TROUBLESHOOTING

Maintaining an LC System with Ease

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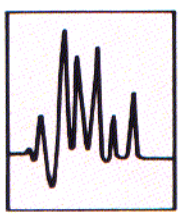

I am frequently asked for advice on how to keep an LC system running with the least amount of effort, prompting me to suggest a few simple rules for preventing most HPLC problems. Although the information is not new, these "Rules for Reliability" serve as a reminder that most HPLC system problems *can* be avoided.

As we saw last month, HPLC analysis of high-molecular-weight compounds can give unusual results (1). In the second part of this month's column, another problem encountered with large molecules will be examined.

RULES FOR RELIABILITY

A lot of space is reserved in this column for discussing unusual problems; they are not only interesting, but they can also broaden our understanding of HPLC techniques. Such discussions might mislead the uninitiated into thinking that reliable LC operation is unusual, when actually there are very few problems that occur on a regular basis. (Reader surveys conducted by *LC•GC* confirm that.) Most problems result from bubbles, dirt, or normal wear, and preventive maintenance is the key to eliminating them. The five rules listed in Table I will help you achieve reliable HPLC operation.

Solvents: Solvents cause the most common pump problems: air bubbles and dirty check valves. If you use the wrong solvents in your chromatograph, you are asking for trouble. Use only HPLC-grade solvents — they are free of particulate matter and chemical contaminants that can cause problems. Water also should be HPLC grade, either purchased from a solvent supplier or prepared in the lab with a water-treatment apparatus (e.g., Milli-Q, Millipore Corp., Bedford, Massachusetts). The mobile phase should usually be filtered through a 0.45-μm filter, especially if it contains buffers, salts, or any other dissolved solid chemicals. If, however, it contains only HPLC-grade solvents, which are filtered at the factory, additional filtering may actually contaminate the mobile phase!

After filtration, reversed-phase mobile phases should be thoroughly degassed, preferably by helium sparging. For isocratic operations that use premixed mobile phases or for high-pressure mixing systems, vacuum filtration is sometimes sufficient, but helium degassing is still a good idea.

Guard columns: Many chromatographers use a guard column for at least some of their work, but system reliability would be improved if guard columns were used for most samples. The guard column, which is placed between the injector and the analytical column, acts as a chemical filter to remove strongly retained materials that might otherwise foul the analytical column and thus shorten its lifetime. Guard columns are available in disposable cartridge designs or as pack-it-yourself kits. A properly selected guard column will not reduce the plate number of the analytical column. If you are running routine assays, your records will soon indicate how long a particular guard column will last. This allows you to replace it, just before it fails, so contaminants don't break through onto the analytical column.

Daily flushing: Perhaps the simplest preventive maintenance step is flushing the HPLC system at the end of each day's work. Flushing removes from the system any buffer residues that could dry, forming abrasive crystals. It also removes strongly retained materials that could gradually deactivate the stationary phase and ruin column performance. The strong component of the mobile phase generally is a good choice for column flushing. For example, if you use acetonitrile–buffer as a mobile phase, flush with about 10 column volumes of acetonitrile at the end of the day. If you are using buffers, however, it is important to flush with unbuffered mobile phase before changing to pure organic; this prevents precipitation of the buffer within the system.

Seal replacement: As pump seals wear out, they shed particulate matter into the mobile phase, potentially causing blockage and wear downstream. Leaky pump seals can cause abrasive buffer crystals to build up and scratch the piston. Seal lifetime varies with type of mobile phase, operating pressure, and pump design, but pump seals rarely last much longer than six months. These parts are inexpensive, and early replacement can eliminate costly downtime. I recommend replacing the pump seals every three months unless there is evidence that they will last longer.

Lamp replacement: Lamp failure is one of the most common problems with UV detectors, yet it can be difficult to diagnose. When sample results become unreliable, lamp failure generally is not suspected until other, less obvious causes have been eliminated. One reason for this is that lamps are quite reliable — deuterium lamps last between six months and a year in most cases. Thus, the lamp is often forgotten until it causes problems. I suggest that deuterium lamps be replaced every six months and mercury lamps replaced once a year. Detectors are now available with extended-life lamp circuits. Also, some manufacturers have begun to equip their deuterium lamps with a built-in meter that measures total running time to help you gauge when to replace the lamp.

If you can make these simple practices second nature, you will find that you spend a lot less time troubleshooting and correcting problems with your HPLC system.

MORE PROBLEMS WITH "NORMAL" OPERATION

Variable retention times that result from normal mixing variations in an LC system have been discussed (1). This month, a reader shared a similar problem (2). To check the purity of a high-molecular-weight polystyrene standard (233 kDa) using gradient elution, an 8.0 cm × 6.2 mm, 5-μm C8 column was used with THF–water as mobile phase. A very shallow gradient was run from 86% THF to 91% THF in 50 min (Figure 1a). It seemed at first that a method had been developed that gave very high resolution, allowing fractionation of a "pure" polystyrene standard! Examination of the collected fractions, however, showed that all the bands were identical. Similar results were obtained on another brand of HPLC instrument.

The experiment described above used 100% water as solvent A and 100% THF as solvent B in a binary gradient. When the experiment was rerun using hand-mixed 86% THF–water as the A solvent and 91% THF–

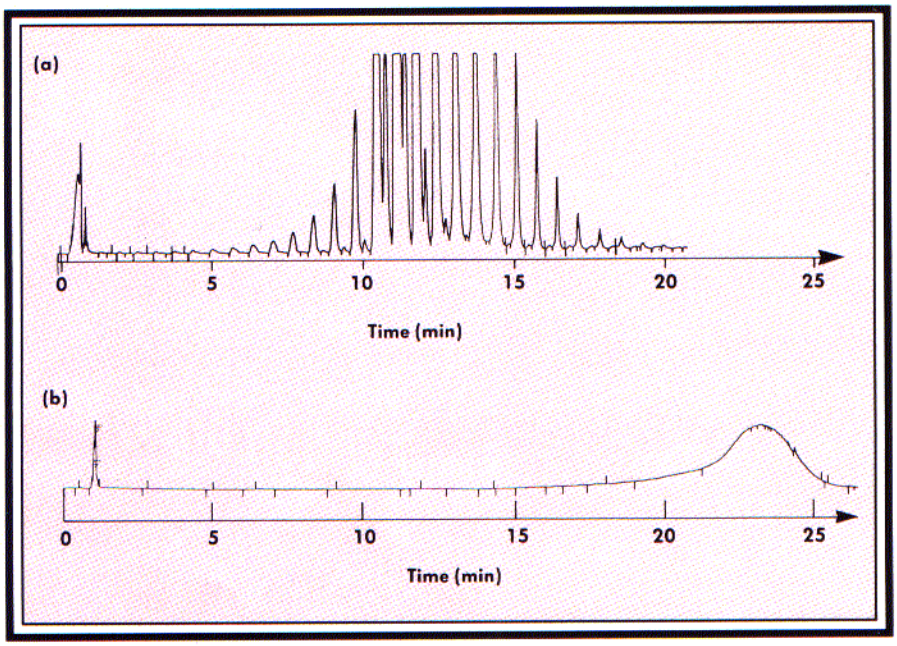

FIGURE 1: Chromatogram of a 233-kDa polystyrene sample. Mobile phase: (a) solvent A = 100% H_2O and solvent B = 100% THF, (b) solvent A = 86% THF–H_2O and solvent B = 91% THF–H_2O; gradient: (a,b) 86–91% THF over 50 min.

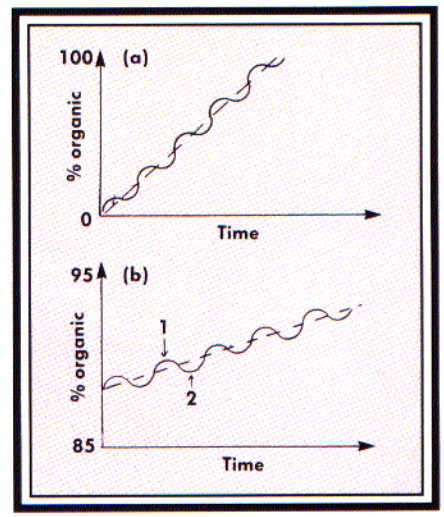

FIGURE 2: (a) Theoretical gradient profile (- - -), profile actually observed (—); (b) same as (a) but for a shallow gradient (mobile phase strength at point *1* is greater than at point *2*, even though point *1* occurs earlier in the gradient).

TABLE I: RULES FOR RELIABLE HPLC OPERATION

Use only clean, degassed solvents
Use a guard column
Flush the system daily
Replace the pump seals regularly (every 3 mo)
Replace the detector lamp early (6 mo to 1 yr)

water as B solvent, with a gradient from 100% A to 100% B, the chromatogram of Figure 1b was obtained.

The conflicting results seem confusing at first, but similar results are commonly found when high-molecular-weight compounds are analyzed by gradient-elution HPLC. For that reason, a discussion of this rather peculiar problem is justified.

The problem resulted from a combination of a compound with a large S value and the normal mixing variations of the HPLC system. As discussed in last month's Troubleshooting column (1), a large S value indicates that retention of the compound is very sensitive to small changes in mobile phase composition. In the present case, $S > 100$. The mixing characteristics of the chromatograph itself provided the second clue. It is usually assumed that a linear gradient really *is* linear — represented by the dashed gradient profile of Figure 2a. A binary gradient is formed by mixing pulses of solvents A and B by means of proportioning valves in a low-pressure mixer or by delivering solvent pulses from reciprocating pumps to a high-pressure mixer. Because mixing is never complete, a small *residual variation* in mobile phase strength remains when the sample reaches the column. In Figure 2a, the solid line shows the residual variability (greatly exaggerated for illustrative purposes). The mobile phase composition changes in a stepwise manner — rapid change, then constant mobile phase strength for a moment, then another rapid change. For a typical gradient (e.g., from 50% B to 75% B over the course of 30 min), these "steps" represent only a fraction of a percent of solvent B and thus are unnoticed.

For shallow gradients, however, the profile looks more like that of Figure 2b, in which the profile, instead of looking like a step gradient, alternates pulses of *stronger* solvent with *weaker* solvent. For example, mobile phase strength at point *1* in Figure 2b is greater than at point *2*, even though *1* is earlier in the program.

Even this amount of variation often goes unnoticed for most applications involving low-molecular-weight compounds. That is because compounds with small S values are relatively insensitive to small changes in mobile phase strength. For example, with nitrobenzene ($S = 3$, MW = 123), a 0.1% change in mobile phase strength would change t_R for a 23-min peak by only 0.15 min (0.6% change). As discussed last month (1), however, a 0.1% change in organic for a 37-kDa band results in a difference of ~4 min (>15% change) in t_R.

With isocratic separations of high-molecular-weight compounds, small changes in mobile phase composition result in variable retention times from run to run (1). With gradient elution, however, it is as if t_R were varying *within* a run. A pulse of stronger solvent carries the front part of the sample band through the column rapidly, but the weaker solvent pulse that follows stops the next portion of the band, thus separating two parts of the same band. As the process is repeated, the segments at the front of the band travel through the column more quickly than later portions. In the case illustrated by Figure 1a, the short-term changes in mobile phase concentration were large enough, relatively speaking, to elute the sample as separate bands. When the solvents were premixed by hand, mixing was improved because the solvent pulses were far more alike. The band was broadened but did not split into separate peaks (Figure 1b).

Mixing-pulse "echo" has been acknowledged for a number of years (3), and LC manufacturers have successfully minimized the problem under normal operating conditions. When your high performance liquid chromatograph is run with high-molecular-weight samples or with very shallow gradients, however, be aware of the potential for special problems.

REFERENCES

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