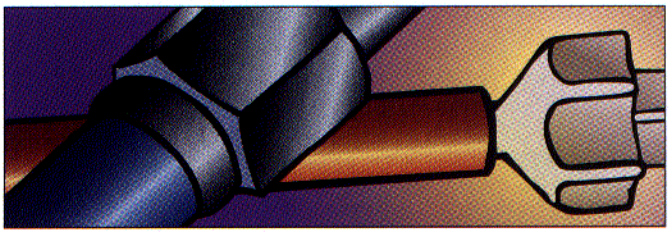
LC Troubleshooting



Readers' Questions about Mobile-Phase Additives

John W. Dolan

What you put in a column doesn't always come out the way you expect.

This month's "LC Troubleshooting" column contains several questions related to mobile-phase additives. Several readers mention their problems with mobile-phase additives, and another reader requests help in isolating suspected sample degradation on the column.

REMOVING ION-PAIRING REAGENTS

Q: I use a mobile phase that contains octylamine (40 mM), acetonitrile (12%), and water adjusted to pH 3.0 with phosphoric acid. I have a hard time removing this mobile phase from my column, and some carryover in the liquid chromatography (LC) system seems to occur as well. What should I use to wash out the mobile phase? I have tried 50:50 (v/v) water–methanol and the above mobile phase without the octylamine with limited success.

JWD: First, I hope you are adjusting the pH of the mobile phase before adding the acetonitrile. Adjusting the pH after you add acetonitrile will give you inconsistent results because the pH meter won't give reliable readings when organic solvent is present. You should adjust the pH of the aqueous phase and then add the organic solvent, ignoring what happens to the apparent pH.

Regarding your desire to remove ion-pairing reagents such as octylamine, I have found that a mixture of 50:50 (v/v) organic solvent–buffer is quite effective. I normally use methanol for ion-pairing applications because of its better solubility characteristics, but acetonitrile also should work. For buffer, use 100 mM phosphate adjusted to pH 6. This combination of intermediate pH, intermediate organic solvent, and relatively high buffer concentration seems to be effective at removing ion-pairing reagents. Because buffers commonly precipitate in acetonitrile, I would premix and then filter the flushing mixture rather than relying on the LC system for mixing. Flush the system with 20 column volumes (approximately 50 mL for a 25-cm column) to remove the old ion-pairing reagent.

To obtain the best results, after you have used ion-pairing reagents with a column, you should never use that column in any mode except ion pairing. You may never be able to wash all the ion-pairing reagent from the column, and, as a result, separations in other modes may be compromised and exhibit abnormal selectivity. You should be able to change from one ion-pairing reagent to another safely, but don't use the column without an ion-pairing reagent.

SEPARATION OF BASIC COMPOUNDS

Q: I need to develop a method for some strongly basic organic compounds, and I am not sure where to start. Could you give me some pointers?

JWD: When working with ionic compounds, our first choice is to use conditions under which the compounds are not ionized. With acids, you can lower the pH to 2–3 and start there. With many bases, however, the mobile phase must have a pH that is higher than 8 to suppress ionization. At high pH, the silica in the column will dissolve, so this option is not good. I usually take a threefold approach to the analysis of bases.

First, start with a column that is likely to give you the best results. The newer, type B silica columns designed for use with basic compounds are the first choice. Many of these columns are commercially available; they often are described as base deactivated or basic, with accompanying recommendations for use with basic compounds. Use one of these columns with a C8 or C18 bonded phase.

Second, because it is impractical to operate at a pH high enough to suppress ionization of the bases, work at a low pH to suppress the ionization of the silanol groups on the silica surface. Starting at pH 2.5–3.0 is reasonable when you use 25 mM phosphate buffer.

Third, optimize the separation of the sample first using low-pH conditions with acetonitrile or methanol. Vary the organic solvent content systematically to get the best separation under these conditions. You can use a stepwise isocratic optimization starting at 100% organic and decrease the organic concentration in 10% steps until the retention is reasonable, then make minor adjustments to fine-tune the mobile phase. An easier alterative is to use one of the software packages designed to assist method development.

After you have determined the best low-pH separation, you probably will find that the bases still are eluted too early. Next you should explore using ion-pairing reagents. Add hexane sulfonate to the mobile phase at approximately 50 mM to see the effect of ion pairing. You should see the retention times of the basic peaks increase. Next you will need to fine-tune the important variables: ion-pairing concentration and pH.

When you validate your method, you should check the influence of percent organic solvent, ion-pairing reagent, and pH to be sure you know what will happen if any of these variables change.

Some additional pointers for ion pairing may be useful. To remove the ion-pairing reagent, follow the instructions in the answer to the previous reader's question. Ion-pairing mobile phases equilibrate much slower than traditional reversed-phases, so you should allow at least 20 column volumes for equilibration (roughly 50 mL of mobile phase for a 25-cm column). Because ion pairing is very sensitive to equilibrium changes, you should thermostat the column and use small injections ($\leq$50 µL) with mobile phase as the injection solvent. For additional advice on developing an ion-pairing method, consult section 9.5 of reference 1.

POTASSIUM CHLORIDE VS. TRIETHYLAMINE

Q: I have heard that potassium chloride is a reasonable alternate for triethylamine as an additive to get better peak shape for basic compounds. Is there any truth to this?

JWD: I have not heard of this approach, but I'm not surprised that it has some effect. Triethylamine improves peak shape by interacting strongly with the acidic silanol groups on the silica surface. Overloading the surface with triethylamine reduces sec-

ondary retention of other amines and thus improves tailing.

Peak tailing also is improved by lowering the pH of the mobile phase with a buffer such as potassium phosphate. The low pH suppresses the ionization of the silanols, and the potassium acts as an ion-exchange counterion to also reduce silanol activity. The normal ionic strength order holds here, so potassium is a stronger counterion than sodium, which is why potassium phosphate provides somewhat improved results compared with sodium phosphate as a buffer in reversed-phase LC.

With the above interactions in mind, it seems reasonable that potassium chloride would have some positive effect in reducing silanol interactions, but I'll stick with low pH plus triethylamine.

ON-COLUMN DEGRADATION

Q: I suspect that my sample is degrading on the column because I see a strange peak shape. Only one peak is problematic, and it has a partially resolved peak on the front side. How can I determine if this situation really is degradation?

JWD: Before you begin hunting for on-column degradation sources, confirm that the peak-shape problem is unrelated to the injection solvent, a blocked frit, or a column void. Try injecting a smaller sample volume. An injection of 20–50 µL of sample using mobile phase as the injection solvent should be acceptable, but if mass overloading is a problem, even this injection volume could be a problem. Try reducing the mass of sample injected by a factor of 10. To check for column problems, replace the column with a new one and see if the problem persists.

As a last check, be sure that degradation is not occurring before the sample reaches the column. Degradation of delicate samples can occur while they sit on the autosampler awaiting injection. A cooled autosampler tray may help.

After you have eliminated the obvious alternatives, you should look for sources of on-column degradation. An easy way to identify on-column degradation is to change the operating conditions so that degradation will be accentuated. You may already suspect what is causing the degradation, and you should change this variable first. The time the sample is in the column can be important if the column is acting as a catalyst to accelerate degradation. You can examine this variable by reducing the flow rate to allow the peak to stay on the column longer. A twofold reduction in flow rate should increase the degradation if the on-column time is the cause. Stopping the flow for 1–2 min after injection and restarting is an alternative, but this procedure can produce confusing results because of system disturbance during start up.

Temperature is another likely problem source. Increase the temperature by 10 °C and rerun the separation. Again, you should see increased degradation if temperature is the source of the problem. Other variables to examine include mobile phase pH and any additives.

The column inlet frit can be a source of unwanted reactions on the column. You may be able to replace the stainless steel frit on the column with a PEEK or titanium frit. If you replace the frit, be sure that the new frit is exactly the same size as the old one. If the frit is too thin, the column packing may be disturbed, and if the column is reversed, packing could leak from the column.

After you have identified the source of degradation, you will have to modify your method to minimize the impact of degradation. Two alternatives are to change the conditions so that degradation no longer occurs or to change them so that sample reaction goes to completion. For example, you may be able to perform the separation at a higher temperature and shift the reaction so that only the end product is detected.

BASELINE PROBLEMS WITH TFA

Q: I use trifluoroacetic acid in my mobile phase with UV detection at 210 nm. I obtain a blank baseline when I first open a bottle of trifluoroacetic acid, but after several weeks, the baseline contains spurious peaks. How can I avoid obtaining these peaks?

JWD: In my experience, trifluoroacetic acid is rather unstable from a chromatographic interference standpoint. After a bottle has been opened and exposed to air, the contents begin to degrade. This process can be slowed by reducing exposure to air (oxygen). I have tried using a nitrogen-filled glove bag with some success. The present practice in my laboratory is to purchase trifluoroacetic acid in 1-mL sealed ampuls. Although these ampuls are more expensive than bulk trifluoroacetic acid, they eliminate the type of problems you have experienced. Sometimes the quality of trifluoroacetic acid varies between vendors, so you should test reagents from more than one source to find the best one for your application.

DEGASSING TIP

I recently heard of an interesting twist on degassing. Some workers had a sample that was sensitive to oxygen, so they needed to remove the oxygen from the mobile phase. In addition, they were working with a poorly retained compound that was eluted very close to the column dead time. With helium sparging and a refractive index detector, they observed a large refractive index positive–negative peak pair at the injection point, presumably resulting from a mismatch between the sample and mobile phase. By sparging the mobile phase with nitrogen and then vacuum degassing with an on-line degasser, they were able to eliminate the oxygen and reduce the nitrogen below the level that caused pump problems. The result was a mobile phase that matched the sample closely in refractive index and nearly eliminated the column dead time disturbance.

REFERENCE

(1) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley & Sons, New York, 1988), pp. 227–251.

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of the Editorial Advisory Board of LC•GC. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC, *859 Willamette Street, Eugene, OR 97401, e-mail 76631.2547@compuserve.com.*