Troubleshooting

Celebrating 20 years of "LC Troubleshooting."

Honoring Readers

This installment of "LC Troubleshooting" marks the completion of my 20th year as column editor. My first installment appeared in the October 1983 issue of *LCGC* — then named *LC Magazine* (1). At approximately 11 columns per year, that makes 220 installments. Wow! People often ask how I come up with ideas for columns month after month, year after year. It's quite easy — you, the readers of "LC Troubleshooting," continue to inspire me with your feedback and questions. We'll probably never solve all of the liquid chromatography (LC) problems, so my job is secure as long as you keep your cards and letters coming. (Actually, nearly everything comes by e-mail today, so save the postage.)

Many installments of "LC Troubleshooting" are inspired by one or more questions that get blended into a thematic column — pumps, air bubbles, tailing peaks, and so on. Others are based upon feedback from the courses I teach. A few are contributed in entirety by experts in fields that are not my strong points: pH in organic solvents, evaporative light-scattering detectors, and other specialty topics. Now and then, I dip into the mailbag and select a potpourri of questions. Some of these questions and comments are published as written, but sometimes I embellish them a bit to fill in details that were not included or to expand the questions and cover a broader range of applications. I remove anything proprietary in nature and generally keep the questions anonymous — to protect the guilty. It seems fitting to devote this anniversary to reader input: a bit of feedback, an e-mailed problem, and a question posed during a recent course I taught in Europe. Thanks for all the comments you've provided throughout the years.

But You Forgot . . .

You folks are really good reviewers, and you often point out an aspect of a problem that I overlooked in a particular discussion. Usually, I incorporate this information in a subsequent column, but sometimes — as is the case here — I get a valuable tip that should be included in its entirety. Here's a good addition to a recent column (2), contributed by John Allen (3):

Negative peaks: I don't believe you mentioned that a UV-absorbing impurity in the mobile phase that is not present in the diluent can cause negative peaks in much the same manner as indirect detection. For example, consider an absorbing impurity that is present in (or is added to) the mobile phase and that normally would be eluted at 2 min in that mobile phase. Injecting a sample of the mobile phase without the impurity would yield a negative peak at 2 min. The region of absence of the impurity moves down the column, just as the impurity would in a normal chromatogram. To diagnose this problem,

- inject the diluent to confirm that the negative peak does occur,
- inject a sample of mobile phase to confirm that the negative peak is absent, and
- separately inject the reagents used in the mobile phase to determine which one gives the response corresponding to the negative peak.

Some workers use the mobile phase as the sample diluent to avoid seeing this kind of negative peak, but I have seen a negative peak arise when a different batch of mobile phase was used as the diluent because it was cleaner than the mobile phase prepared by another analyst.

Editor's note: *This situation is one more reason why the best chromatographic performance for a method usually is observed when the sample is injected in a small volume and when mobile phase is used as the sample diluent.*

When Is My Column Dead?

Q: Can you give me your opinion about how the column plate number reflects the status of a column? We are trying to set criteria for the retirement of an LC column. From your experience, what percentage loss of the original column plate number (when it is new) indicates that the column has

John W. Dolan
LC Troubleshooting Editor

failed? Or should we use the plate number at all to measure column quality? What are the criteria at your laboratory for the disposal of an LC column?

A: Well, you asked for my opinion, and that's what I'll give you. (I'm sure this will generate a flurry of responses.) Personally, I do not use the column plate number as an indicator, except as a gross overview of column performance. I do use the plate number to assess system performance (see below), but that is not what you asked.

I believe chromatographers should concentrate on resolution — how well does the column separate the peaks that they need to have separated? After all, peak separation is what chromatography is all about. Yes, we need to have reasonably narrow peaks for good quantification limits, but this seldom is a challenge with modern columns. Another thing to consider is that the plate number influences resolution only as a square-root function; that is, a 50% loss in plate number reduces resolution by only approximately 7%. By the time you have lost 50% of the plates, you are sure to see other more-significant symptoms of impending column death. Finally, in my experience, most workers use only a fraction of the available plates. For example, a new 150 mm × 4.6 mm, 5-μm d_p column might test at 14,000 plates in a manufacturer's column test. I usually use a shortcut to estimate the number of plates that can be expected with real samples as

$$N \approx 3000\ L/d_p \qquad [1]$$

where N is the column plate number, L is the column length in centimeters, and d_p is the particle diameter in micrometers. So, a 150 mm × 4.6 mm column might generate approximately 9000 plates, which is roughly 30% less than the original factory plate number. In practice, however, my observation is that most methods require only one-half this number of plates. Is there room for improvement in these methods? Yes, but column plate number is not the most important parameter in a separation.

In my laboratory, my co-workers and I tend to use a combination of pressure, resolution, and peak shape to determine the quality of a column at any point in its life. Pressure measurements are simple. We simply record the pressure at the beginning of a series of runs. Or, if you are fortunate, your LC system will do this measurement automatically. Generally, we like to have methods that generate no more than approximately 2000 psi with a fresh column and no more than 2500 psi under any conditions. Although modern LC systems can handle much higher pressures, system wear and leaks are much more problematic as system pressure is increased. So, when the pressure reaches approximately 2500 psi, we replace the 0.5-μm porosity frit in the in-line filter installed between the autosampler and the column. If this change doesn't help, the column frit or column itself may be fouled and probably is due for replacement. I think most workers will agree that excessive pressure is one of the most common causes of column failure.

Resolution is the primary measure of column quality that we use. As long as two peaks are baseline separated, chromatographers will have few problems quantifying the analytes. Baseline separation is seen with resolution (R_s) of approximately 1.5 for symmetric peaks; a value of 1.7–2.0 is desirable to obtain a little extra separation. If peaks tail significantly, more resolution will be necessary. The advantage of resolution as a quality measurement is that it can be estimated visually and very quickly. In our laboratory, every reported analytical result gets 100% visual review of the chromatograms by a second staff member. It is very easy to see when two peaks run together or when the valley between two partially resolved peaks changes. So even without going to the trouble of reviewing calculated resolution values, the eyeball method easily catches resolution problems.

Another measure of column quality is peak tailing. Column manufacturers select test compounds that will exhibit good peak shape and, therefore, are of little use in determining peak tailing. On the other hand, a great many real-world samples contain analytes that tail to one degree or another. Of particular interest are nitrogen-containing compounds, which tend to interact strongly with unbonded silanol groups on the silica backbone of most modern columns. As a column ages, the bonded phase is lost, and tailing tends to increase. As opposed to resolution problems, which often stand out, increased tailing can be an insidious change that goes unnoticed. For this reason, it is best to have an upper limit of acceptable tailing; for example, a *United States Pharmacopeia (USP)* tailing factor of no more than 1.8.

The best time to establish rejection criteria is during method development and validation. Usually, you will have tried several columns and analyzed enough real or mock samples during this process to know what happens when the separation degrades. I prefer to leave the limits as broad as possible at the beginning. With experience, you might find that you can tighten the system-suitability requirements, but relaxing the requirements after a method has been validated can be difficult to justify. Before running real samples, analyze several mock samples as a system-suitability test each time the method is set up to ensure the method meets your acceptance criteria.

Earlier I mentioned that the column plate number is useful as a system check. If you put a new column on your LC system and inject the test sample under the column manufacturer's test conditions, you should get the same results as those obtained from the manufacturer's test for the column. Because the manufacturer's test systems often are designed for maximum performance, your results can yield plate numbers that are, perhaps, 10% less than the manufacturer's stated value. If your observed values are much less than this figure, your system might have a problem that needs to be addressed. Injection problems, extracolumn dead volume, poor connections, and detector problems are the most common problem sources. In my laboratory, this assessment is part of a biannual system check we perform on each LC system.

Early Peak

Q: I see a peak in my runs that has me puzzled. I'm using a 150 mm × 4.6 mm, 5-μm d_p C8 column with a methanol–buffer mobile phase run at 1 mL/min. The peak in question has a retention time of 1.1 min. To me, it looks as if this peak is before the solvent front, which I see as a sharply rising peak at 1.55 min. The rest of the peaks in my sample appear to be normal, with retention times of 3–10 min. Can you explain why this peak comes out before the solvent front?

A: You are observing the behavior of a peak that is excluded from the stationary phase. Perhaps you will recall the general rule that allows the estimation of the column volume (V_m) for a 4.6-mm i.d. column as

$$V_m \approx 0.1L \qquad [2]$$

where L is the column length in centimeters and V_m is the column volume in milliliters. Thus, for your system, $V_m \approx (0.1)(15) \approx 1.5$ mL. This amount is converted to the column dead time (t_0) by dividing by the flow rate (F) in milliliters

per minute. For your method, $t_0 \approx 1.5$ mL $\div$ 1 mL/min $\approx$ 1.5 min. You observed the solvent front at 1.55 min, which is within the expected error of this estimation.

Normally, sample peaks are eluted after t_0, and this situation is what you observe for the normal peaks in your runs. If a sample peak does not have full access to the pores in the column, it will be eluted in a volume of mobile phase that is less than the dead volume of the column, thus producing peaks that come out before t_0. Although all the peaks are supposed to come out before the void volume in size-exclusion separations, they should be eluted after the void in reversed-phase separations.

Exclusion from the pores in reversed-phase separations can result from one of at least two causes. First, if the molecules are too large to have full access to the pores, exclusion can occur. This situation can happen with proteins or other large molecules analyzed on traditional particle columns with pore sizes of 80–120 Å. (This reason is why most columns for protein analysis have pore sizes of 300 Å or larger.) Another possible cause of early elution is ion exclusion, which can occur if the stationary phase has a charge that is the same as that of the sample molecule. For example, if ion pairing is performed with a negatively charged additive such as one of the sulfonates, the column surface will have a negative charge. Negatively charged sample molecules can be excluded from the pores and eluted before the solvent front.

If the peak that is coming out too early is a peak of interest, I strongly recommend modifying the method to obtain larger retention times. If the peak is a compound that is of little interest, I wouldn't worry about it too much.

Thanks for the Feedback

Once again, I want to say thank you to all of you readers — some of you have been with me for the past 20 years, and others might be reading this column for the first time. Some contact me regularly, others only once, and most of you are just consumers. As with any product, the quality of this column is improved with each bit of feedback you provide. Thanks for hanging in there.

References

(1) J.W. Dolan and V.V. Berry, *LC Mag.* **1**(7), 406–407 (1983).
(2) J.W. Dolan, *LCGC* **21**(6), 540–544 (2003).
(3) John R. Allen, personal communication, June 2003.

John W. Dolan

"LC Troubleshooting" editor John W. Dolan is vice-president of BASi Northwest Laboratory of McMinnville, Oregon; a training consultant for Rheodyne LLC, the LC Resources Training Group, of Walnut Creek, California; and a member of LCGC*'s editorial advisory board. Direct correspondence about this column to "LC Troubleshooting,"* LCGC*, 859 Willamette Street, Eugene, OR 97401, 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.