Troubleshooting

But I Can't Change the Method

Some tips for transferring a validated liquid chromatography method from one laboratory to another.

John W. Dolan
LC Troubleshooting Editor

The following question is typical of many that readers e-mail to me: "I am trying to transfer a validated method from another lab. The method uses a 150 mm × 4.6 mm, 5-μm d_p, C18 column at a temperature of 35 °C. The mobile phase is 30:70 acetonitrile–20 mM acetate buffer at pH 4.5 and a flow rate of 1.0 mL/min. The method calls for a 35-μL injection of sample dissolved in acetonitrile. The first peak comes out at 2.1 min and the second peak at 4.5 min. The problem I've encountered is that the first peak is badly distorted, sometimes even split. The second peak isn't so bad, but it is broader than it should be. If I reduce the injection volume to 20 μL, the peak shape is usable, but the people who developed the method insist that I use 35 μL because it works that way on their system. Because the method is validated, I can't make any changes. What can I do?"

The problem of using a liquid chromatographic (LC) method that works for someone else but not for you is very frustrating. Not being allowed to adjust it to make it work is even more frustrating. In this month's "LC Troubleshooting," I would like to use this method as an example of a seemingly impossible situation that can be solved with a little work on your part.

What Could Be Wrong?

The first thing I like to do when confronted with a situation such as this is to list the possible problems that come to mind when I review the data. For this method, I spot two potential problems that alone or in combination could be responsible for the behavior observed.

First, the injection uses too large a volume of too strong a solvent. As a general rule, if the injection solvent is stronger (more organic in reversed-phase LC) than the mobile phase, peak distortion can occur unless the volume is minimized. I like to use a guideline that if the injection solvent has more than about 20% more organic solvent than the mobile phase, the injection volume should be kept to a maximum of about 20 μL. If 100% strong solvent is used, a 10 μL maximum injection is a better idea. (You might get by with larger volumes of stronger solvents, but you should test this thoroughly.) I'm not surprised that you observe peak-shape problems upon injecting 35 μL of sample in 100% acetonitrile into a 30% acetonitrile mobile phase. What surprises me more is how the original lab was able to validate the method under these conditions.

The second potential problem has to do with the retention of the sample components. I encourage everyone to check the retention factors (k) of the peaks in every run. For isocratic separation:

$$k = (t_R - t_0)/t_0 \quad [1]$$

where t_R and t_0 are the retention time of the peak and the column dead time, respectively. We are given the retention times of the two peaks, but not t_0. The column dead volume for a 4.6-mm i.d. column can be estimated as

$$V_M \approx 0.1\ L \quad [2]$$

where V_M is the column dead volume in milliliters and L is the column length in centimeters. So for the current column, L is 150 mm or 15 cm, and 0.1 × 15 = 1.5 mL. Convert V_M to t_0 by dividing the volume by the flow rate: 1.5 mL/1.0 mL/min = 1.5 min. Thus, k = 0.4 for the first peak and 2.0 for the second. Ideally, one would like $2 < k < 10$ for a separation, but this isn't always possible. A situation in which $1 < k < 20$ usually is acceptable. When k is less than about 1, resolution can be overly sensitive to small changes in mobile-phase organic, and the interference from the unretained material at the beginning of the chromatogram can be a problem. Injection effects can be more dramatic when conditions for $k < 1$ are used.

What Now?

The important thing at this stage is to find out the root cause of the problem. The "you are not allowed to change the method" command just has to be ignored. No, you might not be allowed to change the method, but unless you know what the problem is, you won't be able to use it as is.

Another thing to consider is why the method worked in the other lab and not yours. We've already speculated on two possible causes — the injection solvent and the injection volume. If possible, you might double-check with the other lab to see if their injection volume really is 35 µL. Mistakes can be made. For example, could they be programming the autosampler for 35-µL injections but have a 20-µL loop installed? If their LC system is an older system, it might be plumbed with excessive lengths of large-diameter (for example, 0.010-in. i.d.) tubing, whereas your system might have smaller extracolumn volume. How about well-made connections? Could there be sufficient extracolumn mixing taking place to effectively dilute the injection solvent? Have you been supplied chromatograms for comparison? I've had several experiences in which chromatograms are described as normal but upon visual examination they would never meet my criteria for normal. So maybe the other lab has terrible looking chromatograms, but thinks that they are okay.

At this point, I would perform some injection-related tests. First, I would make a series of injections (in duplicate) with different injection volumes to determine the effect of the injection volume on peak shape. You have preliminary information that 20 µL will work satisfactorily. So I would make injections at 15, 20, 25, 30, and 35 µL with the sample dissolved in acetonitrile. Ideally, you would inject the same sample mass for each injection, but I think the problem is related to the solvent, not sample mass, so you probably could inject different volumes of the same sample. This experiment will help you identify the largest sample volume that provides acceptable results.

Next, I would determine the effect of the sample solvent. Make a series of samples with 100, 80, 60, 40, and 20% acetonitrile. Again, ideally one would want these with the same sample concentration, but because the problem is likely to be related to the injection solvent, not sample concentration, you might want to dilute a single sample concentration to the desired injection solvent concentrations. Inject 35 µL (in duplicate) of each solution. This will give you an idea about the largest injection solvent concentration that can be used with a 35-µL injection and still produce acceptable results.

I think what you'll find is that either approach will help mitigate the problem. Now you have to decide what to do with the data you've gathered.

Table I: Allowed method adjustment parameters (2,3)

Parameter	Adjustment	OR	Comments
pH	±0.1		
Buffer	±10% concentration	±0.1 pH units	Whichever is smaller
Mobile phase	±30% relative	±2% absolute	±2% absolute limit
Flow rate	±twofold		
Injection volume	±twofold		Any reduction meets system suitability
Column temperature	±5 °C	>5 °C	>5 °C only to correct equipment differences

Permissible Changes

What changes are permissible with a validated method? As a first reaction, many workers would say, "no changes." However, let's step back and look at the method more carefully. How accurately are the different variables controlled in the normal laboratory? The method calls for 30% acetonitrile, but how accurately is the mobile phase prepared? Is ±1% reasonable? How about the pH? Most workers control the pH no better than ±0.1 pH unit unless the buffer components are weighed. The temperature? The setting on the oven might be 45 °C, but what is the true temperature? The oven might vary by ±1–2 °C from the setpoint. In my laboratory, we've observed that a block heater, an air bath oven, and a Peltier-heated oven provided column temperatures that vary by several degrees even though they were all set at the same nominal temperature (1). You can see that no matter how well you control the conditions, some variation will result.

In my laboratory, we've decided to take the approach that variations to a method are permissible if they correspond to the magnitude of changes that are expected from normal experimental error. This approach has been recommended by others, as well (2,3). For example, some of the allowed changes based upon references 2 and 3 are shown in Table I.

Let's see how this might apply for the current method. We would like to increase k as much as possible to move the first peak away from the solvent front to minimize the potential for interference from materials at t_0 and hopefully reduce the injection effect. Using the guidelines listed in Table I, we could adjust the pH ±0.1 units or the buffer composition by 2 mM (10% of 20 mM) if this would help, but we have no information on the effect of pH or buffer at this point. We do know that a reduction in mobile phase organic will increase k. We are allowed to reduce the acetonitrile to 28% (the lesser of 30% of 30% or 2% absolute). This also will increase retention times somewhat, but because k is not influenced by flow rate, we could increase the flow rate to compensate for the change in retention, while maintaining the new k-values. We could reduce the injection volume to the 20 µL that originally was indicated would work, or some other value if the injection volume experiments suggest this. A decrease in column temperature will increase retention, and thus k, by 1–3%/°C. I would make the full 5 °C decrease in temperature for the maximum increase in k from this parameter. The peaks are well separated, so I doubt that a 5 °C change in temperature will compromise the resolution.

So you can speculate that one or more of these changes would move the method into a usable region. The trick here is to have predefined the allowable variations in the method that will not disqualify it from validation. Without such preexisting guidelines, you still could make a strong argument that normal variations in the method operation should allow for ±0.1 pH units, ±1% organic, and ±2 °C in temperature. Flow rate has little practical impact on most isocratic separations unless the resolution is marginal or the pressure is too high. I think any reduction in injection volume should be easily defensible as long as the

limits of detection can be met. I would run a standard curve and a sufficient number of replicate injections at high, mid-range, and lower method limits to show that system suitability, as well as method precision and accuracy, can be obtained under the adjusted conditions.

Planning Ahead for Change

In addition to having a standard operating procedure in place that allows for specified small changes to validated methods, the validation process should be designed to anticipate required method changes. A thorough validation will examine the effects of changes in each of the important parameters in the separation. During method development, you should determine the impact of each variable, then demonstrate during validation the changes that can be made while keeping the method within specifications. For example, you might find that the method is fairly insensitive to percent organic and temperature, so changes of ±5% acetonitrile and ±5 °C can be made with no impact on method precision or accuracy. However, you might also find that the method is very pH sensitive and that the pH needs to be controlled within ±0.05 units or the critical resolution will be compromised. This means that you will have to prepare your buffer by weighing the components instead of the traditional pH meter adjustment technique. Once the limits of the variables have been determined, the method then can be written with the allowable variations in parameters included. This way, the laboratory that uses the method for routine analysis will know which changes are permissible and which ones are not. Yes, it might take a little more work during method development and validation, but you will understand the method better and it will be a more robust method for use by others.

Conclusions

Rarely are you not allowed to make *any* changes in a method, because you often make unintentional changes due to operator skills, instrument differences, and by nature of the variability of the techniques you use to prepare reagents. When you encounter a problem, such as the current one, that seems to have no allowed solution, step back and look the method over carefully. First, speculate on what is the possible source of the problem. You will almost always have a better chance of convincing "the powers that be" of the need for a change when you are armed with empirical evidence, so perform some experiments to determine if you can correct the problem with minor changes to the method. The reduction of injection volume seems like the simplest solution to the current problem. Even though the *k*-values were too small and the injection solvent was too strong, a smaller injection seemed to provide a fix that allowed the method to be used.

The best approach is to develop methods that have been tested for robustness and are written to include certain small changes that can be made to keep the method working properly. A standard operating procedure that contains allowable changes to a method, such as those recommended by references 2 and 3, will give you further support when a small but unanticipated change is needed to bring the method back into compliance.

References

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John W. Dolan

"LC Troubleshooting" Editor John W. Dolan is Vice-President of BASi Northwest Laboratory of McMinnville, Oregon; a Principal Instructor for LC Resources, Walnut Creek, California; and a member of LCGC's editorial advisory board. Direct correspondence about this column to "LC Troubleshooting," LCGC, Woodbridge Corporate Plaza, 485 Route 1 South, Building F, First Floor, Iselin, NJ 08830, e-mail John.Dolan@Bioanalytical.com.

For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.