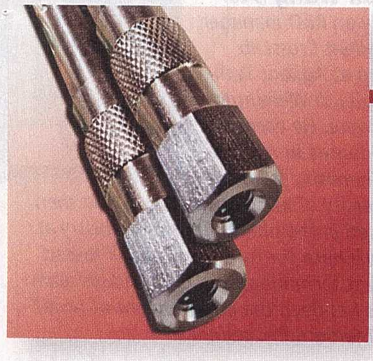

LC TROUBLESHOOTING

LC Column Problems Everywhere

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No matter where you live or who you work for, you're likely to encounter one or more of these liquid chromatography (LC) column problems at some point.

I recently returned from a tour of teaching liquid chromatography (LC) classes to users in Minnesota, the United Kingdom, Poland, and Malta. One thing that always impresses me on such trips is that no one group has a corner on the LC problem market. The same problems pop up in most laboratories, no matter where they are located, the role of the laboratory (for example, analytical, forensic, production, research), what industry is involved, or the brands of instrumentation used. As I teach, I tend to jot down questions as they come up, because these give me some of the topics for this "LC Troubleshooting" column. This month I share some of these question topics (and their answers) that are brought up in a majority of the classes I teach.

Column Regeneration

We would all like our LC columns to last forever, but that just isn't realistic. As I discussed in an earlier article (1), approximately three-quarters of all users get at least 500 injections through a column before it fails, and nearly half exceed 1000 injections. I've stated many times that I believe the break-even lifetime of a column is about 500 injections—with typical analysis costs, the contribution of a column to the per-sample cost is 2% or less with a 500-sample column lifetime.

One of the ways to maximize the column's lifetime is to prevent or correct for buildup of unwanted contaminants on the column. The column cleaning process often is called column regeneration. The simplest way to remove such materials is to *increase the eluting strength of the mobile-phase strong solvent*. For reversed-phase LC, the strong solvent usually is acetonitrile or methanol. When considering the effect of solvent strength in reversed-phase mode, I like to use the Rule of Three as a guideline. The Rule of Three states that the retention factor, *k*, will drop by a factor of about three for each 10% increase in the strong solvent (B-solvent or %B). For well-retained compounds, which is the case for strongly retained contaminants, we can consider that retention time will also change by threefold for a 10% change in %B. For example, let's consider a 10-min isocratic method that uses 60% acetonitrile as the mobile phase and has contaminants that are eluted at 100 min. These contaminants normally would start being eluted in the 11th injection, and with a retention that strong would likely be so broad that they wouldn't show up as peaks, but only as broad humps or a rolling baseline. So at the end of the batch of samples, we'd want to flush all the remaining contamination from the column. If we switched to 70% acetonitrile, we'd expect the retention to drop by threefold to $100/3 \approx 33$ min. A 20% change to 80% would cause a 3×3 = ninefold change, and running at 100% acetonitrile should reduce retention by approximately $3^4 = 81$-fold. This would cause our 100-min peak to be eluted at $100/81 < 2$ min. It is good to take a cautious approach here, because the Rule of Three is an approximation based on an "average" compound of ~400 Da, and each additional 10% change in %B will result in a propagation of errors. However, even with allowance for such uncertainties, a 10–15 min flush with 100% acetonitrile would be very likely to elute anything that was going to come off the column with acetonitrile. This example uses an isocratic method, but you can easily understand that you would get similar results with a gradient method that ran to 100% B with a short hold (for example, 5 min) at the end of each run.

The column flushing process is the same whether we are using traditional LC separation conditions or ultrahigh-pressure LC (UHPLC). For universal application, we should consider flushing in terms of column volume and flow rate rather than time, and it will apply for all circumstances. Take for example, the two most popular column sizes: 150 mm × 4.6 mm for LC and 50 mm × 2.1 mm for UHPLC or LC–mass spectrometry (LC–MS) applications. The 150 mm × 4.6 mm column has a volume of ~1.5 mL, whereas the 50 mm × 2.1 mm column has a volume of ~0.1 mL. A flow rate of 1.5 mL/min and a 10-min flush for the isocratic method discussed above would result in a 1.5 mL/(1.5 mL/min) = 1 column volume/min flushing rate, or 10 column volumes of total flush. The 50 mm × 2.1 mm column operating at 0.5 mL/min would take only 0.2 min/column volume or 2 min to flush 10 column volumes of solvent through the column.

I generally recommend attempting a 10–20 column-volume flush with 100% of the mobile-phase strong solvent to clean the column. If this flush isn't successful, I don't think it is worth the trouble going to any more work trying to clean the column unless there are extenuating circumstances. As mentioned above, after a 500-injection threshold is reached, I don't feel that the column owes me anything, and additional work at cleaning the column may have diminishing returns. There are techniques to use stronger solvents (for example, methyl *tert*-butyl ether or dichloromethane), but extra care needs to be taken to ensure solvent miscibility, additional time is needed, and the cleanup efforts may have marginal results. The more extreme the cleanup technique, the more risk there is that the "cleaned" column will not return to a comparable chemical state, so even if you are successful at removing the contaminants, you may not get the same separation with the "regenerated" column.

If your method falls into one of the categories where the column lifetime is <500 injections or the column cost is exceptionally high, such as for chiral columns, you may want to consider one or both of two additional techniques that you can find detailed in reference 1. The first is to use a guard column. Guard columns will extend the life of an analytical column, but they are fairly expensive, often costing 20% or more of the cost of the analytical column. Guard columns will work for most method applications. You can do the math and figure out if it is worth adding a guard column or not. The second technique is to add sample cleanup steps. Sample cleanup can be very effective and sometimes is essential if column lifetimes are otherwise too short. However, sample cleanup can remove things from the sample that you want to analyze. For example, cleanup may not be an option if you are trying to analyze impurities in a product. Sample cleanup also can rapidly add expense to the chromatographic process that eclipses the cost of the column. If a reversed-phase column costs $500 and you get 500 injections before it fails, the cost per sample for the column is $1. Solid-phase extraction (SPE) cartridges or well-plates can cost $2–3/sample plus solvents, supporting equipment, and technician time. As a result, SPE may not be an economically wise choice in trying to reduce overall analysis costs. On the other hand, if sample cleanup results in improved data quality, the added cost may be easily justified.

Air Problems

I occasionally get a question about the negative impact of air on an LC column.

This question is usually about one of two problems: injections of air, or pumping air through the column as a result of running a reservoir dry. Let's consider both of these next.

What happens if you inject air instead of sample or have air bubbles in the sample that is injected? This injection problem could result if the autosampler was not adjusted properly, the amount of sample in the sample vials was insufficient, or for some other reason. Most of the time, an injection of a few microliters of air will have no observable effect—the increased pressure in the column will compress the air into solution, and it will pass harmlessly through the column. At the end of the column, when the pressure returns to near-atmospheric pressure, the bubble may come out of solution and can result in a baseline spike when the bubble passes through the detector. However, a slight back pressure on the detector flow cell usually will keep the bubble in solution until it exits the cell. Just be careful that the back pressure does not exceed the limits for the flow cell, or leakage may occur. In one of my previous jobs, I intentionally injected a sample from an air-segmented sample stream. I modified the injector to compress the bubbles into solution before injection, and the method worked quite well.

Sometimes, an injected air bubble may be retained and will show up as a positive or negative peak in the chromatogram (2). If you suspect that injected air is the source of a problem, intentionally make an air injection from an empty vial and see what happens. Then make an injection where you are sure no air is injected. Comparison of the two chromatograms should allow you to determine if injected air is the source of the problem. Usually injected air can be avoided by proper adjustment of the autosampler and ensuring that sufficient sample is in the vials. Rarely is it necessary to degas the samples to remove dissolved air.

A more common question regards what happens when the pump is run dry, such as when you run out of solvent in a reservoir—does filling the column with air ruin it? My first response to this question is to ask what happens when the pump runs dry? Does it really pump the column full of air? The answer, of course, is no. This is because LC pumps are very good at pumping liquid, but do not pump air at all. As soon as the pump loses prime by filling with air, the piston moves back and forth, but no air is expelled from the pump. Yes, you may pump a bit of air into the column, but you won't "pump the column dry." However, you may ruin the pump seals because of increased seal-to-piston friction when operating the pump with no solvent. This can be avoided if you use the lower-pressure cutoff for the pump, so that if a reservoir runs dry, the pump will turn off when the pressure drops below a preset limit.

What happens if the column somehow is filled with air? Does it ruin it? It probably will not harm the column. I remember doing an experiment years ago where we evaluated a column, then intentionally ran compressed nitrogen through the column to remove all the solvent. When we refilled the column with solvent, it performed as it did before drying it out. The primary problem was that pumping degassed solvent through the column to remove all the air took a lot of time.

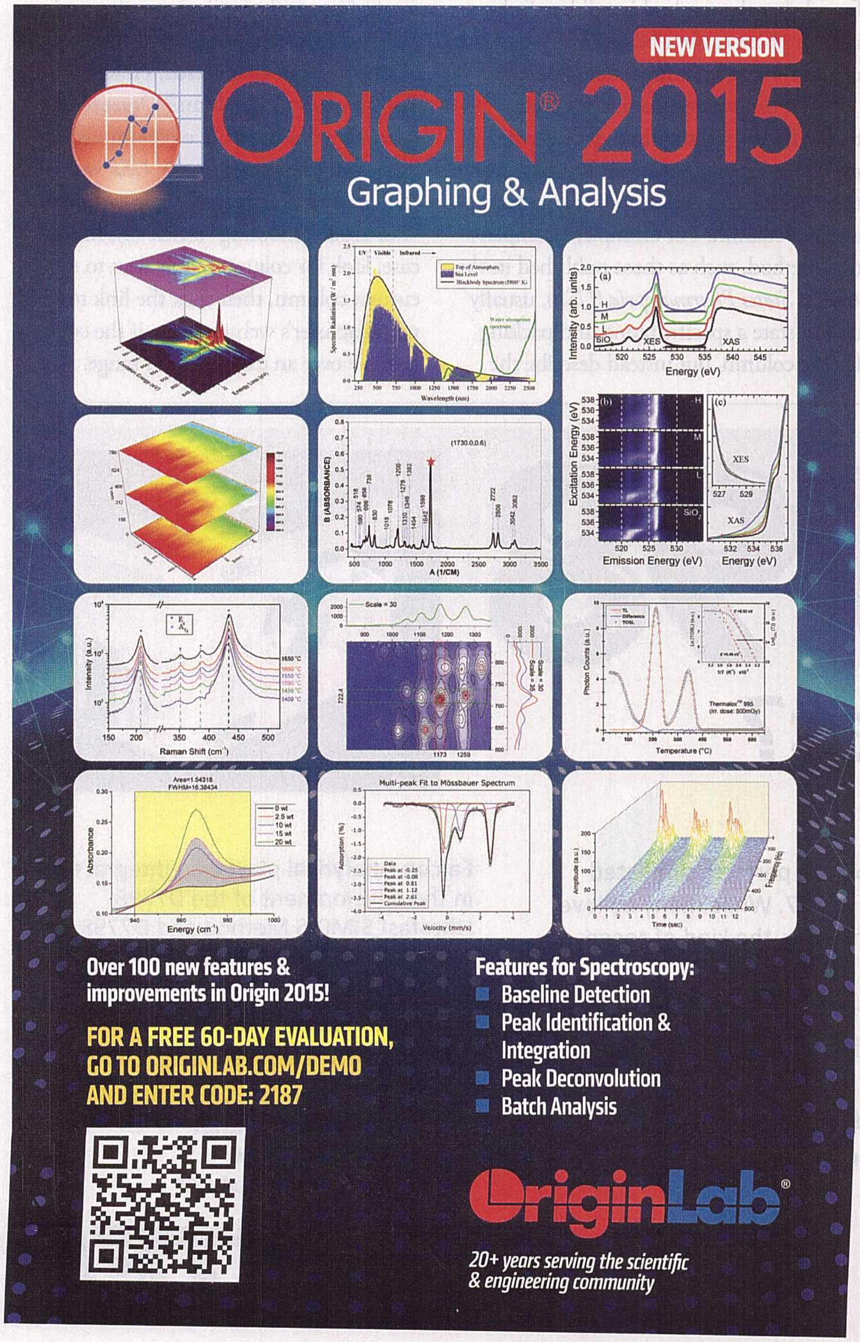
A more likely problem of column damage resulting from air in the column has to do with neither injecting air nor pumping air into the column, but from improper storage of the column. If you store the column with a nonvolatile buffer in it, such as phosphate, and leave the end plugs out of the column, it is possible to evaporate the solvent at the ends of the column. When the solvent evaporates, it will leave a buffer deposit in the column. Solid buffer crystals inside the particle pores will dissolve extremely slowly, if ever, and it is likely that the column would be ruined under these conditions. This problem, of course, is easily avoided by never storing the column when it contains buffer and tightly capping the column when it is not in use.

The Result of "Bad" Method Conditions

There is one question that I can almost guarantee occurs when one of my lectures contains guidelines regarding conditions that should be avoided for a successful method. For example, the standard silica-based reversed-phase columns that most of us use have a recommended pH range of 2–8. Below pH 2, the bonded phase falls off, and above pH 8, the silica dissolves. There are plenty of columns available today that can be used outside this range, but they are specifically designed for extended pH stability. During the lecture I will see someone very busy taking notes or there's an "oh, no" expression on a face. Usually this is followed by a question regarding the method. Most recently, one of the students described a method that required a mobile-phase pH of 10 and he complained about short column lifetimes. A batch of samples comprised approximately 50 injections, and he had to replace the column every three or four batches, for a column lifetime of 150–200 samples. This is definitely a problem. When I investigated the method a bit more, I found that the method is validated, the mobile phase cannot be modified, and the use of another column is not allowed.

What can we do in a situation like this? My first reaction is to examine the method a little more closely. Is it true that the column really cannot be changed to another column? For example, a compendial method, such as those published in the *United States Pharmacopeia* (*USP*), usually do not state a specific brand and packing for the column, but instead describe the column size, particle size, and stationary-phase classification (such as C18). For other methods, often a certain column may be specified, but many times the column specification is followed by an "or equivalent" statement. This is to anticipate that the specified column may not be available and another may work. If either of these cases applies to the method in question, I would suggest substituting the current column for a column with an extended pH range that would cover the pH 10 mobile phase. Of course, a little work will be involved in this substitution. You'll have to show that the separation is equivalent between the two columns, verify that you get the same results from real or spiked samples, and document the change; in many cases, revalidation will not be required. How can you find an equivalent column? One excellent source is a free database of column equivalency that is available on the *USP* website (3); I described this database in an earlier "LC Troubleshooting" article (4). In this case, look for columns equivalent to the current column, then click the link to the manufacturer's website to see if the column is stable over an extended pH range.

If you have to live with the current high-pH conditions and designated column, you may be able to reduce your costs by using a guard column. The guard column will be attacked by the pH 10 mobile phase and will fail, as well, but the goal is to use the guard column as a sacrificial element of the system to neutralize the mobile phase before it reaches the column, hopefully extending the life of the column. You'll have to experiment to see how long a guard column will last and how effective it is at extending the life of the analytical column. For example, it may be possible to replace the guard column after each batch of samples (~50 samples) and extend the useful life of the column beyond the current 150–200 sample limit. The key, of course, is to replace the guard column early so that it provides maximum protection to the analytical column. After you determine the replacement frequency of the guard column, you'll have to check the economics of using a guard column versus replacing the analytical column more frequently. Don't forget to also observe the effect of the guard column on the data quality and consider it in your decision.



Conclusions

We've looked at three questions about LC columns that are encountered by many users at one time or another. Column cleaning is a technique that we should use routinely, but we need to remember to balance the expense and effectiveness of column cleaning with the overall cost of analysis and the quality of the data. Next, we saw that the injection of air rarely ruins the column, but it can be irritating because of the work required to remove the air from the system. A better approach is to ensure that the autosampler is working properly, that there is enough sample in each vial, and that there is enough solvent present in the reservoir for the analysis of the desired number of samples. Finally, we considered how to mitigate problems related to methods that use conditions that are not recommended for good column performance. The best way to avoid such problems is to develop methods that use columns that are stable under the desired operating conditions. If that is not possible, see if there is another column that gives the same separation, but is more stable. Another option is to use a guard column to help protect the analytical column.

References

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(2) Y. Egi and A. Ueyanagi, *LCGC North Am.* **16**(2), 112–118 (1998).
(3) http://www.usp.org/app/USPNF/columns.html.
(4) J.W. Dolan, *LCGC North Am.* **29**(3), 235–244 (2011).

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