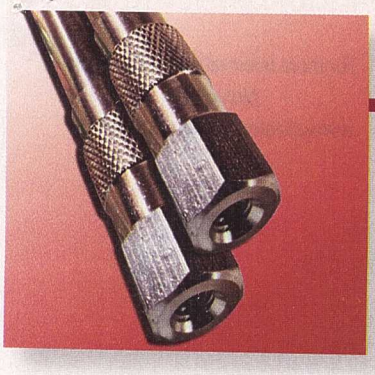

LC TROUBLESHOOTING

Seven Things to Avoid in the Liquid Chromatography Laboratory

Have a problem with your LC system? Maybe applying a little "DDT (Don't Do That)" may help.

John W. Dolan
LC Troubleshooting Editor

Back in the days when my company wrote and supported chromatographic software, I spent my share of time on the telephone with technical support calls. Some of the calls went something like this:

JWD: LC Resources technical support. Can I help you?

Customer: I have a problem with your software crashing.

JWD: Oh, that's terrible. Was there anything in particular that triggered this?

Customer: Sure. Every time I press CTRL-ALT-;-! the software crashes.

JWD: Oh, my, that's not good. We don't like crashes either. I'll pass this along to our programming team.

And then, as a joke, we had a file folder with such complaints that was labeled "DDT" — the solutions were simple: Don't Do That!

As I teach liquid chromatography (LC) classes around the world, I never cease to be amused at some of the creative practices some of the students have invented that can cause problems with the LC hardware or method. Some of these should be classified as chromatographic DDTs. I'd like to share seven of these for this month's installment of "LC Troubleshooting." Consider them as cautions that, if avoided, will make your life in the laboratory a much happier experience. These are presented in *no particular order or ranking.*

DDT #1: Don't pH the Organic

There are three ways to prepare a buffer and adjust its pH: the right way, the easy way, and the wrong way. Let's say we want to prepare 1 L of a pH 4.0 acetate buffer at a concentration of 20 mM. The right way to make this buffer would be to weigh the appropriate amounts of acetic acid and sodium acetate according to the instructions from one of the on-line buffer calculators (such as http://www.zirchrom.com/Buffer.asp) and dilute them to 1 L in a volumetric flask. Alternatively, you could make 20 mM solutions of acetic acid and sodium acetate and blend them together until the measured pH was 4.0. Either of these methods would yield what you intended, approximately 1 L of a 20 mM, pH 4.0 acetate buffer. The easy way to make the same buffer would be to prepare 1 L of 20 mM sodium acetate and then titrate the pH to 4.0 using concentrated acetic acid. This would yield a little more than 1 L of acetate buffer at pH 4.0, but the concentration would not be 20 mM, because concentrated acetic acid was used for the titration. Will it matter? Reversed-phase separations are not very sensitive to the buffer concentration, provided it is above a minimum threshold (5–10 mM in most cases), so it is unlikely that the easy way would give different chromatographic results than the right way to prepare this buffer. However, ion-exchange, hydrophilic-interaction chromatography (HILIC), mixed-mode, and other separation modes in which ionic interactions are important can all be sensitive to buffer concentration. So in these cases, there *may be an observable difference in the separation when the two different buffer preparation techniques are used. Whichever way you choose to prepare the buffer, this technique should be noted in the LC method document so others can get the same results.*

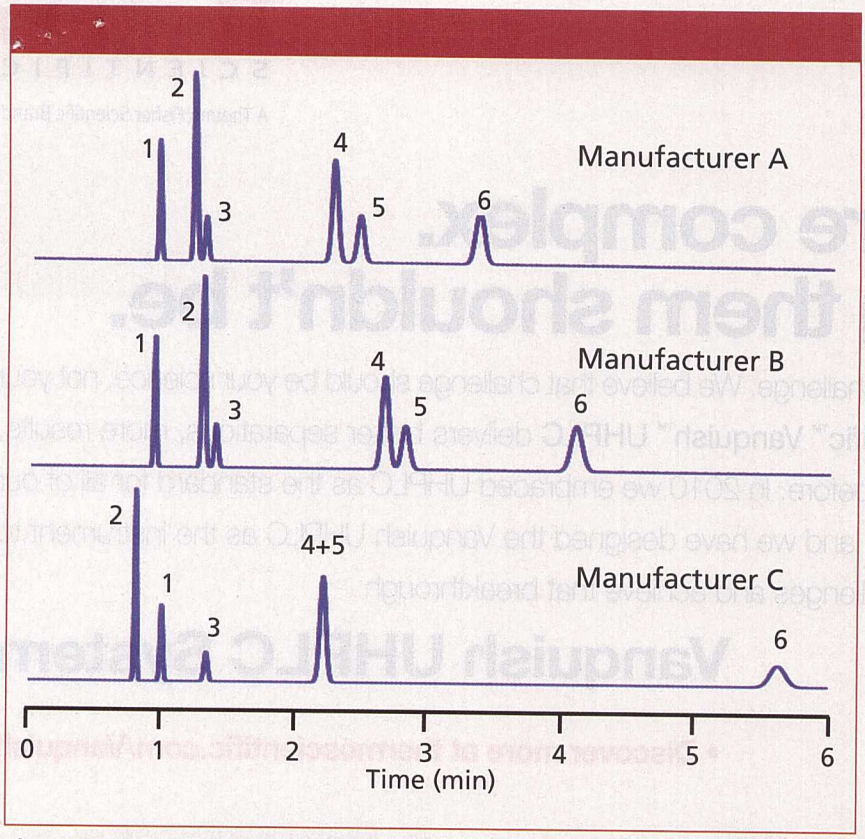

Figure 1: Differences in selectivity between C18 columns from three manufacturers. Based on data from reference 2.

The wrong way to make up the buffer is to adjust the pH of the solution after the aqueous buffer is combined with the organic component of the mobile phase, such as methanol or acetonitrile. When organic solvents are present, pH meters give different results than in aqueous solutions. In addition, these pH readings can vary with the laboratory temperature in ways that are not properly compensated by the pH meter hardware and software. The resulting buffer can give different chromatographic results than the buffer prepared in the first two ways. I remember a consulting project I once had where the method worked in the summer, but not in the winter. The laboratory was in a poorly heated production facility, the pH of the buffer was adjusted after the organic component was added, and the difference in the effective pH was sufficient to cause the method to fail in the wintertime.

In reporting the buffer composition and pH in LC, it is customary to refer to the pH of the aqueous portion of the buffer, and ignore what happens when organic solvent is added. Yes, the apparent pH will change with organic solvent present, but it will change the same way every time. DDT #1 reminds us to never adjust the pH of the buffer once organic solvent is present.

DDT #2: Don't Use the Same Column for Multiple Methods

When you have two LC methods that call for the same column description, it is best not to use the same individual column for both methods. A better approach is to buy two separate columns and use one for each method. While it may seem that there is no harm in using one column for two different methods, sooner or later you'll find cases where the unimportant peaks from one method, such as those from impurities, excipients, or late-eluted materials, will cause problems in the second method. This may be most apparent when one method is for the determination of large analyte concentrations, such as a content-uniformity determination, and the second method is for minor degradants or impurities in another product. However, problems sometimes occur with two different methods for the same product if one column is used for both. By dedicating a column to each method, an added benefit is that your annual column budget may be lower. DDT #2 tells us to use a separate column for each LC method.

DDT #3: Don't Develop a New Method on a Used Column

Let's face it, columns are expensive, and many of us don't have the luxury to put a brand new column on the LC system each time we want to screen it as a potential candidate column for a method under development. Trying a lightly used column during column screening experiments shouldn't be a problem. However, once you've selected a particular brand and model of column as a serious candidate for method development, put an unused column on the LC for your method development work. Too many times, a column that was used for another purpose or method may no longer have the identical chemical properties as a new replacement column (see DDT #4 below). Imagine the frustration if you spend a few weeks developing a satisfactory method, only to find it didn't work with a brand new column. It just isn't worth the risk. DDT #3 will help us avoid unwanted method changes late in the method development process.

DDT #4: Don't Use an Ion-Pairing Column for a Non-Ion-Pairing Method

This caution goes hand in hand with DDT #2 and #3. Ion-pairing reagents, especially the more hydrophobic ones such as the longer-chain sulfonates, are never completely removed from the column during column cleaning or regeneration procedures. For example, in one study (1) only about half of the lauryl sulfate was washed off a C18 column with ~700 column volumes (1 L) of 20:80 methanol–phosphate buffer. The authors were not able to remove all of the ion-pairing reagent even with 100% methanol or isopropanol. Shorter-chain ion-pairing reagents are removed more readily, but it is prudent to assume that once an ion-pairing reagent goes on the column, it will never be completely removed. Traces of reagent remaining from past column history can change the column chemistry sufficiently that the column will never be equivalent to a new column. In such cases, DDT #2 and #3 apply. DDT #4 reminds us to play it safe and not use a column that

has experienced ion-pairing reagents for any non-ion-pairing method.

DDT #5: Don't Assume that All C18 Columns Are Equivalent

At one time we assumed that all C18 columns gave equivalent separations. In fact, the United States Pharmacopoeia (USP) has an "L"-classification scheme that describes LC columns according to their chemistry. An L-1 column is any C18 column bonded to silica or ceramic, with or without endcapping. Experienced chromatographers know that this is not the case, but it may not be obvious to newcomers. An example of this is seen when comparing the chromatograms in Figure 1. Most workers would agree that the column from manufacturer A gives an equivalent separation as that of manufacturer B, even though the retention times are not exactly the same. On the other hand, the chromatogram generated on the column from manufacturer C is distinctly different from those of the other two columns. Yet all three columns are C18 columns. DDT #5 tells us to be careful when using any column other than the specific brand and model recommended for a particular method.

DDT #6: Don't Top Up the Buffer Reservoir

The buffers used in LC separations, such as acetate and phosphate, can create an attractive environment for microbial growth. In our laboratory, we once tested buffers to see how long they would last before they had a significant population of microorganisms. Various buffer bottles were set on a windowsill and monitored over time. Our conclusions were that we probably could use the buffers for two weeks before we had to be concerned, so we took the conservative approach and set the expiration date for all buffers one week after they were formulated. I know that many workers are able to use buffers for more than two weeks, but this depends on the buffer, the pH, and the general laboratory environment. However, no matter what precautions you take, bugs will eventually begin to grow in the buffer reservoir. If you don't replace the reservoir with a clean one each time you add buffer, you are just inoculating the fresh batch with the organisms growing in the remaining few milliliters of the previous one. This is an especially serious problem with ultra-high-pressure LC (UHPLC), because the columns use 0.2-µm porosity frits, which are small enough to become blocked by bacteria. A better practice than topping up the reservoir is to replace the reservoir with a clean one each time new buffer is prepared. DDT #6 reminds us not to inadvertently contaminate freshly prepared reagents.

DDT #7: Don't Pinch Pennies that Cost Dollars

I'm amazed at how often we ignore the economic realities in the laboratory. The most expensive thing in the laboratory is you — if you don't believe it, just ask your boss! This means that we need to be prudent when trying to save money. A couple examples on the extremes of cost will illustrate the point.

First, what do you do about a solvent inlet filter that you suspect is blocked? It is a common practice to sonicate these frits for a few minutes as an attempt to clean them. This is marginally successful, at best, and such cleaning will last only a fraction of the time a new filter will last. Conservatively, a laboratory employee costs $100,000 or more per year (fully burdened with salary, benefits, and so forth), which translates to ~$50/h. How much time can you justify trying to clean a $15 filter before you've spent more on labor than a new part?

A second example is the use of heroic measures to extend column life. I just read a thread on one of the on-line chromatography discussion groups where much advice was being given in this area. This included washing the column with strong solvents, such as dichloromethane, or protein solublizers. These practices almost never return the column to new performance levels, and can take a long time when washing through a series of mutually miscible solvents and back to the mobile phase. It is easy to spend an hour or two on such a task with marginal results. I've said many times before in "LC Troubleshooting" that if you get 500 to 600 samples through a $500–$600 column, it is costing only $1/sample. At that point, the column doesn't owe you anything. As we saw last month (3), nearly three-quarters of users get at least 500 samples through a column before it fails, and nearly half are able to process more than 1000 samples. It is important to flush the column after each batch of samples, and maybe even reverse-flush it occasionally, but anything more than that may not make sense. DDT #7 tells us that we should consider the real cost of trying to refurbish a portion of the LC rather than replace it with a new part.

Conclusions

We've looked at seven bad practices that are alive and well in many laboratories. Let's turn these on their heads and make them into best practices that make sense and help to improve productivity in the laboratory.

References

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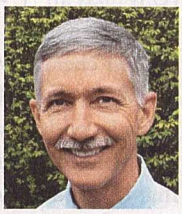

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