TROUBLESHOOTING

Readers' Questions

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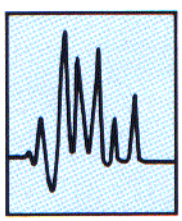

This month, problems with double peaks and lab-to-lab variations in LC separations are discussed. Also covered are an autosampler modification to accommodate submicroliter injections and one reader's experience with rigid-gel reversed-phase columns.

DOUBLE PEAKS

Q: I am analyzing OPA-amino acids by gradient elution using a two-pump system with high-pressure mixing. A 12.5-cm, 5-μm C18 column and a cartridge-type guard column are used. The gradient is from 30 to 80 vol % methanol in buffer (0.1 *M* sodium acetate, pH 7), at 1 mL/min; retention times are in the 10- to 20-min range. A 5-μL sample in dilute HCl is injected.

I consistently observe double peaks for all the bands in the chromatogram. I have changed the column twice and have also changed the injector, but I still have the problem. Do you know what's wrong?

JWD: Your suspicion that there may be column problems is on the right track. You need to isolate the problem more thoroughly, however. Problem isolation is an area in which many workers could use more coaching, so let's look at the process in detail.

Your first guess, that the column is at fault, is a good one. Most problems involving double peaks, especially those that appear throughout the chromatogram, are caused by the column. Another possible cause is an injection-related problem, but you have done a good job of eliminating that possibility: the new injector should take care of any hardware problems, and the small sample size in a dilute aqueous solution should cause no problems, unless very weak mobile phases are used.

The component that you have not changed, which is most suspect, is the guard column. Before proceeding with the systematic checkout discussed below, remove the guard column from the system and run another sample. My guess is that the system will perform normally with the guard column removed. Remember that the guard column is more than a filter — it, like the column, is filled with packing material. Guard columns deteriorate in three ways: First, the frit on the guard column traps particulate matter from the sample or mobile phase. A blocked frit will show up as a pressure increase or, sometimes, as double peaks in the chromatogram (this is true also for the frit at the head of the analytical column). Second, the guard column acts as a chemical filter. Strongly retained materials, such as lipids and pigments, are often trapped at the head of the column, accounting for the yellowish tint that is sometimes observed in the packing at the top of a column. As these materials collect, the plate number of the guard column drops, but this is of little concern because the guard column has no significant contribution to the separation. Problems arise when the guard column becomes overloaded with this chemical "garbage," and breakthrough onto the analytical column occurs. Finally, guard columns fail if the packing within the guard column is disturbed, which happens when a channel or void is formed at the head of the guard column. A void can be caused by the use of aggressive mobile phases (for example, pH outside the 2 to 7 range) or by poor packing technique, certain injector problems, or a number of other conditions.

Generally, it is not worth the trouble to determine the cause of guard column failure unless the lifetime is unacceptably short. In this case, you indicated that the guard column had been in use for a long time; it probably had died a natural death from old age. To minimize the problems caused by guard column aging, keep a sample record, as discussed at the end of this section.

If the system performs normally with the guard column removed, install a new guard column, and you should be back in business. If the peak-splitting problem still occurs without a guard column in place, you need to further isolate the problem by testing system performance under ideal (column-test) conditions.

To test the LC system under ideal conditions, change the mobile phase to 70% methanol–water at 1 mL/min, and allow it to equilibrate for ~15 column volumes (~20 mL or 20 min, in this case). Then inject a 5–10 μL sample of toluene diluted in mobile phase and observe the peak shape. If peak splitting occurs, change to a new column and repeat the test. Three results are possible:

- Splitting occurs with a known good column under these conditions (very unlikely). Check for injection, plumbing, and mobile phase mixing problems.
- The problem does not appear under the standard conditions with the old column. There is a problem with the method, not with the hardware.
- Splitting occurs with the old column but not with the new one. The first column is bad and should be replaced.

When the LC system is set up for column testing, it is also a good time to confirm that the system is performing as well as it can. To do this, compare the column plate number for toluene with that obtained by the column manufacturer (see the column test sheet). If the plate number for a new column in your system is significantly lower than the manufacturer's (by 15% or more), check your system for extracolumn effects. (See the November 1986 Troubleshooting column [1] for a thorough discussion of this topic.)

Finally, a few notes on this case study: I don't know how much time the reader spent trying to solve this problem before he contacted me, but I do know that the problem could have been solved quickly if two simple practices had been followed.

First, the performance of each new column should have been confirmed when the column was received. Column testing helps to identify the occasional bad column that is received, so that it can be exchanged. That way, the reader would have been sure that a good column was used to isolate the peak-splitting problem (remember, he tried two new columns before he gave up). Column testing is also a good way to check the performance of the entire LC system.

Second, the reader would have saved time if he'd kept adequate records on system usage. In this case, a simple record of the number of samples run — from the time the previous column and guard column were installed — would have indicated that the guard column was due for replacement. After you keep column records for several months, you will be able to anticipate column failure points and quickly solve problems caused by column failure, or avoid them altogether.

A PUZZLER ON PEAK RATIOS

Q: Several years ago our plant began working with some new resins. As part of the quality control testing, we routinely ran LC separations on one of these resins. In the beginning, there was no specific requirement

for the testing; we simply ran the tests and recorded the results. Since then, the resin has come into routine use, and limits have had to be set for acceptance. The resin is tested in three different locations, and the peak area ratios of P1/P3 and P2/P2 are calculated. The problem is that the ratios routinely vary slightly from lab to lab, with one laboratory consistently higher than the second lab, which is higher than the third. This trend has continued for some time, even through column changes and other routine maintenance. What could be causing such consistent differences?

JWD: This one has me puzzled. I would like to encourage any readers who have suggestions to contact me.

I first suspected that detector calibration might be the problem (see reference 2), but in a telephone conversation with the reader, I discovered that the same detector model is used on all three systems, and calibration is routinely performed using holmium oxide filters. Thus, detectors are probably not the source of the problem. I also found that each lab was using a different brand of C18 column, although the same mobile phase was used in each case. For the greatest consistency among labs, it would be best to use the same column in each case. When the same mobile phase is used, however, the peak areas should not differ from one brand of column to another.

The remaining variables are sample preparation and calibration. The calibrators in each case should be prepared in exactly the same way. If the calibrators are stable, it is best to have one lab prepare a batch of calibration standards and then split the standards for use in the three labs. This will minimize lab-to-lab variability. If sample preparation of the resin involves extraction, derivatization, reaction, concentration, or evaporation to dryness, one or more of these steps could account for the differences. Again, have one lab prepare all the sample, then divide it among the labs. If the lab-to-lab variability is reduced when the split samples are used, isolate the source of the variability in the sample-preparation technique.

WISP MODIFICATION

Earlier Troubleshooting columns discussed problems associated with autosamplers (3,4). One requirement that is becoming increasingly important is the ability of an autosampler to make injections of 1 μL or less. The Waters Wisp (Waters Chromatography Division, Millipore Corp., Milford, Massachusetts), which is probably the most widely used autosampler, has a minimum injection volume of 1 μL.

A modification of the Wisp to enable submicroliter injections has been discussed (5). As with many instrument modifications, however, the changes mentioned require skills not found in most LC laboratories. A conversion kit for this modification is now available (Aura Industries, Staten Island, New York), and the manufacturer claims it allows Wisp injections down to 0.2 μL in 0.1-μL increments. I don't know how well the kit works, but it might be useful for workers who need to make small injections for microbore or short, small-particle columns. Do any of you have experience with the kit?

POLYMERIC SUPPORTS

A few months ago, I asked for feedback on readers' experience with the new rigid-gel polymeric reversed-phase columns. One reader sent a testimonial (6) (which was also reported elsewhere [7]) to the durability of these columns. The particular assay was for elemental sulfur and required a chloroform–methanol mobile phase. The soft-gel beads that were used in earlier columns swelled greatly in chloroform, so the columns had to be custom-packed with chloroform–methanol as a packing solvent. In addition, these columns were very pressure-sensitive. The rigid-gel polymeric reversed-phase column, however, allowed solvent changeover from water–acetonitrile to chloroform–methanol (via appropriate intermediates) and back without any problems. The reader cautions that the upper pressure limit (6000 psi, in this case) should be respected; he observed column collapse when the pressure went over 6000 psi for several minutes. As a footnote, the reader added, "Since we have put many thousands of soil extracts through one column in the course of 18 months, the news is not necessarily good for the manufacturer — we have had to replace the column only once."

REFERENCES

(1) J.W. Dolan, *LC•GC* **4,** 1086–1090 (1986).
(2) Ibid. 1178–1182.
(3) J.W. Dolan, *LC•GC* **5,** 92–98 (1987).
(4) Ibid. 224–226.
(5) H. Joshua and R.E. Schwartz, *LC, Liq. Chromatogr. HPLC Mag.* **3,** 50–51 (1985).
(6) D. Lauren, personal communication.
(7) D.R. Lauren and J.H. Watkinson, *J. Chromatogr.* **348,** 317–320 (1985).

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