LC Troubleshooting

Avoiding the Pitfalls of Published Methods

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Using a published method may seem like an easy way to get a quick separation, but beware of incorporating someone else's mistakes into your work.

Thousands of liquid chromatography (LC) methods appear in the scientific literature, and we'd like to think of them in the same light as the taste-tested recipes in our favorite cookbook. Unfortunately, published methods often are like grandma's favorite cookie recipe — a hastily scribbled note on the back of an envelope. All of the general ingredients are included, but some key ingredient or process may not be listed. This month we look at some of the problems that can arise when using published methods. I hope that this discussion will make us all a bit more cautious about adapting methods for our own use. We'll also see why some experts suggest that it is more expedient to start from scratch rather than deal with the missing information in someone else's method.

One common way to develop a separation for a new sample is to use historical information from your personal experience or from the scientific literature. You can perform a literature search based on information about the structures of the sample components to determine whether someone else has developed a separation for your sample or its analogues. If you are fortunate, you will find a method that you can modify slightly to meet your needs.

Adopting and adapting someone else's method may sound like an easy way to get the separation that you need, but the path to success often contains surprises. These surprises can be organized into four categories: chemistry, instrumentation, missing information, and quality.

CHEMISTRY PROBLEMS

Chemistry problems arise when the published method uses a column or reagents that are unavailable to you. Although all C18 columns share many general characteristics, their differences are such that you seldom obtain an identical separation with different brands of columns. That is not to say that Brand A cannot successfully separate the same sample that Brand B can separate, just that they are seldom equivalent under identical conditions. For some sensitive separations, batch-to-batch variations for nominally identical columns can alter the separation, even when all other chemical and physical conditions are held constant. The first step in the successful use of a published method is to use the same column (brand and part number). Differences in reagent sources, surface coverage by the stationary phase, silica characteristics, and bonding techniques can result in significant differences in retention and selectivity between various manufacturers' products. If you don't have a duplicate of the referenced column and can't order one, plan on spending time reoptimizing the method for the column you choose.

The second area of concern with chemistry relates to the mobile phase. Under the best circumstances, the mobile-phase conditions often require some adjustment to achieve a satisfactory separation. This can be true even if you use the brand of column specified in the published separation, especially when the article is several years old. Over time, small changes in column-preparation techniques can mean that today's column does not have exactly the same surface chemistry as one prepared several years ago. To compensate for these changes in the column characteristics, you will have to adjust the mobile phase. In most cases, the reagents used to form the mobile phase are interchangeable with equivalent reagents from other suppliers. Be sure to use HPLC-grade reagents when they are available because other grades of reagents can contain interfering contaminants. Proper preparation of the mobile phase is important, and, as is discussed below, missing information can make it difficult to prepare the same mobile phase as described in a literature reference.

How do you overcome chemical differences? Depending on many factors, the separation you obtain may be close to the published method, or it may be totally unacceptable. You will have to adjust the separation conditions to improve a marginal separation — the same process you would undertake if you were fine-tuning a separation you developed yourself.

INSTRUMENT PROBLEMS

The second pitfall of using a published method is the LC instrument itself. Instrument-to-instrument variations can have a significant influence on the separation, even if the same brand and model of instrument is used. For example, if the mobile phase is formulated using on-line mixing and the instruments are maladjusted, the actual mobile-phase composition can differ from the programmed level.

Figure 1 shows an example of the type of problem you can encounter. It shows a standard step test used to check for proper proportioning of solvents. In this test, the A and B solvent reservoirs are filled with 50% (v/v) methanol–water, and B is spiked with 2–3 mL of acetone. With a column installed, the system is run at 100% B, and the detector attenuation and wavelength are adjusted to obtain 90% full-scale deflection. The system is then programmed to pump isocratic steps from 0 to 100% B in 10% increments with a step every 5 min. The step-gradient trace for a properly functioning system (Figure 1a) shows even steps, whereas the maladjusted system (Figure 1b) shows a great error in the step sizes. It is easy to see that a separation run at the same setting for percentage of solvent B on these two systems may produce drastically different chromatograms.

If the separation is performed in the gradient mode, it may be impossible to get the same separation on the two systems. Worn pump seals, leaky check valves, and poor mobile-phase degassing are some other problems that can compromise the mobile-phase proportioning and flow-rate accuracy in any LC pump. It is very important that your LC system is operating within specifications, but you have no control over the system used for the published method, and you seldom know whether the system was operating properly.

Subtler instrument variations come into play if you are using gradient methods. The dwell volume (the volume between the point at which the mobile-phase solvents are mixed and the head of the column) can vary by a milliliter or more even with nominally identical systems

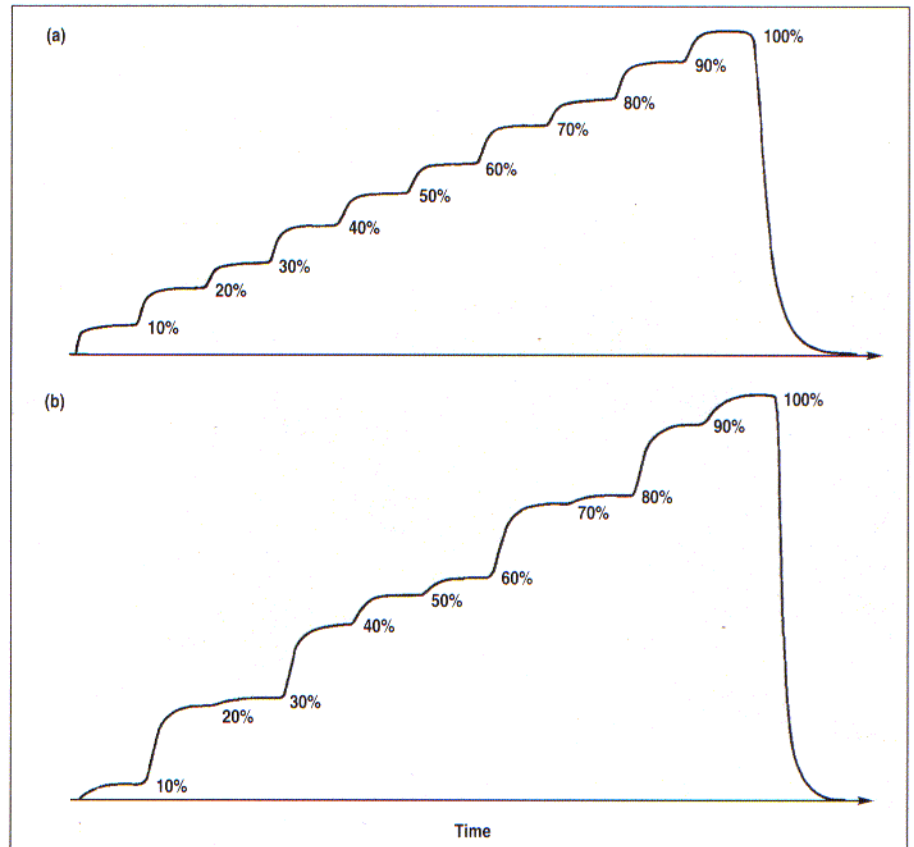

FIGURE 1: Step-gradient plots for (a) an LC system that is functioning properly and (b) a system with proportioning errors. See text for details.

in the same lab. Dwell-volume variation can be caused by differences in plumbing and injector-loop volumes. Brand-to-brand differences in dwell volume can be ≥10 mL.

A larger dwell volume is equivalent to adding an isocratic hold at the beginning of a gradient separation, which can cause differences like those shown in Figure 2. Figure 2a shows a gradient chromatogram of a polynuclear aromatic hydrocarbon sample run on an LC system with a 5-mL dwell volume. Although peaks 3 and 4 are not baseline resolved, they can be quantitated under these conditions. When the method is switched to a system with a 1-mL dwell volume (Figure 2b), peaks 3 and 4 have unacceptable resolution. Dwell-volume differences of this magnitude are common between different brands of equipment. These chromatograms are simulated, so the only variation between them is the dwell volume. Imagine the effects if there were also differences in solvent proportioning and column chemistry. Clearly, differences in dwell volume and instrument performance can affect the separation in unexpected ways.

MISSING INFORMATION

A third source of problems when transferring a method is incomplete reporting of experimental detail. Look carefully at the experimental section of the literature method: How much information is missing? One common omission is a statement of the column temperature. Was the column operated at room temperature, or was it thermostated? A difference of 1 °C can result in a 1–2% difference in retention and unpredictable changes in selectivity (relative peak separation). The system dwell volume is seldom mentioned, but as we saw earlier, dwell volume is very important when gradient methods are used. How was the mobile phase prepared? Was 600 mL of methanol added to 400 mL of water to make the mobile phase, or was methanol added to 400 mL of water until 1 L of mobile phase was obtained? Changes in volume when liquids are mixed can make significant differences in the mobile-phase composition. Was the pH of the mobile phase checked in the aqueous phase or after the organic solvent was added? Measurements of pH when the organic solvent is present generally yield erroneous readings. It is easy to see that commonly accepted techniques in one laboratory may not be used in another laboratory, and thus the assumptions you make to fill the informational gaps may add errors to the method transfer process.

When method information is missing, you can often get by because the missing information is insignificant or you can make a good guess about the methods used. When you see errors in the method, however, your confidence in the entire method erodes. One common error is using a buffer that is out of the acceptable buffering range. For example, the method may report using a phosphate buffer at pH 4.0. The effective range of a buffer is ±1 pH unit from the p*K*. Phosphate has p*K*s of 2.1, 7.2, and

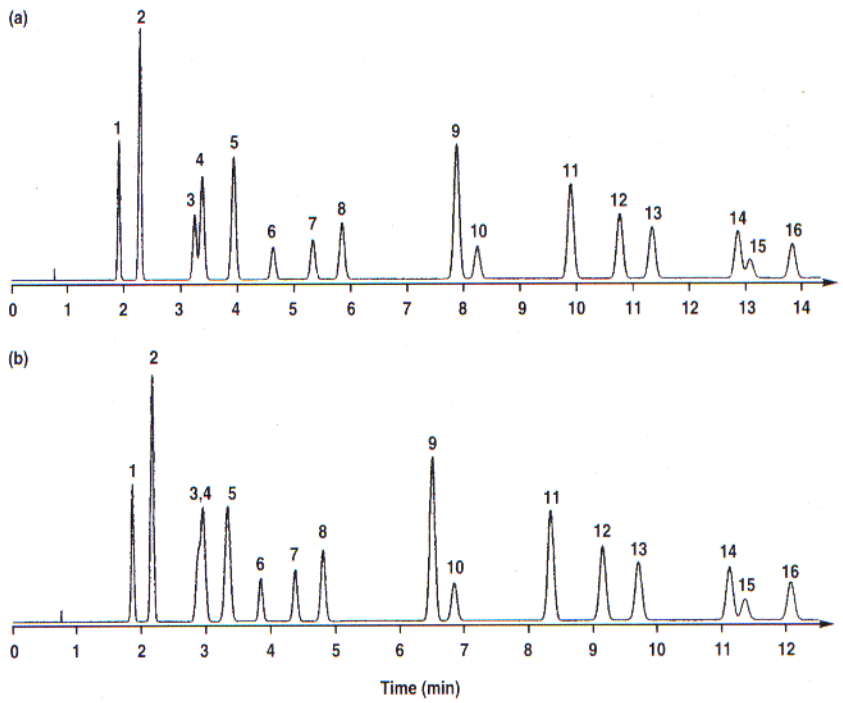

FIGURE 2: Simulated chromatograms for the separation of a polynuclear aromatic hydrocarbon sample using systems with (a) 5-mL and (b) 1-mL dwell volumes. Column: C18; mobile-phase gradient: 55–90% (v/v) acetonitrile–water over 14 min; flow rate: 2 mL/min.

12.3, so it should not be used at pH 3.1–6.2 — citrate or acetate would be a better choice. So if the improper buffer was used, what else could be wrong with the method? You have no way of knowing.

QUESTION OF QUALITY

A final area of uncertainty with methods reported by others is the quality of the work. Methods that have been put through rigorous validation procedures inspire much more confidence than one-off methods. Statistical reporting of sample-to-sample, day-to-day, and instrument-to-instrument reproducibility can give you a feel for the quality of a method. Did the developer take the time to thermostat the column, change the guard column regularly, or fully equilibrate the column? Was the method tested on more than one column? How many real samples could be run before the method needed adjustment or before column replacement was required? When answers to questions like these are contained in the method report, you will be more confident that the method can be successfully transferred to your laboratory.

NOW WHAT?

So I've destroyed your confidence in published methods, and you don't know what to do next. Perhaps I've been a bit hard on literature methods — many good methods have been published, but a lot of work is of negligible value. The problem is telling the two apart.

You certainly can use the scientific literature to find out if your sample compounds have been analyzed before and learn the conditions that were successfully used by others. In many cases, you will have to adjust the method until it works on your instrument with your sample. Many of the method revalidation steps you have to go through are the same ones you would use if you started from scratch — a strong argument for starting over from the beginning and developing a method that you know is valid and rugged. You can use a published method-development strategy, such as the one outlined in reference 1. Alternatively, you can begin with one of the commercially available software packages designed to speed method development.

When you take care to avoid or prevent unwanted surprises, you will have a method in which you have confidence and one that is rugged and reproducible. When you publish the method in the scientific literature or an internal report, remember to take extra care to list all the relevant variables so the next person can have confidence in your method.

REFERENCE

(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 Walnut Creek, California, USA, and a member of the Editorial Advisory Board of LC•GC. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC, *P.O. Box 10460, Eugene, OR 97440-2460, USA.*