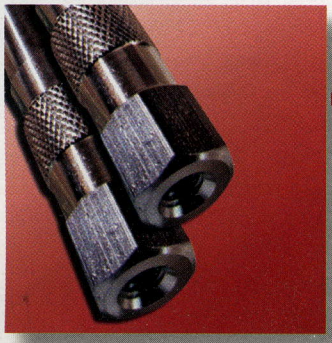

LC TROUBLESHOOTING

System Contamination

Deterioration of the separation can result from many causes. This month's column looks at some techniques to isolate the problem source.

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LC Troubleshooting Editor

Often I am asked how I come up with ideas for "LC Troubleshooting" each month. Usually the problem is not finding a topic, but sorting through possible topics. One of the prime sources of fodder is the regular e-mail inquiries I get from readers. This month's topic is based upon one of these e-mails. As often is the case, the inquiry came loaded with specifics that, although not proprietary, were not intended for public consumption, so I'll generalize a bit on the exact conditions. The inquiry went something like this:

"I've been using a C18 column to analyze phenylisothiocyanate (PITC)-derivatized amino acids from protein hydrolysates. A gradient separation is used with sodium acetate as the A-solvent and acetonitrile as B, running to 100% B over 35 min. Recently, I have been seeing a large baseline disturbance toward the end of the run that interferes with the separation and eventually renders the column unusable. I have replaced all the reagents, the guard column, and the column, but the contaminant is not eliminated.

I do not get regular access to the liquid chromatograph, and because I'm the only person using it, it often is inactive for several days between uses. It appears that the problem peak is always largest in the first few runs of the day and decreases as successive samples are run, although it never goes away completely. The peak is much worse if a blank is injected than if the gradient is run with no injection. I flush the column with 80% B at the end of each batch of samples. I know little of the LC system's history, but I can guess that it has been a long time since any part of the system has been serviced. Can you help me find the source of this problem and eliminate it?"

Service First

The most alarming statement in the entire scenario is in the next to last sentence, where the lack of service history is mentioned. Here, I would follow the advice I've heard given for guitar players — if you can't remember when you last changed the strings, they are overdue for a change. Similarly, if you can't remember when you last serviced your liquid chromatography (LC) system, it is overdue, also. You've read the rule-of-one here many times: Change just one thing at a time. This rule is used to help isolate a problem, because it allows us to avoid a shotgun approach to troubleshooting that might fix the problem, but doesn't give us much information about the problem source. We'll use this rule later in the problem isolation scenario. However, here's one time that I recommend ignoring the rule-of-one and going for a thorough system service. Here are some essential service items and system checks that I recommend.

Reservoir: The mobile phase reservoirs should be cleaned or replaced. This is especially true for the A-reservoir, because acetate is such a good nutrient source for growing microorganisms. Next, I would replace the

inlet frits in the reservoirs (I like ≥10 µm porosity frits for minimum flow restriction). These last a long time, and might be perfectly good, but with an unknown history, they can be a source of contaminants. Once the frits have been replaced, perform a siphon test to ensure that the tubing connecting the reservoir to the pump is unrestricted. Just follow the tubing downstream from the reservoir to the first low-pressure fitting in line, usually at the mixing manifold (low-pressure-mixing systems) or pump inlet (high-pressure mixing). Start a siphon flow. I like to see at least 10 times the required solvent flow when the reservoirs are mounted 0.5 m or more above the pump. For example, if you normally run 1–2 mL/min, you should observe at least 20 mL/min of siphon flow to ensure the pump has plenty of solvent available. If the flow is too low, find the restriction and correct the problem. Finally, take a close look at the solvent transfer tubing between the reservoirs and the pump, especially from the aqueous reservoir (A-solvent), to be sure there is no visible contamination — replace any suspect tubing.

Pump: Replace the piston seals. These should be replaced once a year, whether they need it or not. Worn pump seals can result in leaks, inaccurate or irregular flow rates, pressure fluctuations, and shedding of particles that can block frits downstream or interfere with check-valve operation. Check the owner's manual for the pump and determine if there are any internal frits that should be replaced. The most common place for these are as part of the check-valve assembly between the two pump chambers on an accumulator style pump or at the pump outlet on many systems.

Autosampler: Consult the operator's manual for specific service recommendations for your autosampler, but there are some universal maintenance items that should be considered. Clean or replace the wash-solvent reservoir and replace the inlet frit for the delivery tubing, for the same reasons as mentioned for the solvent reservoirs previously. It is easy to forget to clean or change the wash reservoir, because so little wash solvent is used, but it is best to perform this task on a weekly or biweekly basis. Check the delivery tubing for restrictions. If the wash-solvent delivery pump is a serviceable item, replace the seal or perform other recommended maintenance activities. Thoroughly clean the interior of the autosampler. There often are drips inside that leave contaminating residues. It might be prudent to replace the needle seal, or at least to take the seal assembly apart and clean it in a sonicator.

Now that you've serviced the instrument, you hopefully have corrected the problem. We can go back to the rule-of-one and make changes one at a time to help isolate the problem, if it remains.

The brand of autosampler that was mentioned uses a traditional six-port valve with a sample loop mounted on it for either filled-loop or partially filled–loop injection. Although the injection valve rotor can last >100,000 cycles, there is no way to know its history in the current case, and a worn rotor can be the source of sample carryover. For this reason, I would replace the rotor seal. Be sure to label each of the pieces of tubing connecting to the valve and draw a diagram to guide proper reassembly. Sonicate all the valve parts in methanol to help clean them, install a new rotor, and reassemble the valve. Take particular care to ensure that all the tubing connections are seated properly. Any small gaps between the tube ends and the injector body can serve as tiny reservoirs to cause sample carryover.

Detector: Most detectors do not require routine servicing, but it would be wise to check the operator's manual for any recommendations for your particular detector. Detector cell contamination is unlikely to be the source of the present problem, and cell-cleaning techniques (other than flushing with mobile phase at the end of a batch of samples) can be more likely to damage the detector than to correct nonexistent problems.

Performance Tests: At this point, it would be a good idea to run a system performance check, such as that described in reference 1 (available in the archives section of www.chromatographyonline.com). I like to run this test once or twice a year to ensure that the LC system is performing as it should. Correct any additional problems that are highlighted by these tests.

Documentation: Before you put the instrument back in service, document the service activities that you performed. I find that this is best done in a notebook that is kept with the system. I prefer a looseleaf notebook that can have sections for different maintenance and testing activities. In each section, I keep several blank copies of various record forms that I have created to speed record keeping as well as later review of the data. Another option is to use a bound notebook, such as a laboratory notebook or theme book. If the notebook is kept with the instrument, it is handy for making notes or checking on past maintenance, and it is more likely to get passed on to the next user.

Problem Isolation

Now that you've serviced the instrument, you hopefully have corrected the problem. We can go back to the rule-of-one and make changes one at a time to help isolate the problem, if it remains. Make up fresh mobile phase and equilibrate the column. I would start with two or three blank, no-injection gradients to see if the problem peak is present or not. If it is present, it is unlikely that it comes from the autosampler, but just to be sure, bypass the autosampler by plumbing the pump directly to the column and repeat several no-injection gradients.

If the problem persists without the

autosampler in the flow path, contaminated mobile phase is a possible problem source. Usually, it is the A-solvent that is the problem. Test this by making two no-injection blanks with the normal equilibration between runs followed by a run with equilibration three times as long before the gradient is run. Compare the second and third runs. If the problem is associated with the A-solvent, the background peaks in the third (long equilibration) run should be approximately three times as large. If this is the case, try substituting each of the mobile-phase components (including water) for new ones, one at a time. Do not neglect the possibility of inadvertent contamination of the mobile phase from the pH meter. This problem, although not common, can occur if the pH probe is dipped in the bulk buffer while adjusting the pH (2). Eliminate the possibility of this contamination source by checking the pH of an aliquot of buffer, then discarding the aliquot, rather than dipping the probe into the bulk mobile phase.

If the problem persists only when an injection is made, you need to isolate the problem source within the autosampler. I would try injecting different volumes of a blank sample to determine if the problem is associated with the sample or some other part of the autosampler. For example, you can inject 10, 20, and 30 µL of blank and see if the problem peak grows in proportion to the volume. If this occurs, it is something in the blank that is causing the problem. Once again, you'll have to isolate the problem source by substituting each of the reagents used to make the blank with fresh ones until you eliminate the problem.

Another area to be aware of is inadvertent contamination of your samples or mobile phase from dirty glassware. I know this is a particular problem with some of the protein hydrolysis procedures and subsequent PITC derivatization. For example, fingerprints or other organic matter can be a source of contamination. Glassware that has not been thoroughly rinsed is another possible source of contamination from detergent residues. Some workers rinse all their glassware in acid during the washing process and then rinse with organic solvent before use. A contaminated solvent filtration apparatus can be a problem. This is checked easily by preparing mobile phase without filtration.

Finally, adjustment of your LC method can help to convert this problem to the nonproblem category. You indicated that the large baseline disturbance occurred near the end of the run. I wonder if it is possible that this represents something that is not completely eluted from the column. Thus, it builds up over time as part of it is eluted in one run and part in the next, along with the contaminant from that run. It might be possible to eliminate the problem by placing a hold at the end of the run when you are pumping 100% B. This can allow sufficient time for the problem hump to be eluted. Alternatively, try using a stronger wash cycle at the end of each run. For example, rather than stopping at 80% acetonitrile, increase the solvent strength to 90% or even 100% acetonitrile. This, of course, will require using a stronger B-solvent and adjustment of the gradient program to deliver the same gradient used to elute the sample components.

Conclusions

Although I have not listed every possible cause of the reported problem, I suspect the problem will be isolated and eliminated by the time all the procedures listed earlier are performed. The key to effective problem isolation is to use a systematic procedure so that you can identify cause and effect when a change is made and the problem is eliminated.

More Information Needed

In Jennifer Birchett's recent contribution to "LC Troubleshooting" (3), she mentioned finding microbial growth in the tubing transporting the aqueous phase from the reservoir to the pump (see Figure 1 of reference 3). Since that time, I have had two students in my short courses complaining of similar problems.

This is something that I have never encountered, so I wonder how widespread the problem is. And how do you correct it? Tubing replacement is an obvious answer. Flushing with dilute nitric acid (with the column removed!), as when passivating the system also should be effective. What about dilute household bleach (also without the column)? I would be interested in feedback from you, the readership, on this topic. Do you ever observe it? How do you correct the problem? E-mail me at John.Dolan@LCResources.com and put "tubing" in the subject line.

Thanks.

References

(1) G. Hall and J.W. Dolan, *LCGC North America* **20**(9), 842–848 (2002).

(2) M.D. Nelson and J.W. Dolan, *LCGC North America* **16**(10), 992–996 (1998).

(3) J. Birchett and J.W. Dolan, *LCGC North America* **28**(4), 292–300 (2010).

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For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.org.