L C T R O U B L E S H O O T I N G

System-to-System Variation

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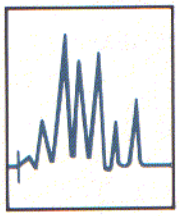

This month's "LC Troubleshooting" addresses readers' questions about problems that can occur when a method is moved from one liquid chromatographic (LC) system to another. These questions also remind us of the often overlooked step in method validation — ensuring that the method will perform satisfactorily on an LC system from another manufacturer.

METHOD TRANSFER PROBLEMS

Q: I am using a method to analyze a pharmaceutical formulation that contains two major components. After successfully analyzing samples with the method for several months, I moved the analysis to another LC system. On the second system, the first peak was about half the size it had been on the first system, whereas the second peak stayed the same — even though the same column was used in both systems and the sample was taken from the same vial. The retention times were the same for both systems. The first system consisted of two pumps with a high-pressure mixer and a variable-wavelength detector. The second system used a single pump with low-pressure mixing and a diode-array detector. Autosamplers normally were used with both systems; however, nominally identical manual injections gave the same results. What could be causing this problem?

JWD: With this kind of problem, it is best to eliminate from consideration the things that cannot be causing the problem. Because both systems used the same column and injection technique, they shouldn't be causing problems. Because the same retention time is observed in both cases, we can eliminate the mobile phase and the pumping and solvent proportioning systems. (We would expect problems with these components to cause retention-time variations.) This leaves the detector and the system's plumbing as possible problem sources.

If we knew the column plate number for both separations we could speed problem isolation, but we can identify the possible causes anyway. First, let's consider the case in which the plate number has decreased. You can identify a decrease in plate number by measuring the bandwidth at a convenient place (such as at half the peak height); broader peaks give a lower column plate number. Recall that the plate number, N, is an expression of the peak variance (σ^2):

$$\sigma_T^2 = \sigma_i^2 + \sigma_c^2 + \sigma_t^2 + \sigma_d^2 \quad [1]$$

where σ_T^2 is the total variance and σ_i^2, σ_c^2, σ_t^2, and σ_d^2 are the variance contributions by the injector, column, tubing, and detector, respectively. We know that for a given system configuration, the contributions by the injector, the plumbing, and the detector are constant. The longer the bands stay in the column, the larger is the column's variance contribution, which is why in isocratic operation late-eluting peaks are broad relative to early peaks. Furthermore, under the same conditions (mobile phase, flow rate, injection size, retention time, etc.), the same column gives the same variance contribution no matter what system it is used in. Finally, note from equation 1 that the plumbing and detector contributions to the total variance are proportionately smaller for later peaks. So where does all this lead us? If the plumbing or detector contributions increase (we call this extracolumn band broadening), you would expect the first peak to broaden relative to the second. Because peak area is conserved, you would expect a broader peak to be shorter. Thus, if you observe a lower value of N for the first peak, look to extracolumn band broadening as the source of the problem.

How do we further isolate extracolumn band broadening problems? The tubing that contacts the sample generally is limited to the plumbing between the injector or autosampler and the column, and between the column and the detector. Long tubing runs and large-diameter tubing increase band broadening. Generally, the internal diameter should be $\leq$0.010 in. and the length should be as short as possible. If you suspect any of the tubing, replace it with short lengths of small-diameter tubing. Except for cases in which you must have the minimum possible volume (for example, microbore applications), you should avoid 0.007- and 0.005-in. tubing because of its susceptibility to blockage. If the problem band gets taller and narrower when the tubing is changed, you have isolated the problem. If the problem remains, look to the detector.

There are two possible band-broadening contributions from the detector. First is the detector-cell volume. If a large detector cell is used, broader peaks are expected. Thus, if a 2-μL cell is used in the first system and an 8-μL cell in the second, broader peaks would be expected in the second case. Often you cannot do much about the detector cell, although cells that have different volumes are available for some detector models. Check the manufacturer's literature or the detector operation manual for detector-cell specifications.

The other possible detector problem is the time constant, an electronic filter used to remove excess noise from the chromatogram. If the time constant is too small, increased chromatographic noise results. A too-large time constant, on the other hand, can reduce the peak height. The time constant is controlled either by a switch on the detector (often on the rear panel) or by software control. In either case, the time constant should be no larger than one-tenth of the bandwidth at the baseline. Most workers set the time constant to 0.5 or 0.1 s when conventional 15–25 cm × 4.6 mm columns are used. The problem should easily be corrected if the time constant is the source.

If you determine that extracolumn band broadening is not responsible for the problem you observe, faulty detector calibration could be the cause. If the two compounds of interest have different absorbance maxima, a change in the detection wavelength will change the relative detector response to the two bands. The simple way to check is to observe the peak spectra collected by the diode-array detector; these should immediately indicate whether the use of the wrong wavelength is the source of the problem. Alternatively, make one run with the variable-wavelength detector set 5 nm above the present wavelength, and make a second run with the detector set 5 nm below the present wavelength. Changes in relative peak height from the first run to the second run should give you a clue to whether the wavelength setting is wrong. If the wavelength of either detector is determined to be at fault, you should recalibrate both detectors, following the instructions in the operation manual.

Let's review the strategy. If the first peak with the second LC system is broadened relative to the first system, we suspect extracolumn band-broadening problems. This band broadening results from plumbing problems, the detector-cell volume, or the detector time

constant. If the first peak is not broadened, the detector-wavelength calibration is suspect.

AUTOSAMPLER TROUBLE

Q: I have two LC systems that I use to run the same method. The precision and accuracy of the results seem to be comparable, but one system gives consistently broader peaks than the other. I swapped components until I isolated the autosampler as the source of the problem. In the system with the broader peaks, the autosampler injects 10 μL from a 2-mL sample loop, whereas in the other system the injection is made from a 10-μL sample loop that is completely filled with sample. This band broadening becomes a problem in terms of column life. Using the small-loop system with a new column, the three bands of interest are well resolved, but using the large-loop system with the same column, two of the bands are barely separated. As the column ages, the resolution in both systems decreases, and as a result the useful column lifetime is significantly shorter on the system that uses the large-loop autosampler. What's wrong? I run too many samples to inject each sample manually, and I can't afford a new sampler.

JWD: Injection problems, such as the one you describe, can arise from two sources. The sample band can be broadened either before it gets to the column because of plumbing problems or after it gets to the column because of the injection of too large a volume of a strong solvent. Let's look at these two possibilities.

Band broadening before the column when using large-loop injectors can result from improper plumbing of the loop or from excessive connecting-tubing volume. Any time a partially filled sample loop is used, the loop should be plumbed so that the sample, rather than the remaining solvent, exits the loop first. This is the normal configuration for autosamplers. Verify that your sampler is plumbed properly by comparing the plumbing connections with those described in the operator's manual, paying special attention to the connections at the sample valve. If the loop is plumbed backwards, the 10-μL sample will have to flow through at least 2 mL of tubing before it reaches the column, whereas in the normal configuration the sample goes directly to the connecting tubing and then onto the column. Any increase in the volume the sample passes through increases the width of the sample band as it goes onto the column, and usually produces broader sample bands at the detector, as well.

Excessive lengths of connecting tubing also can cause band broadening, as we saw in the discussion of the previous question. With some autosamplers the length of the tubing connecting the autosampler and the column cannot be shortened to much less than a meter. When using a run of tubing of this length, mistakenly installing 0.020-in.

instead of 0.010-in. tubing can be disastrous; often it is necessary to use tubing with a diameter as small as 0.007 in. to minimize the extracolumn band broadening. Check the tubing sizes in your system. If you are not sure about the size of the tubing, replace it with as short a length of 0.010- or 0.007-in. tubing as will fit. If this is the source of your problem, the peaks should now be much narrower.

If plumbing problems are not the cause of the broad peaks, the problem probably lies with the injection solvent. We commonly say that as long as the injection volume is <25 µL, it doesn't matter what injection solvent is used so long as it is miscible with the mobile phase. This is true in this case as well, but remember that the solvent containing the sample is not the only solvent being injected. Along with the sample, almost 2 mL (the balance of the loop) of the loop-wash solvent is injected. Real problems can be created if the wash solvent is stronger than the mobile phase. Often, methanol or acetonitrile is used as the wash solvent because it is effective at removing any sample residue. But again, the design of the autosampler is important here. If the sample needle and valve plumbing are flushed while the valve is in the *inject* position, fewer problems should occur than when the flushing is done in the *load* position. When flushing is done in the *inject* position, the loop remains filled with mobile phase, and 2 mL of mobile phase is injected with the sample. Flushing in the *load* position, however, leaves the loop filled with the flushing solvent. If the solvent is too strong, the bands will broaden as they pass through the column. Again, you should check the operator's manual to see how flushing is accomplished, and either flush during injection or fill the flushing reservoir with mobile phase. Of course, there may be a much simpler solution — replace the 2-mL loop with a 10-µL loop if the autosampler design allows it.

In specific cases, you need to take special care when selecting the wash solvent. With ion-pair applications you need to use mobile phase to flush the loop because injection of solvent other than the mobile phase can upset the equilibrium of the ion-pair reagent on the column, causing chromatographic problems. Second, with some autosamplers a significant volume of fluid is contained in the sample needle and in the tubing that connects the needle to the sample loop. In some autosampler designs, the contents of these parts — mobile phase or strong solvent — can be injected together with the sample. If this is the case, strong solvent remaining in the sample needle can be important even if the flushing is done in the *inject* position. Finally, you may need to adjust the injection-cycle timing when gradient elution is used. If the sampler is flushed in the *inject* position, the loop will contain mobile phase. If the loop is switched back to the *load* position at the end of the gradient, the loop will contain strong mobile phase; if it is switched after the system is reequilibrated to the initial mobile phase conditions, the loop will contain weak mobile phase. Clearly, the second case is more desirable than the first.

Finally, if you use mobile phase for autosampler flushing, be sure to wash it from the system with unbuffered solvent at the end of the day so that buffer residues don't create additional problems.

In summary, the problem of band broadening with a large-loop autosampler is likely caused by excessive connecting-tubing volume or the injection of too much strong solvent. These problem sources should be easy to isolate and correct.

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Lafayette, California, USA, and is a member of the Editorial Advisory Board of LC•GC.

Bulletins

PACS to offer chromatography courses. Professional Analytical and Consulting Services, Inc. (PACS) is offering a variety of chromatography short courses in 1990. The courses —to be held in Pittsburgh, Pennsylvania — include: "Basics of Gas Chromatography," 22–23 January; "Basics of Capillary GC," 24 January; "Thin-Layer Chromatography," 25 January; "Quality Assurance of Chemical Measurements," 22–23 March; "Capillary Zone Electrophoresis," 16 April; "High Performance Liquid Chromatography," 17–18 April; "Supercritical Fluid Chromatography," 19–20 April; and "Ion Chromatography Principles and Applications," 23–24 April. For more information contact Barbara Nowicki, Professional Analytical and Consulting Services, Inc., 409 Meade Drive, Coraopolis, PA 15108, tel. (412) 262-4222.

Preparative HPLC market expected to grow. The world market for preparative HPLC systems and columns is expected to grow at a 14% annual rate, bringing the annual total to more than $220 million by 1993, according to a market study conducted by Strategic Directions International (Los Angeles, California). The report, titled "Preparative HPLC Systems and Columns," segments the market by industry, geography, and application. Included in the report are market breakdowns, analyses of column techniques, evaluation of market competition, and end-user surveys. For more information, contact David Milligan, Strategic Directions International Inc., 6242 Westchester Parkway, Suite 100, Los Angeles, CA 90045 USA, tel. (213) 641-4982.