LC TROUBLESHOOTING

Mobile Phase Buffer Problems

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It's only chemistry: whether you develop your own LC methods or use those of others, be sure that the mobile phase contains reasonable concentrations of each component. Poorly buffered mobile phases may work today, but they're likely to cause problems tomorrow.

This month's "LC Troubleshooting" is prompted by letters from readers regarding four different mobile phase problems. As we will see, following certain guidelines for minimum levels of buffers and other mobile phase additives is likely to increase the reliability of your liquid chromatographic (LC) analyses.

Where'd the Peaks Go?

The first case involves a method for a new drug compound containing amine functional groups. A C18 column was used with an acetonitrile/buffer mobile phase. The buffer was 10 mM citrate at pH 4.5. Because of peak tailing, 0.1% triethylamine (TEA) was added to the mobile phase to reduce the tailing problem. However, when the sample was run with TEA in the mobile phase, the desired peak did not appear in the chromatogram.

This problem probably is related to the change in the pH of the mobile phase. Adding 0.1% TEA to the mobile phase makes the mobile phase 8 mM in TEA. Adding this much base to the relatively weak citrate buffer is likely to increase the pH significantly. Once the mobile phase pH changes, the ionization of the sample will change, and the retention is likely to change, as well. In this case, the worker was on the right track to add TEA to reduce the tailing, and the level of TEA should be 10–20 mM. However, to maintain the buffering capacity of the citrate, the ionic strength of the citrate should be increased. I would increase the citrate to 30–50 mM so that the pH would remain fairly constant when the TEA is added. The pH stability should be checked by measuring the pH of the aqueous portion of the mobile phase after the TEA is added, but before adding the organic.

Light-Sensitive Mobile Phase?

A reader submitted the chromatograms shown in Figure 1. She obtained chromatograms similar to Figure 1a when she used fresh mobile phase. After the mobile phase had been in use for two or three hours, the retention times began to change and the baseline began to degrade until chromatograms similar to Figure 1b were obtained. The worker found that by protecting the mobile phase from light (by wrapping the reservoir with aluminum foil), the mobile phase remained usable for at least six hours. Although we often encounter light-sensitive samples that decompose unless kept in amber vials, most of us never think about this kind of problem with the mobile phase.

I was curious about what could be degrading in the mobile phase. This ion-pairing method had been developed in another lab, and the mobile phase consisted of 300 mL of reagent-grade methanol, 80 µL of methanesulfonic acid, and 21 mL of water. I never did figure out what was photodecomposing, but several things about this mobile phase should raise a red flag. First, reagent-grade methanol is used. One should always use HPLC-grade solvents, which are specially purified for use in LC systems so that minimal amounts of organic contaminants and UV-absorbing interferences are present. Possibly, something in the alcohol was the source of the problem. It is penny-wise and pound-foolish to use poor quality reagents, because they often cause problems at some inopportune moment. The second problem with this mobile phase has to do with the ion-pairing agent. Methanesulfonic acid is a poor ion-pairing reagent because it has such a short hydrocarbon chain; generally hexane- or heptanesulfonic acid is a preferred reagent. Furthermore, the concentration of ion-pairing reagent (3.8 mM) is so low it is not likely to do much good at all. It is recommended to use ion-pairing reagents in the 50–200 mM concentration range, adjusting the concentration if necessary (1). Finally, because ion-pairing methods deal with ionized samples, some form of pH control is needed for good reproducibility.

Although there is a good deal of wisdom in the "if it ain't broke, don't fix it" school of chromatography, this is a good example of a separation system that is just waiting to cause future problems, even if it does work today. If you are working with a method such as this, either stop and correct the potential problems or be very diligent about watching for problems with the separation.

Buffering-Range Problems

I often see mobile phases reported such as 45:55 methanol/20 mM phosphate buffer, pH 4.0. Although this looks fine, pH 4 is outside the effective buffering range for a phosphate buffer. In general, you can obtain effective buffering within ± 1 pH unit of the pK_a of the buffering compound. Thus, for phosphate — whose pK_a values (Table I) are 2.1, 7.2, and 12.3 — we can have useful buffers of about pH 1–3 and 6–8 for LC use (pH 12 is too high for use with silica-based columns). Phosphate doesn't give us the desired buffering capacity in the pH 3–6 range. This doesn't mean that the method mentioned is no good — perhaps all it needs is to have the mobile phase adjusted to pH 4 to work properly (this is the case for many assays). In other words, it may be the pH, not the buffering capacity of the mobile phase, that is important. However, when buffering is required, phosphate, even though convenient to prepare and compatible with most samples, may not always be the best choice for a buffer. You could fill the gap in the phosphate buffering range by using an acetate buffer (pK_a = 4.8, Table I) in the range of about pH 3.8–5.8.

An alternative solution is to use citrate as the buffer. Citrate has three pK_a values in the normal working range for reversed-phase LC (Table I). Thus, you could get a buffer in the range of pH 2–6.5 by using citrate. I have heard that citrate is "hard" on LC pumping

TABLE I: pK_a Values for Common Acids

Buffer	pK_a	Buffering Range*
Phosphate	2.1	1.1–3.1
	7.2	6.2–8.2
	12.3	11.3–13.3
Acetate	4.8	3.8–5.8
Citrate	3.1	2.1–4.1
	4.7	3.7–5.7
	5.4	4.4–6.4

* effective buffering range ~ ±1 pH unit from pK_a

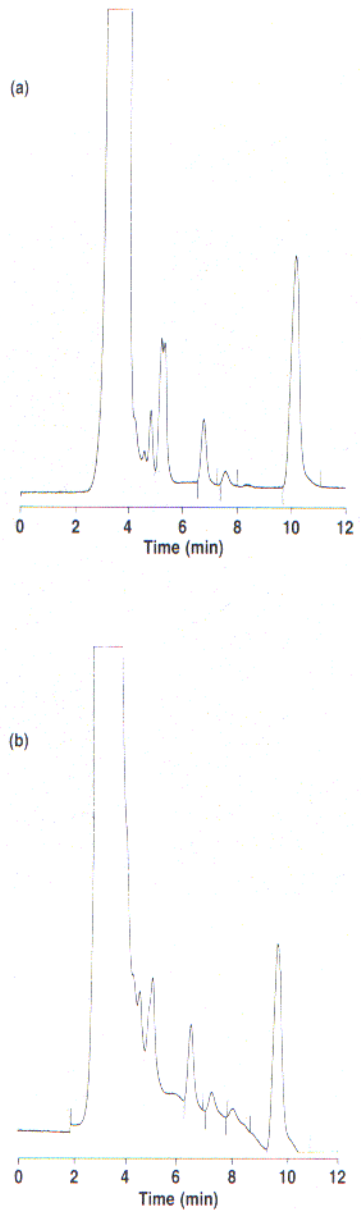

FIGURE 1: Chromatograms resulting from the use of (a) fresh mobile phase and (b) the same mobile phase after a 3-h light exposure. See the text for details.

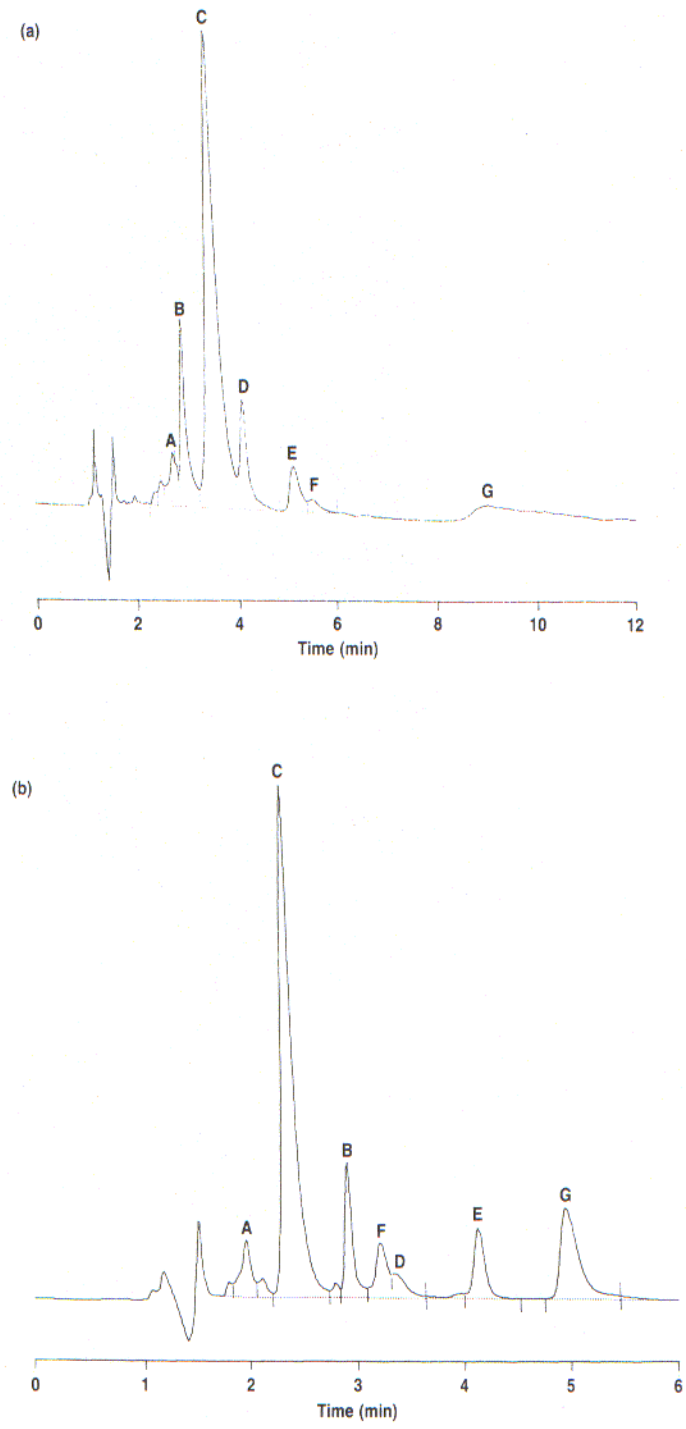

FIGURE 2: Chromatograms of a fermentation broth sample using (a) 8.3 mM buffer and (b) 25 mM buffer. See the text for details.

systems, but I have no evidence other than hearsay. We use citrate routinely in our laboratory with no problems (we do rinse the system with unbuffered mobile phase at the end of each day). I would appreciate hearing from any readers who have experienced problems related to the use of citrate buffers (see note at end of this column).

A final point to keep in mind when adjusting the pH of the buffer: make sure that the buffer can be prepared in a reproducible manner. For example, "20 mM" phosphate buffer, pH 6.5, might be prepared differently in different labs. One lab might prepare a 20-mM solution of Na_2HPO_4 (pH ~ 9.5) and add phosphoric acid until pH 6.5 was obtained. Another lab might prepare the same 20-mM Na_2HPO_4 solution and mix it with a 20-mM NaH_2PO_4 solution (pH ~ 4.5) until pH 6.5 was reached. If the separation were such that the ionic strength was important, these two buffers might give significantly different results. Why? The first buffer is no longer 20 mM, but has a higher concentration, because of the addition of concentrated

phosphoric acid. Whereas the second method produces a more accurate buffer concentration, the first method is reproducible and should be transferable from one lab to another *if* the directions clearly state how to prepare the buffer.

The Importance of Buffer Concentration

The final example of buffer problems concerns the quality control analysis of a fermentation broth for several proprietary antibiotics. The analyst was using a method developed in another part of the company. The mobile phase was easy to prepare: just add 1 g NaH_2PO_4 plus a fixed volume of TEA to 900 mL of water, adjust the pH with dilute phosphoric acid, and dilute to 1 L.

> ***Adding a mobile phase component "for good measure" or using a noneffective reagent can create problems.***

This buffer was mixed with the appropriate volume of organic solvent to form the mobile phase. Although this method gave good separations for small samples of standard compounds, peaks for fermentation-broth samples were broad, with poor sensitivity and marginal resolution, as shown in Figure 2a.

When you encounter a problem such as this, you should check to be sure that the mobile phase composition is reasonable. The 1 g/L of phosphate gives a concentration of just 8.3 mM phosphate, generally considered much too low for good buffering performance in LC. (It appears that the buffer composition was designed for ease of preparation, without much regard to its effectiveness!) When this potential problem was discovered, the buffer concentration was tripled to 25 mM (still easy to prepare — 3 g/L), and the results shown in Figure 2b were obtained. Note the differences between the two chromatograms. The higher buffer concentration increased the column plate number of the main peak by a factor of two (from $N \sim 1300$ to $N \sim 2800$); narrower peaks improve resolution and increase sensitivity. All the peaks show similar improvements in peak shape and band width. Note, however, that changing the buffer strength did change the selectivity (relative peak spacing) for this sample. For example, peak B elutes before the large band in Figure 2a, but after it in Figure 2b. Such changes are not unusual, and may cause problems in some cases. Fortunately, in this case a slight increase in the percentage of organic solvent in the mobile phase pulled peaks D and F apart, giving a satisfactory method that can be completed in less than 5 min.

In Summary...

We can learn a good deal from these four examples. The first and last cases illustrate the need for maintaining an adequate buffer strength for the sample (including any sample matrix injected with the sample). If you are not sure, follow the advice of experts (1) and start with 50-mM buffer. Don't use less than 20-mM buffer unless you have a good reason. Remember that other additives, such as triethylamine, can change the pH of the mobile phase, so check the pH of the aqueous portion before adding organic modifiers.

The second case reminds us to use only HPLC-grade reagents in our mobile phase. Lower-grade reagents may work, but they also are more likely to cause problems. And be sure to use the appropriate reagents. Adding a mobile phase component "for good measure" or using a noneffective reagent can also create problems.

Finally, the third example should remind us that, although we may be chromatographers, we also need to be chemists. The rules of pH are just as true in chromatography as they are in laboratory titrations. When we forget to think of our separations in terms of the chemistry involved, we increase our chances for problems with our methods.

And one last reminder: Don't forget to flush any buffered mobile phase from the LC system when it is shut off at the end of the day. Switch from buffered mobile phase to unbuffered mobile phase, then flush with 100% organic solvent to remove strongly retained materials from your reversed-phase column. Failure to remove buffers from the system can result in the formation of buffer crystals in the pump, drastically reducing pump seal life. To reduce problems (for example, blocked frits) associated with microbial growth in the mobile phase, make fresh buffer solutions daily and wash the mobile phase reservoir regularly.

References

(1) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley & Sons, New York, 1988).

"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. *Correspondence concerning this column can be sent to "LC Troubleshooting,"* LC•GC, *P.O. Box 10460, Eugene, OR 97440, USA.*

Bulletin

E. Merck and Hitachi sign contract. Laboratory-instrument manufacturers E. Merck (Darmstadt, Federal Republic of Germany) and Hitachi (Katsuta, Japan) have signed a 10-year contract involving development of instrumentation for HPLC. The Merck-Hitachi range of modular HPLC instruments includes autosamplers as well as fluorescence and UV detectors.