Troubleshooting

Reader Questions: Mobile Phase

What mobile phase conditions will give good results?

In this month's "LC Troubleshooting," we look at two questions submitted by readers that relate to mobile phase. The first deals with a mobile phase for a validated liquid chromatography (LC) method that appears to be the source of retention time variation. The second relates to recommended starting conditions.

Irregular Retention Times with Ion Pairing

Recently, a reader contacted me with a problem he encountered when he used a validated ion-pairing method. The conditions seemed normal enough: 54% acetonitrile combined with 46% ion-pairing reagent in water. A pH of 4.5 was obtained by addition of glacial acetic acid. The column was a name-brand 250 mm × 4.6 mm, 5-µm particle C18 column operated at 1.5 mL/min and 45 °C. Before running a batch of 40–50 samples, the column was equilibrated with mobile phase and system suitability was run to check for proper retention and adequate response. Once samples were run, baseline drift was seen on an occasional basis but was not a concern. However, retention times changed on a random basis — sometimes shorter and sometimes longer. Other methods on the same instrument worked satisfactorily, so the instrument itself was not suspect.

As I have mentioned in this column many times over the years, ion-pairing methods are among the most troublesome LC methods one can encounter. Recall that the ion-pairing process relies on an equilibrium between ion-pairing reagent free in the mobile phase and that which is adsorbed on the stationary phase. The equilibrium is rather slow. In my experience, it can be two or more times slower than traditional reversed-phase methods. For example, the rule of thumb for mobile phase equilibration of a reversed-phase column is 10–20 column volumes, but it may take 50 column volumes to equilibrate an ion-pairing system. Furthermore, anything that upsets the equilibrium can change retention times. For example, a change in the column temperature or mobile phase percent organic will affect the partition of the ion-pairing reagent between the stationary and mobile phases, so column thermostating and isocratic operation are necessary for reproducible separations.

When I look at a problematic ion-pairing method, I start by examining parameters that might not be controlled fully. Temperature usually is the first suspect, but in the present case, the column is thermostated, so temperature changes are unlikely to be the cause.

Another possibility is that the mobile phase pH is not consistent. I tend to be suspicious of mobile phases that are formulated merely by pH adjustment rather than the use of a true buffer. In the present case, adjustment of the pH with acetic acid falls in this category. Sometimes, the lack of buffering capacity makes no difference, but in other cases, a small change in pH can be significant. I would formulate this mobile phase using acetate buffer adjusted to pH 4.5 instead of just adding acetic acid. Depending upon the nature of the sample and sample solvent, the buffering capacity of the mobile phase might not be sufficient to adequately buffer the sample. If this were the case, a shift in retention time is quite possible. After all, ion pairing relies on the ionic nature of the sample for retention and if the sample ionization changed, retention could change also. So my first recommendation is to make the aqueous portion of the mobile phase in, for example, 25 mM acetate buffer at pH 4.5. I would also make sure that the final sample diluent was matched as closely as possible to mobile phase — ideally the mobile phase itself.

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Although the baseline drift was more of an annoyance than a problem, it can give us some ideas about a possible problem source. One possibility that comes to mind could occur with on-line mixing of the mobile phase. Acetonitrile is notorious for being a poor solvent for salts, buffers, and ion-pairing reagents. Depending upon the mixing configuration (low or high pressure) and specific mixer design, it is possible to have the acetonitrile–reagent interface create a momentary reagent precipitate. If this did not readily redissolve, check-valve operation could be compromised by particulate matter. Even though other reversed-phase methods worked properly, one could still have such precipitation problems with ion pairing. This is one reason why many ion-pairing methods use methanol rather than acetonitrile — methanol is a much better solvent for salts and buffers. Because ion pairing typically is performed in the isocratic mode, hand-mixing the mobile phase should eliminate any potential for precipitate formation.

As I thought more about this method, I realized that I had not calculated the mobile phase concentration of the ion-pairing reagent. Suspecting it to be high (for example, greater than 50 mM), I was surprised to find that the aqueous portion of the mobile phase contained only 3.5 mM ion-pairing reagent. When it was diluted with acetonitrile, it was only 1.6 mM. This is just the opposite of what I expected. Now I suspect that there might be insufficient ion-pairing reagent present for stable operation. Normally, 20–30 mM would be a better choice.

Because the method was validated, the user was restricted in the changes in the method that were allowed. This had him very discouraged, because the changes suggested earlier were not allowed. "But it's a validated method — I can't change anything," is a litany I hear all too frequently. My stance is that if the method is not working well enough to get the required analytical results, it really isn't validated. Furthermore, one needs to evaluate whether or not possible fixes will correct the problem or not. Once armed with data that support better method performance with a method change, one can then evaluate how to "legally" modify the method so it performs as intended. For cases such as this, I recommend making up mock samples and trying the various changes suggested to see what happens. Ideally, one simple change will fix the method. We should change just one variable at a time so that we can know which change really corrected the problem. In the present case, I would make the changes from least invasive to most invasive. First, I would hand-mix the mobile phase to make sure that any precipitation problems were eliminated. Second, I would make sure that the injection solvent was closely matched to the mobile phase so that pH shifts do not occur when the sample is injected. Next would be to formulate the mobile phase with a true buffer — acetate in the present case, adjusted to pH 4.5. If none of these fixes worked, I would increase the ion-reagent concentration by 10-fold. The increase in ion-pairing reagent concentration would likely increase retention significantly, so a higher percentage of organic solvent might be required to get retention in the right region. If any one or a combi-

Continued on p. 667

Continued from p. 664

nation of these changes gave a stable method, one would be able to justify revalidating the method.

Are there alternatives that might solve the problem without requiring revalidation? I would consider the first two recommendations (hand-mixing the mobile phase and using the mobile phase as the final sample diluent) to be minor method changes that do not require revalidation. I would run a batch of mock samples to see if these changes stabilized the retention times and use these data to support a minor method modification without extensive validation.

Selection of Mobile Phase pH

A reader submitted a question regarding selection of the mobile phase pH. The analyte is a zwitterion containing a carboxylic acid function with a pK_a of 6.2 and an amine function with a pK_a of 9.0. He had been advised to start method development with a pH of 2.4 phosphoric acid solution for the aqueous phase. He wondered why this recommendation was made.

In reversed-phase LC, it is the nonpolar nature of the analyte that is primarily responsible for retention. The ionic functional groups obviously make the compound less polar, and one would expect shorter retention times in such cases. For cases such as the present example, one has to consider the pK_a(s) of the compound as well as the recommended operating conditions of the column when choosing an operating pH. Consider three possibilities. First, the mobile phase pH could be lower than the pK_a of the acid. In this case, the acid would be un-ionized and therefore neutral. This is the case when the mobile phase pH is at least 1.5 pH units below the pK_a, which is true for the recommended conditions. So one would expect good retention of the acidic and neutral portions of the molecule; the basic functional group would remain ionized. Second, if the mobile phase pH were between the pK_a of the two functional groups, both would be ionized. This should be the most polar situation for the molecule, and thus produce the smallest retention times. Finally, if the mobile phase pH is at least 1.5 pH units above the amine pK_a, the amine would be un-ionized and neutral; the acid would be ionized. One would expect longer retention times than the intermediate case.

One also should consider the useful pH range of the column when selecting the mobile phase pH. All other things being equal, silica-based columns generally are limited to a pH range of 2–8. If the pH is less than 2, the bonded phase will be cleaved from the silica; if the pH is greater than pH 8, the silica will begin to dissolve. So it can be seen that the recommended pH of 2.4 is greater than 2, and the column should be stable and at least 1.5 pH units less than the lower pK_a of 6.2 (6.2–1.5), so the carboxylic acid function would be un-ionized. At any pH greater than 4.7, both functional groups will be ionized or the pH will be too high for column stability. This is the reason most workers start method development at low pH even if there is an amine function present — the acids are un-ionized and you just have to cross your fingers on the retention of the bases. An additional benefit of low pH is that the unbonded silanol groups on the column packing exist in a condition of ion suppression, so peak tailing due to silanol ionization is minimized.

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All of these factors go together to support the recommendation of starting method development at low pH, such as the pH 2.4 suggested to the reader. My only additional advice is that he use a phosphate buffer at pH 2.4 rather than just adjusting the pH to 2.4 with phosphoric acid. As with the ion-pairing example presented earlier, a buffer will almost always give better results than simply a mobile phase adjusted to a selected pH.

This leads to an additional question that often comes up. What do you choose for a starting pH if you don't know much about the nature of the analyte(s) — whether it is acidic, basic, or neutral? The answer really is the same as for the present example. Start with a low pH and you probably won't go wrong. Ionization of acids will be suppressed, neutrals will be unaffected, and you can't work at a high enough pH to suppress ionization of bases. If you have basic samples, however, there also is good news, because there are several silica-based reversed-phase columns on the market that are stable at mobile phase pH values greater than 8. For example, in my laboratory, we have several methods that operate at mobile phase pH values of at least 9 and column temperatures of greater than 50 °C. We obtain 500–2000 injections per column with these base-stabilized products, which is quite acceptable.

Summary

Sometimes the mobile phase conditions selected for reversed-phase and ion-pairing methods seem either arbitrary or mysterious. The two examples discussed in this month's "LC Troubleshooting" have allowed us to dissect the mobile-phase characteristics to see their importance and contribution to consistent method performance. As a general rule, buffered mobile phases will give more consistent results than when pH is adjusted, but no buffer is present. During method development, it is a good idea to change the method variables individually (pH, organic concentration, buffer concentration, ion pairing, and so forth) in small increments from the best conditions. This will give you an idea of how much tolerance the method has for such variations and give an indication of the method robustness. It also will provide you with clues as to what symptoms might appear when such changes are made inadvertently so you can quickly troubleshoot method problems.

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