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

Late Peaks and Gradient Reproducibility

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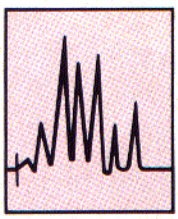

This month, "LC Troubleshooting" addresses readers' questions on late-eluting peaks and gradient method reproducibility. If you would like to submit a question about a problem you've had with your LC system, write to "LC Troubleshooting," The Editor, *LC•GC*, P.O. Box 10460, Eugene, OR 97440, USA.

COLUMN "BLEED"

Q: I observe column bleed, which shows up as an undulating baseline, with a C18 column. I'm using an isocratic method with an acetonitrile–water mobile phase buffered at pH 5. This problem typically appears after I have run the first few samples, then persists for the remainder of the runs. Should I be using a different column or taking special precautions to prevent this problem?

JWD: Several other readers have complained of "column bleed" that appeared when they were using C18 columns for routine assays. But the descriptions I've received of the problem have led me to believe that it is actually one of late-eluting peaks from previous injections. With liquid-coated phases — such as those used in the early days of LC and still used in packed-column GC — true column bleed can be a problem. By "bleed" we mean that some of the stationary phase washes off the column packing and results in a baseline disturbance in the chromatogram. But with bonded-phase LC columns, column bleed is highly unlikely if reasonable mobile phase conditions are used. Because the stationary phase is covalently bonded to the column packing particles, stripping the phase off the particles requires chemically aggressive conditions that break the bond between the stationary phase and the particles. With silica-based packings, mobile phases between pH 2 and 7 are considered safe in terms of packing stability. When you work outside these limits, column degradation will accelerate. When bonded phase is lost, you will observe changing selectivity (relative band spacing), retention, band tailing, and often increased bandwidths. Increased baseline problems may or may not be observed. In severe cases, a void will appear at the top of the column because of the dissolution of column packing.

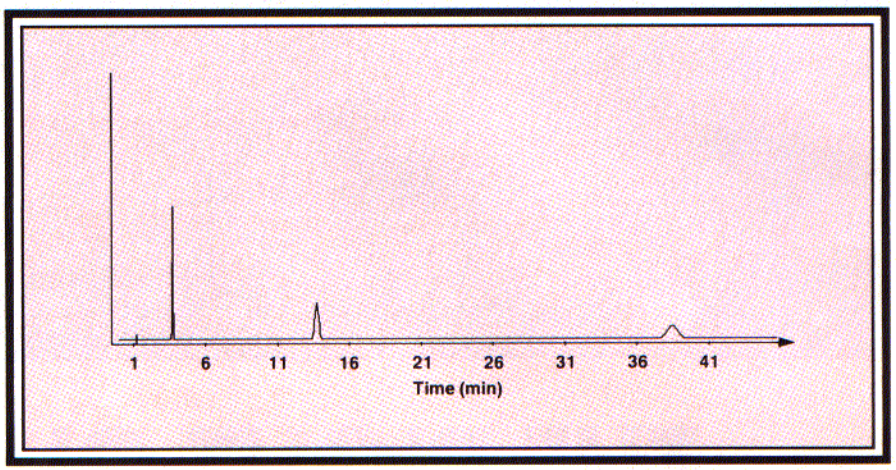

FIGURE 1: Chromatogram showing equal-area bands at $k' = 2$, 10, and 30. (Computer simulation for 25 cm × 4.6 mm column operated at 2 mL/min.)

If your problem is a result of using aggressive mobile phases, you often can extend the column life by using a sacrificial precolumn (saturator column) ahead of the injector. Instead of the analytical column dissolving, this precolumn — an old C18 column or a column hand-packed with silica — dissolves.

True column bleed as described above is not commonly observed in well-designed routine methods. But very late-eluting peaks can produce some of the same symptoms as column bleed — in particular, increased baseline disturbances. The problem can be understood easily with the illustration of Figure 1. Here, we have assumed that all the peaks have the same area, and the method uses a 25 cm × 4.6 mm column operated at 2 mL/min. The first two bands represent the first and last bands of interest, with a k' range from 2 to 10 for good chromatographic behavior. The third band is not of interest and elutes at $k' = 30$.

Notice two things with this chromatogram. First, while the area of the third band is the same as that of the other bands, its height is about 10% of the height of the first band. If the third band were only 10% of the area of the first band, which is more likely if it is an interference in the assay, the band easily could be interpreted as long-term baseline noise. Second, it is likely that the third band in the first run would not be observed until the second or third chromatogram. For example, if an autosampler were being operated on a 15-min cycle, the third band in the first chromatogram would elute about 8 min into the third chromatogram (38 min = 15 + 15 + 8). Each successive chromatogram would have a similar band. If manual injections were used, the band might move around because the time between injections would be expected to vary somewhat. If just one band can show up as a baseline hump several chromatograms later, think of the problems that arise when several late-eluting bands are present!

It is easy to see how the baseline disturbances caused by a sample with several late peaks can be confused with problems caused by column bleed. How can late-eluting peaks be eliminated? Two steps probably will be necessary: improved sample preparation and column flushing. Because late-eluting peaks are much more hydrophobic than earlier peaks (in reversed-phase LC), they usually can be eliminated during sample preparation, either by solvent extraction or by using solid-phase extraction cartridges. It is good to use one of these procedures in sample preparation because it should reduce the need for column flushing to remove strongly retained materials. When isocratic conditions are used, the column can be flushed by switching to a 100% organic mobile phase in order to elute the strongly retained materials quickly. As a

general rule, the retention time should drop by a factor of two to three for each 10% increase in organic, so the late-eluting peaks should be washed off fairly quickly. For example, if Figure 1 represented a chromatogram for a 50% methanol mobile phase, 100% methanol would elute the last band in <5 min. When buffered mobile phases are used, remember not to switch directly to 100% organic, or the buffer may precipitate. With gradient elution separations, you can flush the column at the end of each run by allowing an isocratic hold at 100% of the B-solvent for a few minutes.

Another option in either gradient or isocratic assays is to adjust the sample-injection cycle so that the late-eluting bands come out in an unimportant region of the chromatogram. For example, if the sample of Figure 1 were run on a 19-min cycle, the problem band would elute after the last peak of interest in one chromatogram and before the first peak of interest in the next.

Here's a trick that may help you determine which injection a late band belongs to: The ratio of the retention time to the peak width should be approximately constant for all bands in the chromatogram (under isocratic conditions only). For example, if a peak has a 5-min retention time and a band width of 0.2 min, the ratio is 25. If you observe a broad band eluting in later chromatograms with a width of 2 min, you can use the ratio to determine that its retention time is about 50 min. Now it is easy to see which injection the wide band corresponds to.

In summary, column bleed with bonded-phase LC columns is unlikely as long as the pH of the mobile phase is between 2 and 7. The use of a precolumn can help protect the analytical column from degradation. Late-eluting bands are a likely cause of long-term baseline noise. The user can eliminate these bands by improving sample pretreatment, or at least compensate for them by staggering the injections or flushing the column.

IRREPRODUCIBLE GRADIENT METHODS

Q: I develop gradient elution methods for several laboratories in our company and often have trouble reproducing a method when it is transferred among labs, even if I use the same column. Typically, the spacing of the early bands is different. The resolution of the later bands is satisfactory, but the retention times are not the same from lab to lab. The LC systems are different brands but are configured similarly. Can you help?

JWD: I suspect that your problem is caused by differences in the dwell volume of the various systems. The dwell volume (sometimes called "gradient lag," "gradient delay," or "delay volume") is the LC system's volume from the point where the mobile phase is mixed to the head of the column. For high-pressure mixing systems this volume includes the mixer, the connecting tubing, the injection valve loop, and other precolumn plumbing. For low-pressure mixing, the volume of the pump heads is added to this. The dwell volume ranges from 0.4 mL to about 6 mL for commercially available LC systems; larger volumes may be found when autosamplers, supplemental mixers, or extra plumbing is used. In effect, the dwell volume adds an isocratic hold at the beginning of the gradient. This is illustrated in Figure 2a, where the gradient overlay shows the mobile phase composition at the end of the column. For this example, a dwell volume of 0.5 mL was used. At a flow rate of 2 mL/min, this adds a 0.25-min isocratic hold at the beginning of the chromatogram. The simulation for the same sample with the same gradient (20–60% organic in 10 min) is shown in Figure 2b; however, the dwell volume is 5.5 mL instead of 0.5 mL. In Figure 2b, the gradient reaches the end of the column 2.5 min later ([5.5 mL − 0.5 mL]/[2 mL/min] = 2.5 min). Thus, we might expect the peaks to come out 2.5 min later, such as with the last two bands. The retention of the first bands, however, is not offset by 2.5 min because some isocratic migration has occurred before the gradient reached the column. Also, the bands in the middle of the chromatogram encounter slightly different mobile phase conditions in the two runs, so the resolution differs.

Unfortunately, the calculations used to correct the expected retention time are rather tedious; there is no easy way to make instrument-to-instrument corrections in this manner. When methods are used on LC systems that use the same type of mixing (for example, high-pressure mixing), differences in dwell volume generally do not cause a big problem. When the mixing methods differ between the instrument used for development and the one used for routine work, however, problems such as yours may occur. You can get around the dwell-volume problem altogether by delaying injection by a time equal to the dwell volume. Thus, if you are working with a system that has a 4-mL dwell volume and the flow is 1 mL/min, inject 4 min after the gradient starts. This means that you inject just as the gradient reaches the column. Then, if you switch to another instrument — for example, one with a dwell volume of 1 mL — simply adjust the injection delay to match the new dwell volume. By using this technique, you can remove any instrument-to-instrument variations in dwell volume.

In summary, differences in gradient dwell time among instruments can create problems when methods are transferred among LC systems. There is no easy way to avoid this problem other than timing the injection so that it coincides with the arrival of the gradient at the head of the column.

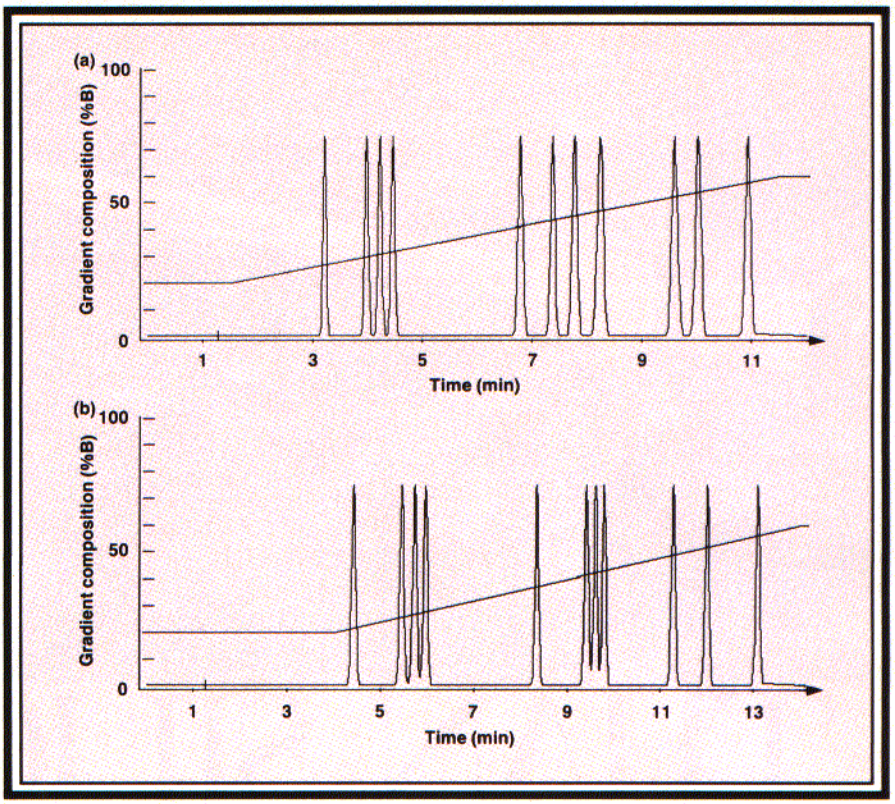

FIGURE 2: Chromatograms for a hypothetical sample run under a 20–60% organic gradient in 10 min. (Computer simulation for 25 cm × 4.6 mm column operated at 2 mL/min.) Overlay shows the gradient composition at the end of the column. (a) Dwell volume = 0.5 mL; (b) dwell volume = 5.5 mL.

"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.