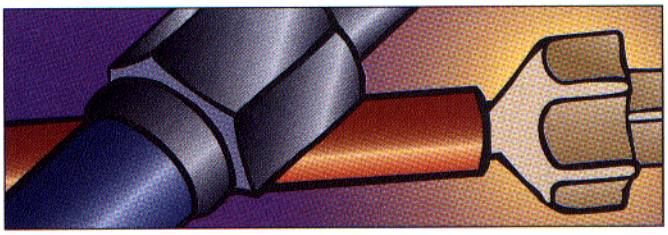
LC Troubleshooting



Method Quality, Standardization, and Peak Shape

John W. Dolan

If you take a hard look at your method, you may find opportunities to improve it.

This month's "LC Troubleshooting" column responds to readers' questions about a variety of liquid chromatography (LC) problems. If a common thread runs through these responses, it is that nearly every method has room for improvement. The key is to determine when it is useful to spend effort to improve a method and when to leave good enough alone — or as we say in my laboratory, "better is the enemy of good enough." I hope that the following questions and answers will provide some practical insight into method improvement.

HOW GOOD IS MY METHOD?

Q: I was given the following validated method to use, and I would like to know if it has any potential problems.

The method uses a 25 cm × 4.0 mm, 5-µm d_p C18 column. The mobile phase is 72:28:0.05 (v/v/v) 0.1 M monobasic potassium phosphate–acetonitrile–triethylamine adjusted to pH 3.0 with 85% phosphoric acid. The temperature is 70 °C, the flow rate is 1.8 mL/min, and the injection volume is 20 µL in acetonitrile. The method uses UV-absorbance detection at 192 nm.

JWD: Let's look at each of the parameters in turn to see where problems might occur. The column is a satisfactory choice, and a flow rate of 1.8 mL/min should provide a reasonable back pressure (I calculate approximately 1200 psi) at 70 °C. I don't like to perform routine analyses at pressures much higher than 2000 psi, because high pressure increases mechanical wear on the system.

The temperature may or may not be a problem. Solvent viscosity will be low at 70 °C, so the column pressure will be relatively low, as mentioned above. This temperature also will generate higher column plate numbers and, thus, narrower and taller peaks, which translate into better resolution and detection limits. However, temperatures this high can cause several problems.

The solvent must be preheated before it reaches the column because a temperature gradient can distort peaks and greatly reduce the plate number. In my laboratory, we found that preheating can be accomplished by placing 50–100 cm of stainless steel tubing in the column oven, plumbed upstream from the injector. The mobile phase passes through the heated coil before it reaches the injector. This process usually is sufficient to preheat the valve and minimize the temperature gradient between the injector and column. If you see distorted peaks, it may be due to poor temperature control of the mobile phase. Add more insulation and a longer preheating coil to improve the temperature control.

Another way to check for temperature problems is to lower the temperature and see if the peak shape improves. If you see inadequate performance at the higher temperature and improvements with a lower temperature, you may have to rework the method. A final problem with higher temperatures is shorter column life. Any chemical attack on the stationary phase or packing material will be accelerated at high temperatures, so you would be wise to turn off the column oven when the system is not in use.

The mobile phase is my biggest concern, because several parameters are different than what I would like. First, the buffer concentration is a bit high. Normally, I like to use a buffer concentration of 25–100 mM.

The pH always should be adjusted before adding the organic solvent.

Your buffer is on the high end of this range. In addition, buffer precipitation problems are more common with acetonitrile than with other solvents, which is another reason to use lower buffer concentrations. Watch carefully for any indications of buffer precipitation, especially when washing the column. For a column wash, start with a 72:28 solution (or 75:25 for convenience) of water–acetonitrile. Wash 5–10 column volumes (10–20 mL) of this solution through the column before washing with pure acetonitrile. This washing will ensure that the buffer is removed.

A second potential problem with the mobile phase is that the concentration of triethylamine is only approximately 3 mM. As with the buffer, I suggest that the concentration should be greater than 25 mM. You probably are obtaining little tailing suppression with 3 mM triethylamine, and worse yet, it may not be fully deactivating the silanol groups on the column, which would cause variable retention and peak shapes. I would increase the triethylamine 10-fold.

The final and perhaps most important problem with the mobile phase is the method of pH adjustment. The pH *always* should be adjusted before adding the organic solvent. The pH meter will give faulty readings when organic modifier is present, and the readings are highly susceptible to changes in temperature and other environmental variables.

The flow rate, as mentioned earlier, should be adequate to yield short retention times and maintain a reasonable back pressure.

Detection at 192 nm will be subject to more noise and potential chromatographic interferences than higher wavelengths, but low wavelengths usually are chosen by necessity, so I suspect you have no choice but to run the detector at 192 nm.

Injecting 20 µL of acetonitrile may or may not create problems. You'll have to check this injection volume experimentally. Too large a volume of too strong a solvent can distort peaks, especially at the beginning of the chromatogram. To test this, try injecting 10 µL of sample or dilute the sample by a factor of two and reinject. If either of these tests yields better peak shape, you may want to adjust the injection procedure.

Although this method may be workable and supposedly validated, I am suspicious. The validation should have included a test of variation of each of the parameters in question. Because of all

the potential problems with the mobile phase, I suspect it was tested inadequately. If it were my method, I would discuss the potential problems with the person who developed the method to determine if some of the parameters could be changed. I would guess that you could cut the buffer strength in half, increase the triethylamine by a factor of 10, and adjust the pH before adding organic modifier without adversely affecting the separation. These changes would help ensure a more robust method when other environmental factors change unavoidably.

INTERNAL vs. EXTERNAL STANDARDS

Q: A lot of methods in my laboratory use internal standards, but I don't think any particular reason is behind the choice of internal or external standardization. How and why is one technique chosen instead of another?

JWD: I don't know of any clear-cut rules for when to use internal and external standards, so let's look at what each method does. An internal standard is chosen to behave in a manner similar to the analyte of interest. Most commonly, internal standards are added before sample pretreatment steps such as solid-phase extraction. Because any losses of sample during sample preparation should be paralleled by losses of the internal standard, the standard will help correct for those losses. Similarly, if injection problems such as imprecision in the injection volume occur, the internal standard should track the analyte, thus enabling you to correct these errors.

Because of these valuable contributions made by the internal standard, you might wonder why internal standards aren't used universally. In fact, analysts commonly make this mistake, and internal standards are used when they aren't needed or worse yet, when they are inappropriate. Let's look at some problems with internal standards. First, an internal standard is chosen for its ability to mimic the analyte, thus many times the internal standard is similar structurally to the analyte, although not necessarily. The behavior of the internal standard in accurately tracking sample loss, especially during pretreatment, is a step that needs to be examined closely during method development but may be overlooked.

You really need to test a method with and without an internal standard.

Another potential problem with internal standards relates to measurement errors. When you add an internal standard, you add measurement errors to the total uncertainty of the system. That is, whatever error is present due to weighing, diluting, and dispensing the internal standard is additional uncertainty that might be absent with an external standard. When the chromatographic peaks are measured by the data system, an internal standard adds additional uncertainty because of the error in measuring the internal standard peak. As long as the measurement errors introduced by adding the internal standard are small compared with the overall method error, it is likely that these errors are unimportant. The impact of these errors should be examined, however, especially for trace analysis, in which any additional error can be important.

A final factor to consider is that by adding an internal standard, you are placing additional demands on the method in terms of resolution. Adding an internal standard means that the system must perform additional work to ensure that no interfering peaks are present to compromise the measurement of the internal standard. Avoiding interfering peaks is a special challenge with biological samples such as plasma that have a seemingly infinite number of minor peaks. Sometimes you can overcome minor interferences by adding larger amounts of internal standard, but this addition may or may not be appropriate.

In conclusion, you really need to test a method with and without an internal standard. If the internal standard provides better results, by all means use it, but be aware of the additional problems that may occur as a result of your choice.

CHECK-VALVE PROBLEMS

Q: I am running an LC system with acetonitrile–methanol–phosphate buffer mobile phases and a C18 column at 4800 psi. Every four–six weeks the check valves need to be replaced. It seems like the mobile phase is causing some kind of contamination of the check valves. Can you tell me what is happening and how it can be corrected?

JWD: You don't mention the buffer concentration. You should watch for a couple of things. First, the pressure is quite high. We don't like to run routinely above approximately 2500 psi in my laboratory. High pressure accelerates physical pump wear, which suggests that you would have fewer problems if you lowered the flow rate, used a shorter column, or increased the temperature.

Second, are you flushing the column and system regularly? You must wash buffers from the system occasionally, or increased wear can occur. Scratched pistons and buffer buildup increase seal wear, which in turn fouls check valves. Be sure to flush the buffer from the system daily (wash with acetonitrile–methanol–water). I like to keep the buffer concentration less than 50 mM if possible. Again, higher concentrations of buffer will increase the likelihood of precipitation, which in turn can cause check-valve problems.

WHAT'S WRONG WITH PEAK TAILING?

Q: I hear a lot about efforts taken to reduce peak tailing, but it isn't clear to me that tailing peaks are problems, as long as the resolution between peaks is sufficient. What am I overlooking?

JWD: In a way, you are right — if peaks are well resolved, peak tailing is less of a concern. However, tailing peaks create several problems. Let's look at a few of them.

First, integration is more difficult with tailing peaks. Data systems determine the start and end of a peak by monitoring the change in the slope of the baseline. With a tailing peak, the

point at which the peak returns to the baseline is less clear than for a symmetric peak. If you have a data system that prints tick marks at the start and end of a peak, you'll notice that they wander around a bit between replicate injections if the peaks tail. For most samples, this wandering is of little practical importance because the areas under the tails are very small in proportion to those under the entire peaks, so the error should be small. However, anyone looking at the fluctuating position of the tick marks will have less confidence in the data than if the peak-end location is constant.

A second concern with tailing peaks relates to the limit of detection. All other factors being equal, the area of a peak should remain constant. Therefore tailing or broad peaks will be shorter. At the limit of detection, the peak height — not the area — is the limiting parameter, so tailing peaks will have poorer detection limits than their nontailing counterparts.

Anything you can do to increase the ruggedness of a method will pay back in fewer problems in the long run.

A third uncertainty relates to the presence of minor peaks in the run. If you use your method for stability studies or the analysis of degradants or contaminants, you will be looking for minor peaks in the range of 0.1% of the primary peak's area. These small peaks easily can hide under the tail of a large peak. If this is the case, two errors can exist: the major peak will be reported as larger than it really is, and the minor peak will be missed. Well-shaped peaks are much less likely to hide minor peaks in the chromatogram.

Fourth, tailing peaks mean longer run times for the same resolution. If you want baseline separation between two adjacent peaks, the difference in retention times must be larger for tailing peaks than for symmetric ones. Longer runs mean more time and more solvent, translating into more-expensive analyses.

Tailing peaks result from two or more retention mechanisms. When this situation happens, your method is more likely to be subject to variables beyond your control. You will have a less-rugged method, with particular susceptibility to changes after column replacement. Anything you can do to increase the ruggedness of a method will pay back in fewer problems in the long run.

Finally, symmetric peaks or peaks with only minor tailing are much more aesthetically pleasing than tailing peaks. Although the artistic characteristics of the chromatogram rate fairly low on the requirements for a good method, I believe most workers will have more confidence in a method with well-shaped peaks than in one with tailing peaks.

So, in conclusion, we have a lot of reasons to want to minimize peak tailing. Although tailing rarely can be eliminated completely, reducing peak tailing using standard techniques — such as using low-pH mobile phases and adding triethylamine — is easy and likely to improve peak shape markedly.

SUMMARY

Many LC methods have one or more marginal parameters in terms of method ruggedness. During method development, you should perform tests to evaluate method robustness. These tests should check the method for deliberate changes in important parameters such as pH, mobile-phase organic-solvent content, temperature, and column batches. This testing will help you to understand your method's soft spots. In this way, you either can adjust the parameters to make the method more rugged or can watch for changes in the method that indicate the need for future adjustments.

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