Stay Away from the Cliffs!

Does your method have a safety factor?

In my home state of Oregon, we have many miles of beaches along the Pacific Ocean. One of my favorite ways to enjoy the view is to stand on one of the bluffs overlooking the ocean. In such places, there often are signs that read "Stand Back from the Edge," and these signs might be accompanied by a fence or barrier to reinforce this suggestion. In many places, the cliffs are undercut by erosion, and it is impossible to tell from above whether or not the surface is stable or if it might cave in at the next step. For this reason, the signs provide sage advice — I have no desire to have my next step be my last.

Perhaps we should have a similar sign posted on the wall of our laboratories, reminding us to build a safety factor into our methods so that we don't encounter unexpected and unpleasant surprises in routine operation. In this month's "LC Troubleshooting," I would like to examine a couple of liquid chromatography (LC) methods that could be operating too close to the edge.

Too Much . . .

The first method is one I encountered several years ago when I was visiting a pharmaceutical production facility. One of the chemists brought me a problem that she had observed with a method that she had used successfully for several years. This was a method used in a cleaning validation. After completion of a production batch of drug, the stainless steel reaction vessels were cleaned prior to their next use. This method was used to ensure that there were no drug residues on the reactor walls. The procedure was quite simple: rinse the walls with methanol, then inject 50 µL of the methanol wash solution. The chromatographic setup used a reversed-phase column and a mobile phase of 50:50 methanol–buffer. After successful use of the method for years, one day, the analyte peak was split into a doublet. To correct the problem, the chemist had tried a new column, a new batch of mobile phase, and even a different instrument, all with no success. What could cause such a problem?

The problem source should be fairly obvious. When the injection solvent is stronger than the mobile phase, one needs to be careful to keep the injection volume small, or peak distortion can occur. As a general rule, one can inject approximately 15% of the volume of the peak of interest without undue effects, *if* the mobile phase is used as the injection solvent. When stronger solvents are used for injection, smaller volumes are required. Let's see what this means for the current method. I don't remember all the details of the method, but let's assume that the column was a 150 mm × 4.6 mm column packed with 5-µm diameter (d_p) particles and operated at a flow rate of 1 mL/min. Also, let's assume that the peak of interest was well-behaved with a retention factor k of 2. To determine the injection size relative to the peak volume, we must calculate the peak volume.

First, calculate the retention time of the peak. Recall that the retention factor is:

$$k = \frac{t_R - t_0}{t_0} \quad [1]$$

where t_R is the retention time and t_0 is the column dead time. For 4.6 mm i.d. columns, t_0 (in min) can be estimated as

$$t_0 \approx 0.01\ L/F \quad [2]$$

where L is the column length in millimeters and F is the flow rate in milliliters per minute. So for our column, $t_0 \approx (0.01 \times 150)/1 \approx 1.5$ min. After solving equation 1 for t_R, we can estimate the retention time as 4.5 min for a retention factor of 2.

Next, we need to determine the peak width. We don't have the actual measurement, so we can estimate the width from the column plate number N. For an estimate of realistic column performance with real samples, I like to use the relationship

$$N \approx 300\ L/d_p \quad [3]$$

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LC Troubleshooting Editor

where d_p is the packing particle diameter in micrometers. This means that our 150-mm, 5-μm column should generate $N \approx (300)(150)/5 \approx 9000$ plates. Let's round this to 10,000 for ease of calculation.

Now we need to determine the peak width for the analyte peak. Recall that the plate number is defined as:

$$N = 16\ (t_R/w)^2 \qquad [4]$$

where w is the peak width at baseline, obtained by drawing tangents to the peak sides and measuring the width between the tangents at the baseline. Solve equation 4 for w and we can calculate a width of 0.180 min for a retention time of 4.5 min and plate number of 10,000. At a flow rate of 1 mL/min, this translates into a peak volume of 180 μL. If we were injecting the sample in mobile phase, the 15% guideline used earlier suggests that a maximum injection volume of approximately 25–30 μL should be usable without peak distortion. As mentioned previously, when a stronger injection solvent is used, a smaller injection volume is necessary. A 50-μL volume of 100% strong solvent certainly is too large a volume of too strong a solvent for this method.

So how do we correct this problem? The simple solution would be to reduce the injection volume; perhaps 5–10 μL would work. But the chemist indicated that 50 μL was chosen because any smaller injection would place insufficient mass on the column for an adequate detection limit. Whereas small injection volumes are required for injection solvents stronger than the mobile phase, often quite large injection volumes are possible when weaker solvents are used. The practical solution for the present problem was dilution of the sample fourfold with buffer, so now the injection solvent contained 25% methanol instead of 100% methanol. To maintain the mass on column, the injection volume was increased from 50 μL to 200 μL. The resulting separation gave adequate peak shape and satisfactory method performance.

Why did the method fail suddenly after several years of satisfactory performance? I don't know. Rather, my question is why did the method work *without* problems for several years? This is a good example of a method that is operating "too close to the edge of the cliff," with insufficient robustness to allow for normal variations in method conditions.

As an aside, the process used earlier to calculate and estimate chromatographic parameters can be useful to check for reasonable performance of any method. Without some point of reference, it is not possible to determine if the peak widths you obtain are reasonable or not. If the peak widths you measure are much larger than the results from the estimations, you might not be getting the performance that you should from your column. Check for extra-column effects: large-diameter tubing, excessive tubing lengths, and poorly assembled fittings. Broader peaks require longer runs for the same resolution, and shorter run times generally are preferred.

. . . Or Too Little?

I encountered another method recently in which significant changes in peak spacing were observed, sometimes with peak order

reversal, when a new column was used. The sample was a mixture of acidic and basic compounds. The column was a Type A C18 reversed-phase column using a mobile phase of 20:80 methanol–0.01 mM phosphate buffer (pH 2). On one column, the first peak, an acid, was eluted close to t_0, followed by a basic compound, then another acid. On a second column, the first acid was retained a bit more, and the base now eluted after the second acid.

When I examine a problematic method, I first look for conditions that send up a red flag for me. This method has two of those red flags. First, it used a Type A silica column. For legacy methods, use of a Type A column might be justified because of the cost to revalidate the method with a newer Type B stationary phase, but it is not justified for a method that is under development, such as this one. Type A silica was used for most columns developed before approximately 15 years ago. This silica was characterized by significant metal contamination and a very acidic surface. This resulted in badly tailing peaks for basic compounds and much larger column-to-column variability than we find acceptable today. The newer Type B silica has a very low metal content and is processed in such a way that the surface is much less acidic than the older silicas. These columns are much more reproducible and, for the most part, generate chromatograms with little or no tailing for basic compounds. I strongly believe that one should never start the development of a new method on a Type A column unless there are very compelling reasons.

The second red flag for this method is the buffer concentration, 0.01 mM. My first reaction was that this was a typographical error, and the chemist meant 0.01 M. However, a concentration of 0.01 mM was confirmed. The buffer pH of 2 is a good choice for this method. A low-pH mobile phase provides two benefits in separations such as this. First, it suppresses ionization of acidic sample components, thus increasing their retention under reversed-phase conditions. Low pH also suppresses ionization of the unbonded silanol groups on the silica surface, making them less acidic. Suppression of silanol ionization tends to reduce band tailing for bases. However, if insufficient buffer is present, these two processes do not take place or can vary significantly with small changes in the column or operating conditions.

I suspect that the primary problem in this case is insufficient buffering. Even with LC–MS methods, in which buffer concentrations generally are minimal, a minimum buffer concentration of 5 mM is common. I would suggest changing the buffer concentration to 10 mM from 10 μM, and I think a dramatic improvement in the method would be seen. The change in retention of the first acid peak suggests that its ionization was not suppressed fully, so its polarity, and thus retention, changed. A difference in silanol acidity between two different Type A columns is not unexpected, and if the buffer were too weak to suppress the silanol ionization, significant retention variability would be expected, especially for basic compounds, which interact strongly with acidic silanol groups. When you start out with the cards stacked against you, the likelihood of obtaining a good method is significantly reduced. With this many problems with the method, I would change the odds and switch immediately to a Type B silica column, increase the

buffer concentration, and adjust the mobile phase to get the desired selectivity. This is just one more example of working too close to the edge of the cliff — one day the method will work and the next it won't unless robustness is designed into the method.

When All Else Fails

Recently, I was asked about a problem related to sample carryover. With one brand of equipment, the method gave unacceptable carryover after injecting a high concentration sample. The user tried all the standard tricks to alleviate such problems — a change in wash solvent, different wash volumes, thorough cleaning, seal replacement, and so forth — to no avail. When the method was moved to another system, the carryover disappeared. Another system of the first brand was tried and the problem reappeared. In the final analysis, carryover was observed on every instrument of one brand and model, but not on any other brand or model tried. The user had contacted the instrument manufacturer, who suggested the same fixes that had already been tried. I didn't have any magic answers, either, as my experience with this brand and model of equipment is limited. This is an ideal problem to submit to *Chromatography Forum* (www.chromforum.com), an on-line discussion group jointly sponsored by *LCGC North America* and LC Resources. Knowing the brand of equipment used, I suspect that there will be forum participants from at least a dozen laboratories who use this make and model of LC system. It is likely that one of them will have encountered a similar problem and might have a simple fix to the problem. *Chromatography Forum* also is a good source of general chromatographic information, whether it is troubleshooting, method development, or operational tips. Try it — you'll like it!

Conclusions

This month, we've examined a couple of problems that illustrate the importance of building robustness into each LC method. One would like a method that will tolerate the type and magnitude of changes that are encountered under normal operation, such as different mobile phases, different columns, and different instruments. During method development, before validation, it is easy to check a method's susceptibility to small, intentional changes. Building in such robustness will help to avoid costly and frustrating problems that can be encountered during routine operation. Don't be afraid to ask for advice. I'll be happy to give you my opinion (contact me via e-mail, see below), or check with other chromatographers on *Chromatography Forum*.

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