LC Troubleshooting

Obtaining Separations, Part I: A Look at Retention

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The first goal in separation development is obtaining reasonable retention times.

Whether we develop liquid chromatographic (LC) methods manually or use sophisticated method development software, we go through the same stages to obtain a satisfactory separation. First, we try to get acceptable sample retention times on a selected column and mobile-phase combination. After we obtain the separation, we may need to adjust the selectivity, or relative peak spacing, by using another mobile-phase solvent or adding other reagents to the mobile phase. After we find the separation conditions that provide satisfactory selectivity, adjustments in flow rate, particle size, and column dimensions may improve the method further.

These three steps — obtaining retention, adjusting selectivity, and fine-tuning the separation — followed stepwise, will provide an efficient, satisfactory separation. This month's "LC Troubleshooting" will cover the first step in the process. Future installments will consider the remaining steps.

MEASURING RETENTION

Most of us measure the retention of sample compounds in minutes, but when developing a separation, we should keep track of the *retention factor.* The retention factor, k, provides valuable information about the quality of the separation. The retention factor is unitless and unaffected by changes in column dimensions, flow rate, and packing particle size, so it simplifies comparison of separations during fine-tuning. The retention factor is defined as

$$k = (t_R - t_M)/t_M \qquad [1]$$

where t_R is the retention time of the peak of interest, and t_M is the mobile-phase hold-up time (also called the solvent front or column dead time).

Figure 1 shows the relationship between k and resolution or peak separation. At very low k values, resolution is poor, but at much higher k values, increased retention does not improve resolution. We know this from experience — peaks that are eluted very early are poorly separated and tend to get mixed with extraneous peaks at the solvent front. Likewise, we have observed that separations generally improve as retention increases until peak broadening cancels out any gain. Ideally, we will find the best separations if k is kept in the 2–10 region, but for practical purposes, peaks eluted with k values between 1 and 20 will provide satisfactory results.

PRELIMINARY CHOICES

To illustrate how to determine the best solvent strength, we'll look at a sample of six neutral aromatic compounds. This information allows us to choose starting conditions. Because the compounds are neutral, we won't have to worry about controlling the mobile-phase pH. However, if we knew that the sample contained charged compounds, we would add a buffer to the mobile phase and adjust the pH to prevent the components from becoming ionized. The aromaticity allows us to use UV detection. With unknown analytes such as these, the best column choice usually will be a C8 or C18 column in a 15 or 25 cm × 4.6 mm configuration. I prefer to use methanol– or acetonitrile–water mobile phases because they are simple to use, UV transparent, and likely to obtain satisfactory results. On average, acetonitrile provides a slightly better selectivity than methanol, but the choice is largely up to the user. We'll start with acetonitrile, a 25-cm C18 column packed with 5-µm d_p particles, and a flow rate of 1 mL/min.

OBTAINING A SEPARATION

Where to start? Any mobile-phase composition will do, but to get the separation as quickly as possible, start with a strong mobile phase and make it weaker as needed. Pure strong solvent (100% B solvent) should elute all the compounds quickly, often too quickly, so 90% B is a good place to start. Figure 2a shows the chromatogram for 90% acetonitrile. This solvent is obviously too strong for a mobile phase because all the peaks are eluted as one at the solvent front.

How much do we change the mobile phase for the next try? A guideline called the *Rule of Three* states that the retention factor changes approximately three times for each 10% change in organic. Thus a change of 20% B would change k by approximately nine times (3 × 3). Figure 2b shows a run at 70% B. The peaks move a little, but everything still comes out close to the holdup volume, and just one peak is present. Another change of 20% to 50% acetonitrile finally produces a chromatogram with measurable retention and multiple peaks (Figure 2c).

Now that we have more than one peak, we can begin to improve the separation. Only four peaks are visible, and $k < 1$ for all peaks. Another 10% reduction in organic solvent yields

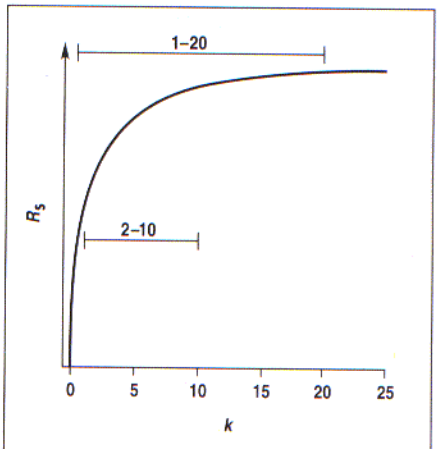

FIGURE 1: Plot of retention factor (R_s) versus resolution.

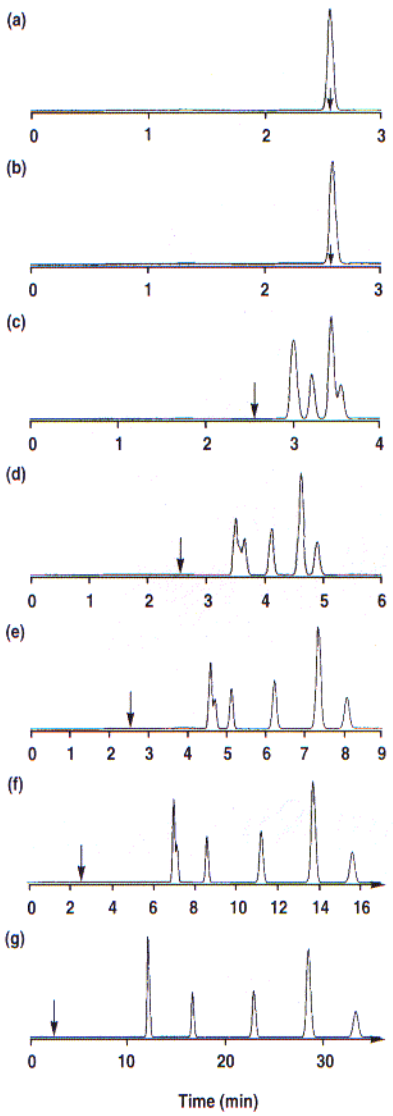

FIGURE 2: Chromatograms for a sample of neutral aromatic compounds run with various mixtures of acetonitrile–water, including (a) 90%, (b) 70%, (c) 50%, (d) 45%, (e) 40%, (f) 35%, and (g) 30% acetonitrile. The vertical arrow marks the mobile-phase hold-up time.

TABLE I: Summary of Results for Various Mixtures of Acetonitrile–Water

% Acetonitrile	*k* Range	t_R Range (min)	Minimum Resolution
90	0–0	2.5–2.5	0
70	0–0	2.6–2.6	0
50	0.2–0.4	3.0–3.5	0
45	0.4–0.9	3.5–4.9	0.6
40	0.8–2.2	4.6–8.0	0.8
35	1.7–5.1	6.9–15	0.8
30	3.7–12	12–33	0.7
25	8–28	23–75	0.2
20	17–67	46–174	0.4

the chromatogram of Figure 2e. We can see all six peaks, but the resolution for the first two is unsatisfactory. The range of retention factors is about $1 < k < 3$.

At this stage, we should make smaller changes in the mobile phase — 5% B changes would be reasonable. Runs at 45%, 35%, and 30% acetonitrile are shown in Figures 2d, 2f, and 2g, respectively. We don't seem to be improving the separation much, and the retention times are getting longer with weaker mobile phases.

COMPARING RESULTS

It is useful to compare the results from the runs we have made so far. Table I summarizes the results of the runs shown in Figure 2 plus a few more runs. Several points should be noted: First, the best resolution for the least-resolved peak pair is ~0.8 for the acetonitrile–water mobile-phase system. Resolution of ~1.5 is a baseline separation between two peaks. For most assays the resolution should be 1.7–2.0, so the separation will tolerate some degradation before it is no longer useful. A resolution of ≤0.8 fails to produce the results we want.

I stated earlier that the most satisfactory separation usually would be obtained in the $1 < k < 20$ range. This statement is confirmed by the data of Table I, in which the best separation corresponds to peaks eluted in the $0.8 < k < 12$ region. This *k* range also provides reasonable retention times. Under the current conditions, the column dead time is ~2.5 min, so peaks eluted before $k \approx 1$ (5 min) likely will be too close to the "garbage" in real samples eluted early in a run. At the other end, $k \approx 12$ would give a run time of ~35 min, which is unreasonably long for a six-component mixture.

We can also use the data of Table I to confirm the Rule of Three. For the most part, we can see that a change of 10% in mobile-phase organic solvent changes the *k* values by approximately four times with this sample. The Rule of Three is based on empirical observations for a large number of compounds, so it isn't exact for any given sample set. As an approximation it is still valid for this sample.

What about starting with a strong mobile phase and making it weaker? If we add up the run times for the chromatograms of Figure 2, then tack on ~30 min/run for equilibration with a new mobile phase, we see that these experiments took about 5 h — roughly a day's work when all aspects are considered. For the present sample, we can see that starting at 50% acetonitrile would have been more efficient, but we had no way of knowing that before we started. With an unknown sample, 50% B could have required a 200-min run time, and then we would have lost considerable time. For manual separation development, working stepwise down from 90% or 100% strong solvent is very efficient. Most of the time, you'll be able to get a good start on the separation in three or four runs.

WHAT NEXT?

The experiments we've run so far (Figure 2) seem to indicate that we can't get a baseline separation for the first two peaks. Is it possible that we've overlooked an intermediate value that would yield better separation? We can visually interpolate between chromatograms to see what the separation would look like for other mobile-phase strengths. Remember, peaks do not jump around in LC — they move in a regular manner as the mobile phase is changed (see Figures 2d–2g). You can see the regular pattern of peak movement by studying pairs of peaks. Even though they move apart on an absolute basis, the relative spacing between peaks 3–5 remains unchanged. However, the spacing for the first three peaks changes dramatically. The third peak moves away from the first two as the percentage of B is decreased. On the other hand, the first two peaks move closer together with a decrease in B. If we continued using weaker mobile phases, peaks 1 and 2 would merge, then peak 2 would be eluted ahead of peak 1 (but the run time would be very long — see 20% acetonitrile in Table I).

By studying the regular movement of peaks for incremental changes in the percentage of mobile-phase B, we can get a good idea of how the separation will appear for interpolated (and to a certain extent, extrapolated) runs. With this sample, we clearly will not get a satisfactory separation of the first two peaks in any reasonable time with the acetonitrile–water mobile phase we have chosen. The best overall separation seems to be about 35% acetonitrile (Figure 2f), which provides the best resolution, a good *k* range, and reasonable retention times.

The next step is adjusting the selectivity or relative peak spacing of your sample by changing the chemistry of the system. You can do this easily by changing to a different organic solvent such as methanol or tetrahydrofuran. We will examine these changes next month.

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