LC Troubleshooting

There's always one more question.

Now What's Going On?

Most chromatographers can expect to encounter certain problems at one time or another during the course of their work. For example, what about those mysterious negative peaks that appear from time to time? Or an increase in column pressure accompanied by peak distortion? Or a gradual increase in peak width over time? This month's installment of "LC Troubleshooting" addresses each of these problems with specific examples from readers' laboratories.

Negative Peaks

Q: Can you suggest the source of a negative peak in my chromatograms? I'm using a C18 column with a mobile phase of 97:3 (v/v) phosphate (25 mM, pH 2.5)–acetonitrile and UV detection at 205 nm. When I make a blank injection, I see a small negative peak at nearly the same retention time as one of the peaks of interest in my separation.

A: Sometimes I wonder why negative peaks are not encountered more often in liquid chromatography (LC) separations. A negative peak is simply the elution of a compound that generates less detector response than the mobile-phase baseline. In your case, the source of the problem peak is not related specifically to the sample, as you've shown by making a blank injection. You could try to further isolate the source of the peak by separately injecting each component of the injection solvent. For example, if the sample is processed in several steps of solid-phase or liquid–liquid extraction, skip one or more of the steps and observe the resulting chromatogram. After you have isolated the source of the problem, you could try an alternate source or higher purity reagent and eliminate the problem peak.

Dissolved air in the injection solvent can be another source of unexpected peaks. This problem is easy to isolate — just degas the sample either with vacuum or by bubbling a little helium through the sample before injection. An air peak can behave in a manner similar to any other chromatographic peak, and although it might seem surprising at first, the retention of air peaks can change with a change in the chromatographic conditions.

Because the negative peak represents a chemical compound in the sample, it can be moved in a chromatogram in the same manner as any other peak. The problem is similar to any case in which users find two closely eluted peaks in a run. Try changing the percentage of organic solvent in the mobile phase by a few percent, adjusting the pH level by 0.5 pH units, or changing the column temperature by 5 °C. If a change in one of these variables affects the peak spacing between the negative peak and the peak of interest, use the information to further adjust the relative retention times.

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For example, if increasing the pH moves the peaks together and lowering the temperature pulls them apart, try decreasing the pH and lowering the temperature in combination to obtain the desired separation.

Some workers intentionally use conditions that generate negative peaks. This *indirect detection* can be useful for detecting compounds that have very poor UV absorbance when refractive index detection is unavailable and evaporative light-scattering detection cannot be used. To create conditions in which indirect detection can be used, add a nonretained UV–absorbing compound such as benzoic acid to the mobile phase. The UV–absorbing compound will elevate the baseline so that

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almost anything that is injected has a lower UV absorbance than the background, and negative peaks will result. Most data system software is not designed to handle peaks that drop more than approximately 10% below the baseline, so remember to select negative peak logic to electronically reverse the peak polarity.

Column Pressure Buildup

Q: I am using an LC method to determine nucleotides in cellular extracts. The column is a 125 mm × 4.6 mm, 3-µm d_p C8 column used with a 100% aqueous mobile phase of phosphate buffer (50 mM, pH 5.75) and a flow rate of 1.0 mL/min. I filter the mobile phase through a 0.45-µm porosity filter and degas it each day. Under these conditions at room temperature, the pressure on a new column is approximately 1500 psi. To avoid having to reequilibrate the column each day, I lower the flow rate to 0.2 mL/min and recirculate the mobile phase when the system is not in use. In a few days, the pressure increases to 1650 psi with no change in retention or separation quality. Then, from one day to the next, the pressure rises to more than 2000 psi, the peaks become distorted, and the retention decreases slightly. I have tried this separation using different brands of columns and have encountered the same problem. Can you give me some suggestions on how to correct this problem?

Don't forget to wash the mobile-phase reservoir occasionally. It is pointless to filter out bacteria only to reinoculate the mobile phase with bacterial residue from the reservoir walls.

A: Increased system pressure and split or distorted peaks are classic symptoms of a partially blocked frit at the head of the column. I suspect that the problem you have observed is the result of a buildup of microbial growth in the mobile phase. Aqueous buffers are notorious for being culture media for bacteria and mold. For this reason I strongly recommend that buffers be replaced at least on a weekly basis. By recirculating the mobile phase, you have created a system that continuously filters the mobile phase as microbial growth occurs. The problem with this kind of filtration is that the filter (in other words, the column) costs $500!

You can do several things to minimize the problem. First, reduce the length of time you use the buffer. For convenience, you might make a concentrated buffer solution and keep it in the refrigerator, dilute a portion of it, and adjust the pH to obtain the mobile phase needed each day. Pass the mobile phase through a 0.22-µm porosity membrane filter before use. The 0.45-µm filters commonly used for mobile-phase preparation are great for removing particulate matter, but the smaller 0.22-µm filter is necessary to remove bacteria. Be sure to select a filter material that is designed to work with aqueous solvents; otherwise you will find the filtration process painfully slow. And don't forget to wash the mobile-phase reservoir occasionally. It is pointless to filter out bacteria only to reinoculate the mobile phase with bacterial residue from the reservoir walls.

If you've been using the LC system only with this mobile phase, it is possible that the entire system is contaminated with microbial growth. I would remove the column and replace it with a piece of connecting tubing. Next flush out the buffer with clean water and then flush it with acetonitrile or methanol to sterilize it. Alternatively, you might want to use a dilute bleach solution. Flush back to water and then replace the column and add fresh mobile phase to a clean reservoir.

Another technique that can help prevent bacterial growth is adding a small amount (for example, 0.1% by volume) of sodium azide to the mobile phase. Generally, the azide will not affect the separation, but you should check to see if it causes any changes.

You didn't mention it, but I suspect that the problem you have observed is more pronounced with a 3-µm d_p column than with one containing 5-µm particles. This difference is not because of the particle size but rather the porosity of the frit used to hold the particles in the column. A 5-µm d_p column uses a 2-µm frit at each end, whereas a 3-µm particle column uses frits of 0.5-µm porosity — and these get blocked much more quickly. I recommend installing a 0.5-µm in-line filter between the autosampler and the column on every LC system. This frit will trap anything that otherwise would get stopped by the inlet frit on the column. It is easy and inexpensive to change. As soon as the system pressure begins to rise, replace the frit and put the system back in service.

Generally, microbial growth in the mobile phase is a problem only when the buffer is not replaced and the reservoir is not washed on a regular basis. When more than approximately 20–30% organic solvent is used in the mobile phase, chromatographers rarely observe microbial growth.

Editor's note: *In a follow-up communication, the reader shared that microbiological testing of the mobile phase showed severe bacterial contamination. The pressure problems have been eliminated by taking additional care in mobile-phase preparation, including filtration through a 0.22-µm filter, and ending the practice of recirculating the mobile phase.*

Separation Deterioration

Q: I was wondering if you could give me some ideas about why my separation gradually deteriorates from the first sample to the last one in a run sequence. I'm using a 150 mm × 4.6 mm, 3.5-µm d_p C8 column operated at 45 °C with a 65:35 50 mM ammonium formate–acetonitrile mobile phase and mass spectrometry (MS) detection. The peaks in the first run or two are sharp, but they broaden gradually as more runs are performed.

A: One possible cause of this problem is deterioration of the stationary phase over time, although the observed degradation is more rapid than I would suspect. You didn't mention the pH of the mobile phase; ammonium formate mobile phases can be used from the pH of formic acid (pK_a = 3.74) to the pH of ammonia (pK_a = 9.24), but it is used most commonly at pH levels higher than pH 6. At levels lower than pH 4, formic acid often is used alone, and acetate buffers are favored in the pH 4–6 range. In the pH 6–8 range, no good buffer is MS compatible, so ammonium formate often is used, even though it has little buffering capacity.

A loss of bonded phase can occur at pH levels lower than pH 2 by hydrolysis of the Si–O–Si bond linking the bonded phase to the silica support. You cannot obtain sufficiently low pH with ammonium formate to cause problems in this pH range. At pH levels higher than pH 8 (or pH 7 for some columns), the solubility of silica is sufficient

that the stationary phase can dissolve. Because this is a chemical process, the dissolution rate increases with temperature and with certain buffers — and it can vary from one brand of column to the next. Silica dissolution at high pH generally is less of a problem with the newer, Type B (high-purity) silica columns and with columns that are endcapped. The particular brand and model of column that you mentioned is a Type B endcapped column. I have used the same model of column occasionally under similar conditions without problem, so I suspect that the pH is not the source of your difficulty.

Another possible problem source is contamination of the column by sample components. For example, if you are using a protein precipitation procedure to prepare a plasma sample, the residual burden of proteins and other potential contaminants generally will be much larger than for samples prepared by solid-phase or liquid–liquid extraction. Try injecting a series of matrix-free standards to see if something in the sample matrix is causing the problem. If the matrix-free samples can be injected without the observed peak broadening, I would next try additional sample cleanup. If you are using protein precipitation by adding organic solvent for sample preparation, you might be able to add an additional precipitant such as zinc sulfate, which can provide more-effective precipitation. Then centrifuge or filter the samples before injection.

The problem also could be related to frit blockage, which I have discussed above. Using an in-line filter might help to mitigate the problem. A regular flushing with a strong solvent such as acetonitrile also might help to increase column life.

Conclusion

No magic bullet exists for avoiding or solving LC problems. Generally, I take the divide-and-conquer approach: pinpoint several possible problem sources, and try to eliminate one or more potential causes with a few simple experiments so that you can focus on the real problem. Sometimes the first step is to determine if the problem is related to the analytical method or to the instrument. You can make this determination by installing a new column and running the column manufacturer's test; if it passes, you know the instrument is working satisfactorily, and you can focus on the method. With method problems, it often helps to start by injecting a series of standards to determine if the problem is related to the separation conditions or to the sample. Think about the potential problem sources and try to design experiments that will eliminate a large number of potential causes, and then subdivide the remaining suspects until you find the root cause.

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