TROUBLESHOOTING

Problems Resulting from Normal System Variability

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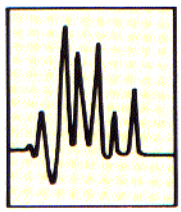

Three problems that result from the normal variability that occurs within an LC system or an LC method are examined in this month's Troubleshooting column. One reader had difficulties with retention-time reproducibility, another had a method-calibration problem, and the third reported a discrepancy in column performance. In each case, the problem can be solved by adjusting the method so that results are not degraded by the normal variations of the system.

RETENTION REPRODUCIBILITY

Q: I am having trouble obtaining stable retention times for my protein separation (molecular weight ~37,000 Da), as you can see by the enclosed chromatograms (Figure 1). I am using a 300 Å–pore C4 column (25 cm × 4.6 mm, 5-μm particles) with 23% *n*-propanol–50 m*M* ammonium phosphate buffer (pH 7) as mobile phase mixed on-line with a low-pressure mixer. The flow rate is 0.5 mL/min. If I use 22% organic, the bands are retained on the column, and with 24% organic, they elute at t_0. The chromatograms show nine consecutive 30-min runs; as you can see, retention times vary. If I check the system with a standard test probe (toluene), retention is very stable. What is causing this problem, and how can I correct it?

JWD: The phenomenon that you have encountered is the result of variations in mobile phase mixing. The reason that you observe the problem with your sample, but not with low-molecular-weight compounds, is illustrated in Figure 2. When retention (log k') is plotted versus the percentage of organic in the mobile phase, straight-line plots (as in Figure 2) are obtained. The (negative) slope S of a plot is characteristic of a compound and generally can be related to molecular weight (MW): compounds with higher molecular weights have larger slopes. Small compounds, with MW < 1000 Da, typically have $2 \leqslant S \leqslant 4$. Large molecules such as proteins have $S > 100$. Two examples are shown in Figure 2 ($S = 3$ and $S = 100$).

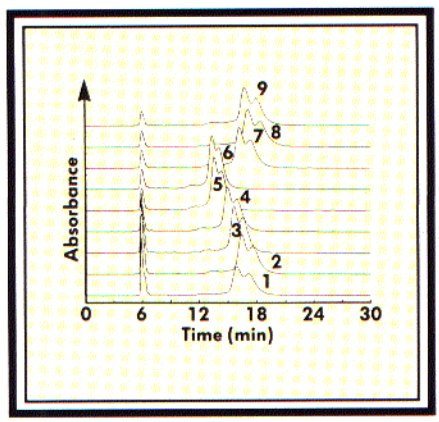

FIGURE 1: Nine consecutive chromatograms of replicate analyses of a 37,000-Da protein.

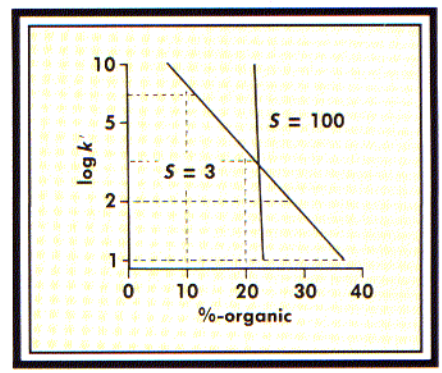

FIGURE 2: Hypothetical log k' versus %-organic plots for a low-molecular-weight compound ($S = 3$) and a high-molecular-weight compound ($S = 100$).

TABLE I: RETENTION PREDICTIONS FOR SAMPLE OF FIGURE 1

Input data
mobile phase = 23% organic, t_R = 17 min
mobile phase = 24% organic, t_R = 6.1 min
Predictions*
S = 204
mobile phase is 23.1% organic, t_R = 12.8 min

*** DryLab 5 (LC Resources, San Jose, California) computer simulations**

In your chromatograms, k' varies from about 1.3 to 2.0 (e.g., for $t_0 = 6$ min and $t_R = 18$ min, $k' = [t_R - t_0]/t_0 = [18 - 6]/6 = 2$). With a small molecule ($S = 3$), k' approximately halves (doubles) for a 10% increase (decrease) in organic (Figure 2). Thus, ~3% change in organic would be necessary to effect the 0.7 units variation in k' that you see (if the sample molecule were small). For the high-molecular-weight compound ($S = 100$), however, k' changes by a factor of 10 with a 1% change in organic. Consequently, a change in organic of less than 0.5% could account for the observed variation in retention.

Table I lists retention predictions based on your data. The S value is greater than 200, and a 0.1% change in organic results in about a 4-min change in t_R, which agrees with your experimental data. It is clear that, with high-molecular-weight samples such as yours, major changes in retention can result from minor changes in mobile phase composition. The same mobile phase variation will have an insignificant effect on low-molecular-weight samples, as is confirmed by your observations for toluene.

Small variations in solvent proportioning by the low-pressure mixer are probably causing your problem. A typical LC pump specification for the precision of proportioning is ±0.1%. As shown above, that much variation could account for the retention variation that you observed; we really shouldn't be surprised by the results. The problem is not

unique to one brand of liquid chromatograph; next month another reader's observations when using a gradient method with a different LC system will be discussed.

The problem should be corrected because retention variations of the magnitude seen in Figure 1 can result in misidentification of peaks. If a data system were used, peak identification would be based on retention times, and the two peaks in Figure 1 might be incorrectly identified. You can eliminate the problem by manually preparing the mobile phase — a premixed mobile phase will be homogeneous and will produce constant retention times.

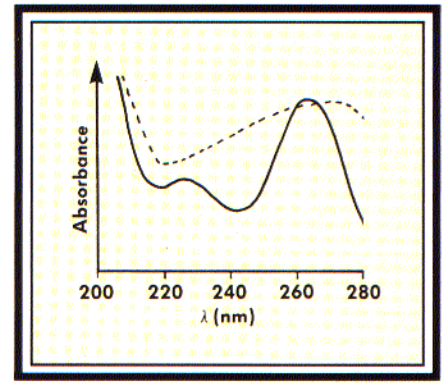

FIGURE 3: Hypothetical UV absorbance spectra for two compounds. (- - -) contaminant; (—) product.

PEAK RATIOING

Q: A problem with a quality-control assay is causing us to reject good batches and accept bad batches of product. We use a *purity factor* as a criterion of batch quality. The purity factor is the peak-height ratio between our compound of interest and an impurity in the product. We validated the method in our lab using one detector, but, when a lab in Europe ran the assay using a detector from another manufacturer, they rejected several product batches. The problem is worst at a detector wavelength of 254 nm, and although it improves at 230 nm, there are still variations. What could be causing this problem? Why is it wavelength dependent?

JWD: I suspect that the problem is related to wavelength calibration of the detector, which is illustrated by the two hypothetical UV absorption curves shown in Figure 3. At a wavelength of 254 nm, the absorbance of one compound, the contaminant (dashed line), is at a plateau, but the other compound, the product (solid line), shows a large change in absorbance in this region. A small change in wavelength can result in a large change in the ratio of product to contaminant. At 260 nm, for example, the ratio for the same sample would be higher than it is at 254 nm.

At 230 nm, in the hypothetical spectrum of Figure 3, the product absorbance is on a plateau, and the contaminant absorbance is on a slope, although the slope is not as steep as in the previous example. Thus, smaller variations in the ratio would be expected in this region.

I suspect that some of the variations you see between labs result from calibration errors. It is easy to see how calibration errors of a few nanometers could cause major differences in the purity factors for the same sample — thus, if one lab's "254 nm" was actually 252 nm while the other lab's was 256 nm, conclusions based on the purity factors could vary significantly. Recalibrating all the detectors with the same calibration standard should reduce or eliminate the problem.

Operator technique could also be responsible for some of the variations. Wavelength selection with most variable-wavelength UV detectors is accomplished using a mechanical linkage to rotate the diffraction grating. Mechanical "slop" in the linkage can cause different results from nominally identical settings. For example, if you turn the wavelength selection dial *down*, say from 275 nm to 254 nm, the grating would end up at a slightly different angle (and therefore will select a slightly different incident wavelength) than if you'd started at a 240-nm setting and *increased* to 254 nm. Depending on the design, age, and adjustment of the particular detector, variations of several nanometers could result. For that reason, it is best to approach the wavelength from the same direction each time. I generally turn the dial to 10–15 nm below the desired setting, then dial up. Consistent technique makes the setting more reproducible (although accuracy may be no better!).

Although use of a "standardless" method, which relies only on an absorbance ratio, might be a convenient way to test for purity, clearly the technique has pitfalls of its own. A much better method is to quantify both the product and impurity (using standards) *before* taking the ratio. That would eliminate problems caused by wavelength variations, yet would not complicate the assay procedure very much.

DIOL COLUMN

Q: I was puzzled by a discrepancy I found while evaluating a diol column using the three phthalate esters (dioctyl, diethyl, and dimethyl phthalate) with 0.5% 2-propanol–heptane as a mobile phase. Dioctyl and diethyl phthalate gave good plate numbers (75,000 plates per meter), but dimethyl phthalate gave a broadened and slightly tailing peak with ~20,000 plates per meter. Next, I tried a mobile phase of 1.0% 2-propanol–heptane; the column performed well for all peaks. In each case, the column was thoroughly equilibrated and the standards were pure. I'm worried that if the column behaves like that with standards, it may give anomalous results for real samples. Do you know what might be causing the problem?

JWD: Band broadening of some, but not all, peaks in a chromatogram can be caused by several factors. As discussed last month, extracolumn effects cause more broadening in early bands than in later ones (1). Similarly, an injection solvent that is too strong will broaden early peaks. I doubt that either of those is causing your problem, and we can also rule out a contaminant that almost coelutes if you are sure that your standards were pure. All of this suggests that interactions with the column packing are the most likely cause of the problem.

Diol columns, like other bonded-phase columns, retain sample components by at least two mechanisms. Interaction between the sample and the diol phase is (one hopes) the primary retention mechanism, but, as with any silica-based column, secondary retention by interaction with the silica surface also occurs. In reversed-phase systems, the silica is often endcapped and amine modifiers are added to the mobile phase to minimize the extent of silanol interactions. For a normal-phase system, a polar modifier, such as propanol or water, often is added to the mobile phase to deactivate the column. Your observation of plate-number improvement with increased propanol content of the mobile phase supports the hypothesis. (A good discussion of normal-phase retention mechanisms can be found in reference 2.)

Why is dimethyl phthalate problematic, while the other two esters are not affected? My guess is that the larger compounds provide better shielding of their carbonyl groups than the methyl ester, which is also more polar and, thus, much more prone to silanol interactions.

CONCLUSIONS

It is clear that problems can arise with LC methods if the system is operated in an unreliable performance region. All LC system components have performance specifications, and if they are considered during method design there should be no problems. Remember also that a new system should perform to its manufacturer's specifications, but an older system is expected to deviate from specifications as the components age. To ensure that this will not cause problems in the future, it is wise to develop methods that do not push the limits of the system.

REFERENCES

(1) J.W. Dolan, *LC•GC* **4,** 986–990 (1986).
(2) L.R. Snyder, *LC, Liq. Chromatogr. HPLC Mag.* **1,** 478–486 (1983). ■

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Readers are invited to contribute their troubleshooting tips to this column or to submit topics or questions for discussion in future columns. Write to: The Editor, *LC•GC*, P.O. Box 50, Springfield, OR 97477.