LC TROUBLESHOOTING

Buffer Precipitation Problems

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Buffer precipitation can be an irritating problem. As with so many other LC problems, prevention is the best medicine.

Two readers submitted questions about buffer precipitation problems. Sooner or later, most chromatographers encounter buffer precipitation problems in one form or another. The two questions discussed here illustrate some of these problems and how they can be eliminated.

EXCESSIVE BASELINE SIGNAL

Q: After using a method for a while without any problems, I suddenly observed a very high baseline. The method uses gradient elution with electrochemical detection (glassy carbon, +0.7 V vs. Ag–AgCl). Mobile phase A is 20:80 acetonitrile–buffer, and mobile phase B is 60:10:30 acetonitrile–methanol–buffer. The buffer is 150 mM sodium acetate (I need high ionic strength for separation)–10 mM citric acid–1 mM EDTA. I add acetic acid to adjust the buffers to pH 4.7 and 3.5 for A and B, respectively; after I add the organic solvents, the final pH is ~5.0 for both mobile phases.

Problems began when I prepared new batches of mobile phases A and B. When solvent B was pumped alone, I observed a very high baseline (30 to >100 nA, instead of the usual 2–3 nA). The problem persists, even though I tried the following steps.

First, I thought incidental contamination might be the problem, so I prepared another batch of mobile phase. The new batch yielded the same results as before.

Next, I checked all the components of solvent B to try to identify the dirty component. Each combination (water–acetonitrile, water–methanol, water–acetonitrile–methanol, and buffer with acetic acid) produced a normal baseline.

Then I opened new bottles of acetonitrile and methanol and prepared new buffer. The new mobile phase B made no improvement. I made up new mobile phase A, and it was OK, too.

Stepwise, I tried other fixes, including swapping the inlet lines for reservoirs A and B, eliminating filtration and vacuum degassing, filtering solvent B again (0.22-μm filter), cleaning the working electrode, and replacing the reference electrode. None of these actions improved the baseline.

The column (15 cm × 4.6 mm, 3-μm d_p C18) is almost new, and I do not think it is responsible for contamination of the system because, although high, the baseline produced by 100% solvent B is perfectly straight. I'm out of ideas. Can you help?

JWD: Your problem puzzles me, also. I consulted an expert in electrochemical detection, and still no clear answer is forthcoming. I see some additional steps you might take, however, and some improvements in your methods that also might help.

According to your description, excessive absorbance occurs for solvent B, but only when all of the components are mixed. It appears that solvent A is OK. The first thing I would try is to put a new column on the system. The only way you can get a high signal with B but not with A (assuming the reagents are from the same source) is either if B is contaminated and A is not, or if B washes something off the column that A does not. Because B is the stronger solvent, it is feasible that it is washing a contaminant off the column. The contaminant could have built up over time or could have been a one-time intrusion. Flushing the column with a strong solvent might help, but first I would try a new column.

If contamination is the source of your problem, where could it be coming from? You didn't mention the sample compounds, their source (for example, metabolites from a physiological fluid), or your injection procedure (solvents, volumes, and so forth). Each of these areas has potential for contaminating the system or upsetting the equilibrium, which could cause detection problems.

You may discover some useful information by lowering the detector voltage to 0.5 V to see if the problem improves or disappears. This voltage may not enable you to get sufficient detection but should give you an idea of the nature of a contaminant, such as whether it is electrochemically active.

Several other things about your method are nonstandard. I don't think these are responsible for the immediate problem, but because they may cause problems in the future you may want to attend to them now.

First, column equilibration can be a problem when solvent B contains additives that solvent A does not (in the present case, B contains methanol, A does not). This difference in mobile-phase composition can affect retention-time reproducibility from run to run. It is best to have all mobile-phase components present in both A and B, even at very low levels. Although this arrangement is not always practically possible, it is an important target to shoot for.

Second, unless you are trying a pH gradient, the pH of both A and B should be the same as measured in the aqueous solution. It really doesn't matter what happens when you add the organics, because the pH measurement with organics included is fairly meaningless. Thus, I would adjust the pH of the aqueous portion of both A and B to pH 4.7 (or 3.5, or whatever you choose) and leave it at that. Also, stay within the normal buffering region for your buffer, which is ±1 pH unit from the pK_a. For acetate, the pH range is 3.8–5.8, so the pH 3.5 buffer that you used is outside the recommended range for acetate (although the citrate additive may extend this range).

It is best to have all mobile-phase components present in both mobile phases, even at very low levels.

Finally, you are running a reverse gradient in salt, EDTA, and acetate. This may be what you want, but I doubt it. Try adjusting the ionic strength of the buffers so that the final (diluted with organic) concentration of both buffers is the same. (You tried to adjust the final pH, which you can't control, and not the buffer concentration, which you can control.) Also, be careful about using too high a buffer concentration — buffers are poorly soluble in acetonitrile, and it is possible to inadvertently form precipitates when high-salt mobile phases (A) and high-acetonitrile mobile phases (B) are mixed.

None of these suggestions are likely to eliminate your problem, but they will result in a more rugged method.

THE IMPORTANCE OF KEEPING RECORDS

The reader solved the problem and sent me the following note:

Q: I changed the column as you suggested, but nothing improved. I got a clue, however, when I was browsing through my HPLC diary, where I record everything I do (it's a boring task, but it has saved me in bad situations before, and I would advise anyone to do it). I noticed the same thing had happened when the method was being set up. At that time, I halved the buffer concentrations and everything returned to normal, so I did it again and the problem disappeared. (I had returned to the higher concentration at one point because I couldn't adequately resolve two peak pairs.) Moreover, I noticed that the bottles of bad mobile phase B that had been sitting for about a week contained some precipitated salts on the bottom. Both times this problem occurred, the lab was quite hot (>25 °C). Do you have any further suggestions?

JWD: The occurrence of a solubility problem at higher temperatures is the opposite of the expected effect. I suspect that the higher temperatures caused sufficient organic solvent evaporation to cause precipitation.

PRECIPITATION IN THE PUMP

Q: We observe a problem with buffer precipitation in our LC system. We are mixing a 90:10 acetonitrile–buffer mobile phase using 100 mM phosphate in the A reservoir and acetonitrile in the B reservoir. (We would like to use the same setup for a gradient we plan to develop soon.) The system consists of two single-piston pumps and a high-pressure mixer. The buffer precipitates inside the tubing that feeds acetonitrile from the pump to the mixer. Gradually, the precipitate creeps back down the line into the pump head. We have changed pump seals and check valves several times, but have observed no improvement. We can remove the precipitate by pumping water through the system from either reservoir. We can reproduce the problem on another system of the same brand, but no precipitation problems occur when we use low-pressure mixing on another brand of system. What can be causing the problem, and how can we avoid it in the future?

JWD: My experience is that buffer precipitates in acetonitrile form at the buffer–acetonitrile interface in the mixer, not in the feed tubes. Also, I have seen more problems with low-pressure mixing than with high-pressure mixing.

I can think of a feasible explanation for the cause of your problem, but it would take some additional experimentation to prove. First, it is common for a precipitate to form at the buffer–acetonitrile interface. You can see this by adding buffer, one drop at a time, to a test tube containing acetonitrile. Often a cloudy precipitate will form when the drop of buffer hits the acetonitrile and then will quickly redissolve. The same thing can happen inside the mixer. Because it is unlikely that the A and B pumps are perfectly synchronized, at least part of the time one of the pumps will be refilling while the other is still pumping. As the acetonitrile pump starts to refill, there is a very short interval during which the outlet check valve closes and the flow in the acetonitrile line between the pump and mixer is momentarily reversed. Thus it would be possible for buffer to be forced back into the acetonitrile line and precipitate. Because buffer precipitates are harder to redissolve than to form, some buffer residue may remain in the line. If this process were to continue and the volume of the acetonitrile line were small enough, it is conceivable that the precipitate could work its way back to the check valves. Some pumps have a very short, narrow-diameter tube between the pump and the mixer.

You can check for this problem in your system by significantly increasing the volume of the acetonitrile feed line. I suspect that the line you are using is 0.010 in. i.d. or smaller. I would substitute a longer and wider bore tube so that the total volume is increased by perhaps a factor of eight (double the internal diameter and the length of the tube). Alternatively, plumb a pulse dampener between the pump and mixer to increase the volume of this section of the plumbing. Neither of these changes should affect the system's performance in terms of mixing or accuracy.

How to solve this problem? There is little you can do about the pump design, so if the larger volume tubing doesn't help, you may need to pursue another route. Generally, buffer precipitation problems go away if you premix a little buffer in the acetonitrile and a little acetonitrile in the buffer — 5% and 95% acetonitrile in the A and B phases, respectively, should be sufficient. Because we rarely need 100% buffer or 100% acetonitrile, such premixing is a minor inconvenience in exchange for more reliable system operation.

I am curious about whether this reader's problem is widespread. Please write to me care of *LC•GC* or send me a fax ([503] 835-7930) to share your experiences.

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.

CONCLUSION

These two problems illustrate the importance of taking extra care when using buffer-containing mobile phases. Buffer precipitation has been covered in detail in a previous column (1) and elsewhere (2). The following simple guidelines will keep you out of major buffer problems.

First, keep the buffer concentration between 20 mM and 100 mM for most applications. Below about 20 mM, methods can be overly sensitive to small changes in buffer concentration. Little is gained when buffer concentrations are greater than 100 mM, and such conditions are likely to produce precipitation problems. Plenty of exceptions to this guideline can be found — hydrophobic interaction and ion-exchange chromatography are obvious examples.

Second, keep the pH within the normal buffering range of the buffer being used (±1 pH unit from the pK_a); Table I shows the pK_a values for common acids. Phosphate has some "holes" where the buffer is not effective (for example, pH 3.1–6.2), so these regions should be avoided.

Third, for accurate buffer preparation, prepare two buffers so that they have the same concentration and bracket the desired pH, then mix them until the proper pH is reached. This method avoids inadvertently changing the salt concentration by adding acid or base to adjust the pH. For example, the pH of monobasic phosphate and phosphoric acid stock solutions at 20 mM will be about pH 4.5 and 1.5, respectively. These two solutions can be mixed until the target is reached (for example, pH 2.9). This technique ensures that the concentration is maintained at 20 mM.

If on-line mixing is to be used, system reliability can be improved by premixing organic solvent with the buffer and buffer with the organic solvent. This seems to "take the edge off" the precipitation problem, resulting in precipitate-free on-line mixing.

Finally, heed the advice of the first reader. Although keeping and reading her HPLC diary was "boring," it turned out to be the key to solving her problem. Such is the case with recordkeeping. It is much like insurance — you hope you'll never have to use it, but it isn't worth the risk of being without it.

REFERENCES

(1) J.W. Dolan, *LC•GC* **8**(3), 212–215 (1990).
(2) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (Humana Press, Clifton, New Jersey, 1989).

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