent upon the type of polymer that was extracted, with acid-containing polymers being the worst. The same problem occurred for four or five consecutive columns. The reader hypothesized that the polymer built up on the column over time and irreversibly bound the amine. Washing the column with tetrahydrofuran or methylene chloride does not help, nor does replacement of the guard column. He speculated that a wash with strong acid might help, but was hesitant because of the potential for irreversible damage to the column.

Column cleaning: Those of you who read this column regularly know that I am a strong proponent of considering the LC column as a consumable item. Generally, columns will last 500–1000 injections or more. At this point, the cost of the column amounts to just a few percent of the total cost of analysis (amortized instrumentation, solvent purchase and disposal, sample preparation, labor, and so forth). In such cases, any efforts to restore a failed column, other than a simple solvent flush, usually are not cost-effective. However, in the present case, a 50-injection column lifetime is too short and justifies some time spent trying to solve the problem.

A generic column-cleaning procedure for reversed-phase columns is to wash with successive 50-mL aliquots of aqueous mobile phase, then 100% acetonitrile. If this is unsuccessful, an additional wash with methylene chloride can be helpful to remove very hydrophobic materials. Be sure to wash back through acetonitrile to remove all the methylene chloride before using an aqueous mobile phase again. If you know of specific solvents that will solubilize your sample components, there is no harm in trying them — just remember to use solvents in an sequence such that each solvent is fully soluble in the prior one.

As a general rule, today's silica-based reversed-phase columns can tolerate a mobile phase pH of 2–8. For short-term exposure, mobile phase pH outside these limits can be used (I remember trying to damage a column intentionally once by washing with 10 mL of near-saturated sodium hydroxide — it had no deleterious effect). Often a low- or high-pH wash will help remove components strongly bound to the column.

There is another recipe that I recommend for removing ion-pairing reagents from the column. This is a 100-mL wash with 200-mM phosphate buffer, pH 6, mixed 50:50 with methanol. The high salt, intermediate organic combination seems to be especially effective at removing ion pairing reagents. However, if you use this, be very careful to avoid conditions that might precipitate the buffer. Wash the column with 50:50 methanol–water before and after treatment. I suspected that this recipe might be useful in the present case, because the acidic polymers can adsorb to the column in a similar manner to ion pairing reagents, leaving the acid group exposed for ion pairing with the amine analyte.

In the present case, I suggested to first try several different solvents to wash the column. As far as I know, you can't hurt a column by washing it with solvents, so pick the solvent that is most likely to dissolve the polymer. If this is not effective, the next step would be to try a strongly acidic mobile phase, such as 0.2% trifluoroacetic acid in tetrahydrofuran. Or 0.1 M sodium hydroxide in tetrahydrofuran. If there are any doubts about solubility of washing solutions, test miscibility in a test tube first. Finally, if these do not work, try the ion-pairing flush. The nice thing about the present problem is that the columns are ruined already, so there is no danger of further damaging them by experimental washing procedures. Just be sure to disconnect the detector before flushing so that nothing is washed into the detector inadvertently.

This situation reminds me of a method I used to analyze a basic drug in a tablet formulation. The method required an extraction procedure to remove interferences. The tablet was dissolved in a high-pH aqueous solvent, which converted the drug to its non-ionized form. This solution was extracted with methyl-*t*-butyl ether, so the drug

partitioned into the organic solvent, leaving aqueous-soluble interferences behind. However, a polymer in the formulation also extracted into the organic phase, so a back extraction was performed by shaking the sample with 0.1 N hydrochloric acid and the now-ionized form of the drug partitioned into the aqueous phase, leaving the polymer in the organic. A similar cleanup process might apply in the present case to help remove residual polymer from the sample before injection. A lower polymer load in the injected sample should extend column life.

The results: The reader first tried flushing with a combination of methylene chloride and 0.2% trifluoroacetic acid. This appeared to remove some of the contaminant, as evidenced by a return of approximately half the response of the amine. Next, a mixture of 0.2% trifluoroacetic acid in toluene was tried and 100% of the amine response was recovered. It is clear that a few hours trying various wash procedures was well worth the effort. Now a routine flushing with the 0.2% trifluoroacetic acid–toluene wash solvent can be incorporated in the method at the end of each batch of samples.

Conclusions

We've looked at two problems that, at first, appear to be totally unrelated. However, in both cases, the primary analytical technique relied on reversed-phase separation of a basic analyte and was confounded by unwanted interactions with the column. It is tempting to view the reversed-phase LC process as simply one of hydrophobic interaction between the analytes and the bonded phase. As was discussed previously, however, there are several other interactions that can confound the simple reversed-phase model. You might consider silanol interactions as totally unwanted, but the presence of silanol groups plays an important part in reversed-phase selectivity — many separations are performed routinely on silica-based reversed-phase columns that would be difficult or impossible on columns based upon polymeric particles.

In the first case, we found that it was necessary to stick some of the desired analyte on the column to get a consistent response. In contrast, immobilizing too much material on the column, as in the second case, also can cause problems. Thus, we see once again that it is important to understand the chemistry of the column and of the sample components. Armed with this knowledge, we usually can adjust conditions so that we can get satisfactory performance from the column.

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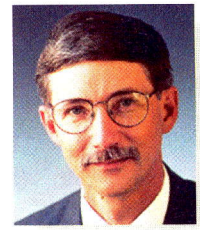

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