LC Troubleshooting

Questions about Ion Pairing

John W. Dolan

Do ion-pairing problems differ from other LC problems?

Recently, I was contacted by a reader who was experiencing problems with an ion-pairing liquid chromatography (LC) method. Ion-pairing LC is not as widely used as reversed-phase LC, but it plays a vital role because it often allows the isocratic separation of samples with a wide polarity range. This month's "LC Troubleshooting" column discusses some user problems that are widely applicable to practitioners of ion-pairing LC.

An analyst was using a method modified for the analysis of substituted adenosine derivatives. The method used a 25 cm × 4.6 mm C18 column operated at room temperature (about 25 °C) in an ion-pairing mode. The mobile phase was 89:11 (v/v) 80 mM triethylamine phosphate (pH 6.8)–methanol at a flow rate of 1.3 mL/min. The user observed several problems with the analysis. For ease of discussion, I'll present each problem separately, followed by suggestions for correcting the problem. As with many LC problems, several symptoms may appear simultaneously. These symptoms may stem from a single problem or several problems, so the suggestions may correct part of or the entire problem.

SYSTEM STABILITY

User: The mobile phase–column system seems to take forever to equilibrate. When I start with a fresh column, the baseline takes several hours to stabilize to a point where samples can be analyzed. As a result, I operate the system at 0.4 mL/min overnight so that I can use the system the following day with minimal additional equilibration. To clean the system or leave it for extended periods, I flush it with 50% methanol–water. However, whenever I flush the system, I then have to equilibrate it for several hours before I can use it again. Is there anything I can do to improve this situation? I experience similar problems after someone else has used the column for another assay, and I try to rerun my method.

John W. Dolan: Let me address your last statement first. I strongly believe (and all the data I have seen support this) that you should dedicate a column to a particular analytical method. There are two strong arguments for my belief. First, you reduce the risk of ruining the column by inadvertently contaminating it with materials from a foreign sample. Second, your column will last longer than one that is switched from method to method. You will use fewer columns in the long run if you assign a column to each method and use it until it fails. Of course, this is sometimes impossible because of budgetary constraints or a method's extremely low sample volume.

I think the slow equilibration problem is related to the nature of reversed-phase column packing materials. Because the reversed-phase surface is strongly hydrophobic, a mobile phase with low levels of organic solvent has difficulty wetting the bonded phase. One way of visualizing this is to think of the bonded-phase surface as either collapsed or extended C18 chains bound to the silica surface. Under totally aqueous conditions, the C18 chains will collapse, minimizing contact with the polar mobile phase. At the other extreme — the lowest energy condition in the presence of strong organic solvent — the chains will be fully extended with organic solvents having full access between the bonded-phase molecules. If you switch from strong organic solvent to strong aqueous solvent, the bonded phase can collapse and trap organic solvent. This trapped mobile phase equilibrates slowly, and it may be the source of your problem.

Several techniques can be used to speed equilibration. First, equilibration is determined by the volume of mobile phase passing through the column, not the time it takes for the mobile phase to pass. Therefore, you can often speed equilibration by increasing the flow rate — as long as you keep the pressure below the method limit (typically 2500 psi). My calculations indicate that the pressure is approximately 1900 psi for your conditions, so you could increase the flow rate to 2 mL/min (if you were willing to run the pressure up to approximately 2900 psi) and reduce the equilibration time by roughly one-third. After the system is equilibrated, return the flow to the normal setting. The system should then be ready to run samples.

A second way to speed equilibration is to increase the temperature. Elevated temperature may or may not improve your separation, but it certainly will reduce the equilibration time. I would try operating at 40 °C during equilibration to see if this would speed up the process. The change to 40 °C will reduce the pressure to approximately 1400 psi under standard conditions. This change will allow you to double the flow rate to 2.6 mL/min with a pressure of approximately 2800 psi. You can return to room temperature for normal operation.

Another technique is to equilibrate the column with a mobile phase containing a higher percentage of organic solvent. This would equilibrate the column with the ion-pairing reagent, yet retain sufficient organic solvent in the mobile phase to ensure rapid equilibration. After the ion-pairing reagent is equilibrated, you can adjust the organic-solvent content. For example, you can prepare two bottles of mobile phase. One would contain the standard 11% methanol ion-pairing mobile phase. The second reservoir would contain more methanol — for example, 50% methanol — with the same concentration of ion-pairing reagent. Equilibrate the system with the 50% methanol mobile phase. This should wet the bonded phase sufficiently to speed equilibration. Once the baseline stabilizes, run a gradient from 50% down to 11% methanol to equilibrate the column with the method's mobile phase. You will probably reduce the total equilibration time using this technique.

I preach that you should flush the column daily and never shut off the LC pump when it contains a buffered mobile phase. Flushing minimizes wear by preventing buffer deposits on pump pistons and other moving parts. You are following this practice by reducing the flow rate when the system is not in use. One way to avoid these restrictions is to leave mobile phase in the column and flush the rest of the system. To do this, just remove the column from the system and cap it tightly. Connect the

injector to the detector with a piece of tubing, then flush the system with nonbuffered mobile phase for storage. Before your next use, reverse the procedure. First flush the system with mobile phase, then hook up the column and reequilibrate it. This should also reduce the equilibration time. You will still need to flush the column occasionally to remove strongly retained material, but you may find removing the column for temporary storage more advantageous in the long run. Storing the column in mobile phase should not affect the column's life, unless the mobile phase actively degrades the column.

Another alternative is to reduce the flow to 0.1 mL/min and recycle the mobile phase. This will keep the system in operation yet will conserve solvent.

COLUMN-TO-COLUMN CHANGES

User: While I was optimizing an extraction procedure, I ran several hundred samples through one column with satisfactory separation. However, when the column died and I replaced it with a new column, the separation no longer worked. The column vendor assures me that they have not changed the column packing, yet I am stuck with a nonfunctional method. What can I do about this?

JWD: Before you rush out and change column vendors, consider that at least three things could affect your separation. First, the original column's chemistry probably changed while you were using it. Second, although the new column is nominally the same as the original, it may differ slightly. And third, your method may not be rugged against small changes. Let's look at each of these in more detail, then see if we can find a solution.

The surface chemistry of all columns changes over the life of the column. Because of the chemical nature of the column packing, bonded phase will be lost gradually and some of the silica support will dissolve. In addition, the column surface tends to collect contaminants over time. Regular column flushing only retards contaminant buildup — it doesn't prevent it. When you used the column to perfect the sample pretreatment, it became contaminated to some degree. Unless the pretreatment removed everything except the target solutes, the column was exposed to matrix materials that reversibly or irreversibly bound to the surface. So if you have gradually changed the column packing over its life, it is unsurprising that a nominally identical column yielded somewhat different separation results. Column-chemistry changes are normal and expected — you have to be prepared to adjust.

Column manufacturers work very hard to deliver a consistent product, but while consistency is possible, columns that are truly identical are rare. Consider some of the many variables that go into making a column: the raw materials and reagents, the bonding chemistry, the packing procedure, and the operator. Compound these variables with changes in the laboratory environment and equipment, and you can see why many consider column production a black art. The manufacturers keep tight controls on everything possible, but I have seen columns with consecutive serial numbers that yield significantly different separations. So column vendors try to ship columns with identical properties, but you should be prepared for small column-to-column or batch-to-batch changes that may affect your separation.

The final variable to consider is that your method may not be rugged against small changes in the column or other run conditions. You should have as rugged a method as possible before putting it into routine use. By rugged, I mean a method in which small and reasonable changes in operating conditions will not compromise the results. If you spend time initially ensuring method ruggedness you will spare yourself troubleshooting time down the line.

So what can you do to overcome these potential problems? Develop a rugged method. The first thing you need to do is to select a system-suitability test. The easiest system-suitability tests are performed with a calibrator, standard, or spiked sample. Set limits of resolution, retention, pressure, and other parameters important to you. Test the method to be sure it passes system suitability before you run samples so that you won't waste time gathering marginal or useless data. Next, determine how rugged the method is to reasonable changes. Ruggedness testing will also provide information about adjusting the method when it no longer passes your system suitability test.

Five obvious tests determine method ruggedness. These tests correspond to normal or meaningful changes in the major variables in the method. You may want to add other tests. First, look at the mobile phase. You should be able to control the methanol content within 1%, but testing for changes at 2–5% will provide more information about adjusting the methanol content to compensate for column changes. Similarly, vary the ion-pairing reagent 5–10 mM to see the impact. You are operating the column at room temperature, which may be okay, but you should check to see how a 5 °C change affects the separation. Similarly, vary the flow rate by 0.5 mL/min to see if it affects anything other than the retention time. Once you understand how each of these four variables influences the separation, you will be able to make minor adjustments in the operating conditions to compensate for gradual column deterioration or some other variable.

Finally, test the columns to determine batch-to-batch and column-to-column variability. Most people use three columns for this test — two columns from the same batch and one from a second batch of packing material. Be sure to specify these requirements when ordering the columns because you cannot always glean this information from the manufacturer's numbering system. Once you run your method on each of these columns, you will have a good idea how your separation will be affected by column replacement. If you can live with these changes, fine, but if they cause problems, you need to incorporate instructions in the method about how to compensate for column differences.

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Depending on the number of samples and the use of your method, you may want to test how the method fares with a different operator, when you move it to another instrument, or when you add any other significant stress to the method's normal operation.

SUMMARY

In the early days of ion-pairing LC in the 1970s, the technique gained a bad name. Analysts encountered numerous problems with short column lifetimes, irreproducible results, and unstable methods. In the last few years, however, ion pairing has regained credibility partly because better column-packing techniques have increased column stability. Our improved understanding of ion pairing has helped us develop operational techniques that can overcome problems such as slow column equilibration. You will obtain the best results with ion pairing if you thoroughly understand how each variable influences the separation and how to carefully control the conditions. As with all separation techniques, your columns will last longer if you dedicate one column to each method.

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