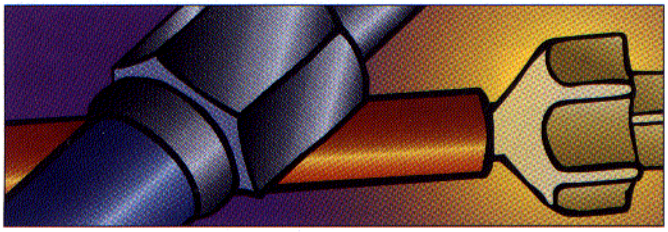
LC Troubleshooting



Improving an Ion-Pairing Method

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How do you improve a method that isn't quite good enough?

This month's "LC Troubleshooting" responds to a reader's question about a problematic liquid chromatography (LC) separation in which ion-pairing reagents were used. We'll see that, although the ion-pairing system is complicated, you can use a systematic approach to develop new methods and improve existing ones.

THE PROBLEM

Q: We are analyzing brain homogenates for morphine and its metabolites using an ion-pairing method, but we have been unable to separate all the peaks of interest from interferences. Can you give us some advice about how to improve the separation? The mobile phase is 2 mM lauryl sulfate, 10 mM sodium phosphate (adjusted to pH 2.1 with phosphoric acid), and 20% acetonitrile. The column is a 7.5 cm × 4.6 mm, 3 µm d_p C18 column operated at a flow rate of 1 mL/min. The UV detector is set at 210 nm. We use a matching guard column.

A: When using ion pairing, the separation depends on a combination of several variables. Usually you must optimize each variable and then check all the variables in the optimum region to get the most out of the system. Let's look briefly at the important variables.

A primary variable in ion pairing is the concentration of ion-pairing reagent on the column. One analytical model describes ion pairing as a process in which the ion-pairing reagent is held on the column to create an in situ ion exchanger. Thus, the charge on the surface controls retention and selectivity. The charge is increased by any process that increases the column loading. Increasing the concentration of reagent will shift the equilibrium toward the column, thus increasing the charge on the column. A longer-chain ion-pair reagent (for example, C_8-sulfonate vs. C_6-sulfonate) will favor partition onto the column, thus increasing the charge. Similarly, reducing the mobile phase's level of organic solvent will shift the equilibrium toward the column, also increasing the charge on the column. So, you can change the column loading three ways.

The choice of ion-pairing reagent is not terribly important. That is, you can get the same results with a higher concentration of a short-chain reagent as with a lower concentration of a long-chain reagent. So changing reagents to change selectivity provides little benefit. Rather, you can choose the reagent chain length to generate the desired charge in the presence of the chosen organic solvent concentration. Generally, it is best to work with 10–100 mM concentrations of mobile-phase additives, and this rule holds for the negatively charged ion-pair reagents. At levels less than 10 mM, small changes in the concentration can make relatively large changes in the system performance, and, in some cases, the concentration may be too low to be effective. At levels greater than 100 mM, the solubility becomes a concern, especially with acetonitrile in the mobile phase. In your case, the 2 mM concentration of the ion-pairing reagent is a bit low for my preferences — I would consider switching to octanesulfonic acid and using a higher concentration.

A second factor in ion-pairing separations is the mobile-phase pH. You are performing an ion-exchange separation, so the pH will control the charge on the sample molecules. You will obtain the best results if the pH is not too close to the pK_a of the sample compounds. Generally, it is best to stay at least 1 pH unit from the pK_a to avoid the problems that can occur when a sample molecule is partially ionized.

A third factor is the organic-solvent concentration in the mobile phase. Because the ion-pairing system uses a reversed-phase column, a mixed retention mechanism will exist. Some of the sample molecules will be retained by ion-exchange (ion-pairing) processes, whereas others will be retained by reversed phase. The result is that the organic-solvent concentration affects the separation in two ways: For nonpolar molecules, higher organic solvent levels will reduce retention in the normal reversed-phase pattern. For molecules that are retained by ion exchange, higher organic solvent levels will reduce the concentration of ion-pairing reagent on the column and thus the charge on the column, also resulting in shorter retention times.

The relative importance of these two retention mechanisms depends on the sample components, so you also should expect selectivity changes when the mobile-phase organic-solvent concentration is changed. The slow equilibration of the ion-pairing reagent on the column means that using gradient elution with ion-pairing separations creates an inherently unstable system. For this reason, gradient ion-pairing separations are not recommended.

The choice of organic solvent can affect the selectivity in ion pairing just as it does in standard reversed-phase separations. Although acetonitrile usually is my first choice for reversed-phase separations because of its usefulness at low wavelengths and somewhat lower viscosity, methanol often is the solvent of choice for ion-pairing separations. Analysts choose methanol because of its superior solubilization characteristics compared with acetonitrile. Ion pairing often calls for relatively high concentrations of mobile-phase additives, so the risk of precipitation is reduced when you use methanol. In your case, you chose to use acetonitrile for its low-wavelength advantages; you will need to watch for precipitation problems if you use higher concentrations of ion-pairing reagents.

Finally, column temperature plays an important role in ion-pairing separations. The expected changes in retention, mobile-phase viscosity (which translates to back-pressure changes), and selectivity hold for ion pairing just as they do in reversed-phase LC separations. In addition, temperature affects the equilibrium of the ion-pairing reagent between the mobile phase and the column. And in some cases, the temperature can be an important factor in peak shape for ion-pairing separations — a phenomenon that doesn't occur with reversed-phase separations. Because of the sensitivity of the system equilibrium to temperature, you should always thermostat

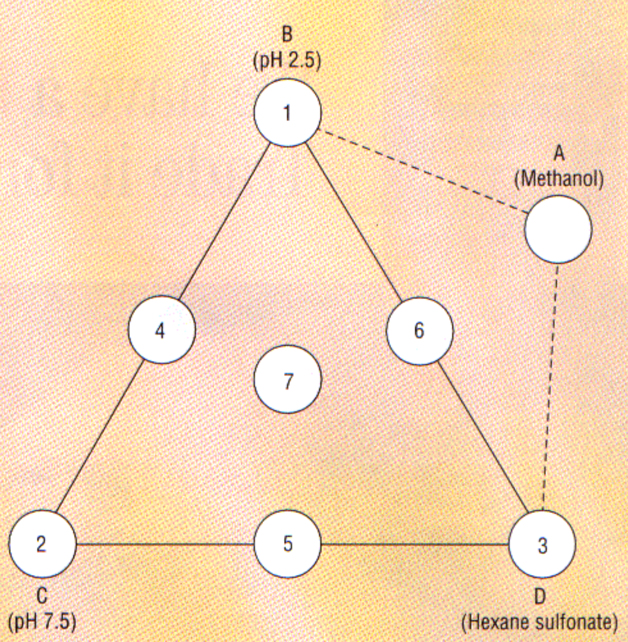

FIGURE 1: Ion-pair method development strategy. See text and Table I for details. (Reprinted with permission from reference 2.)

TABLE I: Mobile-Phase Compositions for Figure 1

Mixture	Type	Composition*
A	Methanol	Methanol
B	Buffer, pH 2.5	50 mL 50 mM phosphoric acid and 50 mM acetic acid; adjust to pH 2.5 with 2 M sodium hydroxide (≈ 1.5 mL)
C	Buffer, pH 7.0	50 mL 50 mM phosphoric acid and 50 mM acetic acid; adjust to pH 7.5 with 2 M sodium hydroxide (≈ 5.7 mL)
D	Ion-pairing agent–buffer†	0.20 M hexane sulfonate in buffer (50 mL) 5 mM phosphoric acid and 5 mM acetic acid; adjust to pH 5.0 with 0.2 M sodium hydroxide (≈ 3.5 mL)

* It may be necessary to add 20 mM triethylamine to all solvents.

† For separating acids, use 40 mM tetrabutylammonium phosphate (instead of hexane sulfonate) as an ion-pairing reagent. With a detector wavelength less than 220 nm, acetic acid must not be used, but phosphate buffers are tolerated.

your column — 35–40 °C is a good place to start.

A STEPWISE APPROACH

With all these variables, it is important to implement a systematic strategy for optimizing an ion-pairing separation. I like the scheme outlined by Snyder, Glajch, and Kirkland (1) that presents a step-by-step process for optimizing a separation. You can adjust these guidelines to help you determine if your separation can be improved.

Figure 1 shows a schematic diagram of the optimization scheme. As always, we should use the KISS principle (keep it simple, stupid) when developing

LC methods. The fewer mobile-phase additives and other system variables we use, the more reliable the method will be.

Let's see how this works: Select a C8 or C18 column — a 15 cm × 4.6 mm, 5 µm d_p column operated at a flow rate of 1.5 mL/min is a good choice. If you use a 25-cm column, you will have to use a flow rate of 1 mL/min to maintain a reasonable back pressure (for example, less than 2000 psi). Because of the temperature sensitivity of the ion-pairing process, it is important to control the column temperature. Prepare the reagents according to Table I. Use a negatively charged ion-pairing reagent such as hexane sulfonate if the sample contains bases; use a positively charged reagent such as tetrabutylammonium phosphate for acids. Detection at wavelengths less than 220 nm can be compromised if you use acetate in the buffer.

The first step is determining if the separation can be obtained without ion pairing. If you don't need to use ion pairing, find a good set of conditions for all sample components.

As a starting point, a low pH produces better reversed-phase separations because the ionization of column silanols and many acids is suppressed. First, explore various mixtures of low-pH buffer and methanol (follow the A–B line in Figure 1) to find the best concentration of methanol; this point defines mobile phase 1 on the map. If the separation is adequate, stop method development (remember, KISS). If the separation is unacceptable, switch to the high-pH buffer (C); the methanol concentration should be roughly the same as for the low-pH system; this point defines mobile-phase 2. If this separation is adequate, stop. Otherwise, compare the best low- and high-pH separations. By studying the chromatograms, you should be able to see which pH is better and if an intermediate pH is likely to provide an intermediate separation that is better than either. If you anticipate a better separation, run intermediate mixtures of mobile phases 1 and 2 to find the best pH (mobile phase 4). Up to this point, you have adjusted only the organic-solvent concentration and pH to find the best combination of these variables. In most cases, this mobile-phase system will be adequate, and you will not need to use ion pairing.

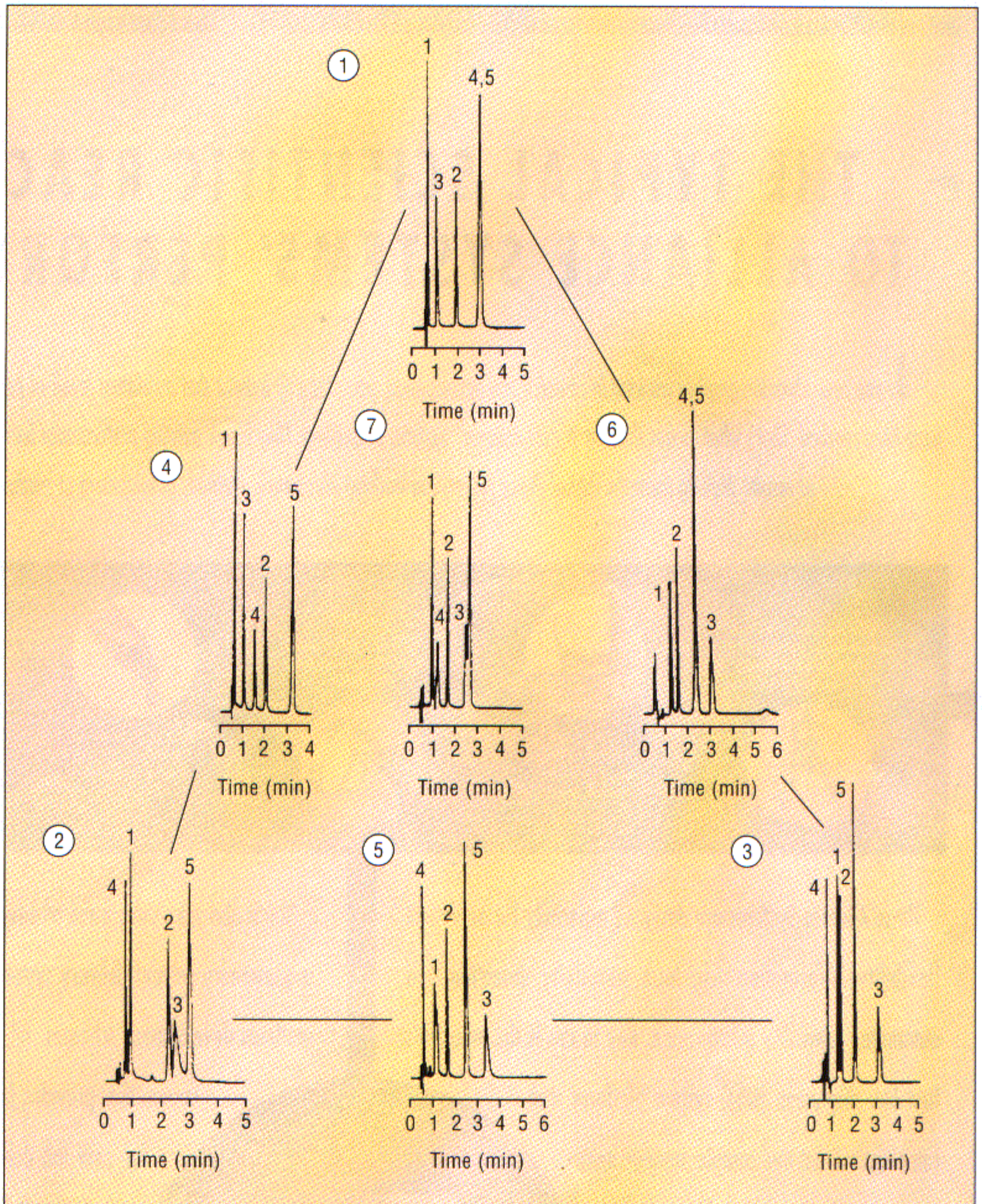

FIGURE 2: Example of results of ion-pair method development. Numbers correspond to experiments of Figure 1. Other conditions are described in the text. Peaks: 1 = phenylephrine, 2 = glycerol guaicolate, 3 = pseudoephedrine, 4 = sodium benzoate, 5 = methylparaben. (Reprinted with permission from reference 1.)

After you have determined the best pH and organic-solvent composition (mobile phase 4) and you are unable to achieve the desired separation, you must add the ion-pairing reagent as a variable. Try various blends of the ion-pairing reagent (D) with the best pH mobile phase (mobile phase 4) to find an adequate separation.

After you have found an acceptable combination of organic solvent, pH, and ion-pairing reagent, change each variable slightly to determine if you truly have found the optimum and if it is a rugged method. I suggest that you explore changes to the organic solvent (±2%), ion-pairing reagent (±5–10 mM), pH (±0.5 units), and temperature (±5 °C).

OR USE THIS APPROACH

I prefer the following stepwise approach because it gets me to an adequate separation in the fewest possible experiments. The original approach to ion-pairing optimization using the strategy of Figure 1 is slightly different, and you may prefer that route.

First, find the best concentration of methanol–pH 2.5 buffer as above (conditions for experiment 1). Then use this concentration of methanol with pH 7.5 buffer and ion-pairing reagent to determine the mobile phases for experiments 2 and 3. A 50:50 (v/v) blend of the mobile phases used in experiments 1 and 2 will define the mobile phase for experiment 4. Similarly, the mobile phases for experiments 5 and 6 are generated by blending the other corners of the triangle. The mobile phase for experiment 7 is an equal blend of the mobile phases used in experiments 1–3. If you have an LC system that will blend three solvents, you can set up the experiments defined by Figure 1 for unattended operation. After the runs are complete, you can lay out the results for study like those of Figure 2.

To find the best conditions of Figure 2 (or your separation), follow the movement of each peak as the conditions change. For example, you can see that peak 4 moves from the last position to the first one as the mobile phases change from experiment 1 to experiment 2. Although the separation of experiment 4 looks good, the first peak comes out too early. Shifting toward the conditions in experiment 7 improves overall retention but compromises the separation of peaks 3 and 5. The best separation is found between the mobile phases used in experiments 4 and 7, as shown in Figure 3. The key to interpreting data such as those presented in Figure 2 is to examine the most poorly resolved peak pairs and see how they change as the conditions change.

Two final precautions for using the strategy of Figure 1: First, make sure the column is fully equilibrated between runs (15–30 mL of equilibration for a 15-cm column) and make duplicate injections to ensure that the retention times are constant. Second, use one set of conditions as a standard to ensure that the column hasn't changed. For example, use the conditions of experiment 1 at the beginning and end of all the experiments (or at the beginning and end of each day for multiple day experiments). If the retention times are constant, the system is stable. You don't want to be led astray by drawing conclusions from a system that is unstable.

PRACTICAL APPLICATION

So how do we apply this information to the problem described at the beginning of the article? I would start by exploring the influence of each variable. First, thermostat the column if it is not already in an oven. Then make systematic changes in each variable to see if you can find improvements. I would check the ion-pairing reagent at 10 and 20 mM to compare to the current 2 mM concentration. Vary the acetonitrile ±5% to check this variable. The reader's system uses low-wavelength detection, so acetate buffer will be problematic, but the phosphate buffer can be checked at pH 3 and 6 to look for improvements. The alternative is to start over — using the strategy outlined in Figure 1 — to explore the importance of each variable in a systematic manner.

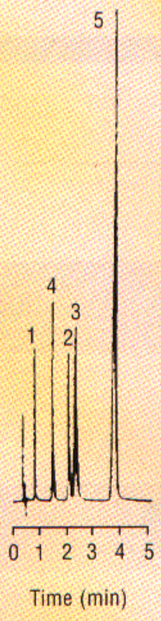

FIGURE 3: Optimum of Figure 2, located between experiments 4 and 7. Other conditions are described in the text. (Reprinted with permission from reference 1.)

REFERENCES

(1) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley & Sons, New York, 1988), pp. 106–114.

(2) "Advanced HPLC Method Development Course Manual," LC Resources Inc. (Walnut Creek, California, 1996).

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