LC Troubleshooting

Injection Problems

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Just getting the sample into the column in a compatible solvent can be a challenge.

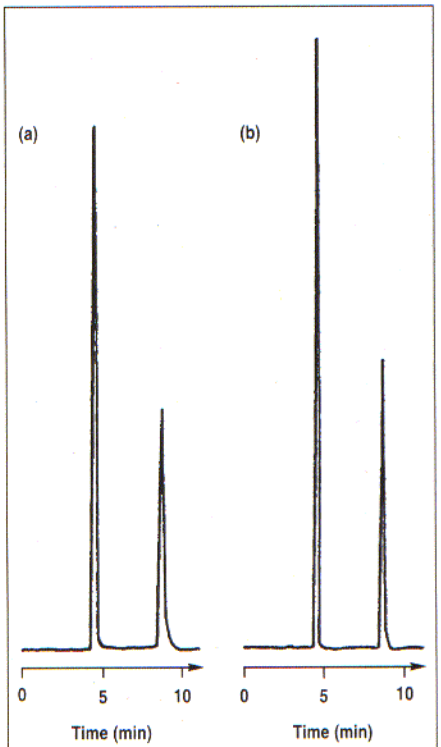

FIGURE 1: Chromatograms from a reversed-phase method using a mobile phase of 18% acetonitrile–water and 30-µL injections of sample dissolved in (a) acetonitrile and (b) mobile phase. (Reprinted from reference 1 with permission.)

The composition and volume of an injection solvent can affect peak shape and retention in liquid chromatography (LC) separations. This month, we will look at some of the injection problems that can occur and consider some guidelines that can help minimize these problems.

Under ideal conditions, the injection solvent would make no difference to the results of a chromatographic experiment because the solvent would be diluted instantly to the same composition as the mobile phase. In practical terms, however, it takes a finite amount of time for the injection solvent to become diluted with mobile phase. Until this happens, the sample molecules in the injection solvent behave as if the injection solvent were the mobile phase.

If the volume and strength of the injection solvent are high enough, the peak shape can be affected, as in Figure 1a. Here we see reduced peak heights and increased tailing when compared with Figure 1b. In Figure 1a, the mobile phase is 18% acetonitrile in water, and 30 µL of sample in acetonitrile was injected onto a reversed-phase column.

We can understand what is happening here by visualizing the chromatography on a molecular scale. When sample molecules are in the mobile phase, they move at a fixed velocity through the column. When they are in a stronger solvent such as the pure acetonitrile used here, they move more quickly. So as long as the samples are in the presence of pure acetonitrile, they will travel as if the acetonitrile was the mobile phase. Gradually the injection solvent will become diluted with mobile phase. If we think of the injection solvent entering the column as a sphere, the outside edges of this solvent ball will get diluted first. As this happens, the sample molecules in this region will be in mobile phase while other sample molecules will remain inside the undiluted injection solvent. Thus, some of the sample molecules will travel at the velocity they would in the mobile phase, and others will travel at the velocity determined by the injection solvent. This difference in migration rates creates a split or distorted injection profile. After this distortion occurs under isocratic conditions, the normal peak shape cannot be restored, so the final peak shape also will be distorted, as we see in Figure 1a.

TABLE I: Guidelines for Injection Solvent Selection

Injection Solvent Strength	Maximum Injection Volume
100% strong solvent	≤10 µL
Stronger than mobile phase	≤25 µL
Mobile phase	≤15% of peak volume
Weaker than mobile phase	Large (see text)

It is the combination of injection solvent volume and strength that is important. As we will see, at one extreme we can inject small volumes of pure strong solvent without deleterious effects, whereas we can inject very large volumes if the solvent is weak enough. Table I summarizes the guidelines for injection solvent volume and strength. We will look at each of these recommendations below. Remember that these are only guidelines — the demands of your separations may allow you to get by with somewhat different conditions.

PURE B SOLVENT

The most demanding situation occurs when the injection solvent is as much stronger than the mobile phase as possible. In this case, you should keep the injection volume as small as possible; I suggest injecting no more than 10 µL when pure strong solvent is the injection solvent. When the mobile phase is greater than 80% B solvent, you may be able to inject larger volumes, because the difference between the injection solvent and mobile phase is smaller. Figure 1a shows what can happen if you use too much strong solvent for injection.

AN INJECTION SOLVENT STRONGER THAN THE MOBILE PHASE

When the injection solvent is no more than approximately 25% stronger than the mobile phase, injection volumes of as much as 25 µL should create no problems. For example, when using 70% methanol as the injection solvent in a 50% methanol mobile phase, you could inject 25 µL of the injection solvent without compromising the peak shape. Obviously, this rule and the preceding one are related. Whenever injecting solvents stronger than the mobile phase, you should examine the chromatogram carefully for possible peak distortion.

MOBILE PHASE AS THE INJECTION SOLVENT

The best case occurs when the injection solvent and the mobile phase are matched. You don't have to worry about dilution, so solvent inhomogeneity is no longer a concern. The injection volume is limited, however. Consider an extreme scenario in which 500 µL of sample would be injected onto a conventional 15 cm × 4.6 mm column. The column volume would be approximately 1.5 mL, so during the injection, the first sample molecules injected would travel almost a third of the way through the column before the last molecules were injected. This large sample injection would cause tremendously broad peaks, if any separation was observable.

The contribution of the injection volume to the peak width can be determined by equation 1:

$$W_{total}^2 = W_{inj}^2 + W_{col}^2 \qquad [1]$$

where W_{total}, W_{inj}, and W_{col} are the widths of the final peak, the contribution by the injection and the contribution by the column, respectively. W_{inj} simply is the volume of the injected sample. Peak widths are measured at the baseline, which allows us to calculate that an injection volume of 15% of the peak volume would broaden the final peak by approximately 1%. This calculation is the basis of Table I's recommendation for using mobile phase as the injection solvent.

We can see in Figure 1b that the peak distortion problem of Figure 1a is corrected when 30 µL of sample is injected in mobile phase. I measure the peak width as approximately 0.5 min; assuming a flow rate of 1 mL/min, this volume would be equivalent to approximately 500 µL. Table I suggests that we could inject as much as 75 µL of sample in mobile phase without problems. This amount would yield an overall peak variance of $500^2 + 75^2 = 505^2$ μL^2 or an increase of only 1% in peak width. A 30-µL injection volume should not cause noticeable band broadening for this peak. Be sure to use the width of the first (narrowest) peak of interest for this calculation — the broadening of this peak represents the worst-case scenario for your sample.

WEAKER INJECTION SOLVENT

When you use a solvent that is weaker than the mobile phase, you can inject much larger volumes than when you select stronger solvents. We can get a feeling of the effect of the mobile-phase strength by considering the *Rule of Three,* which states that a 10% change in the strong solvent (B solvent) will result in approximately a threefold change in retention factor (k) or retention time (t_R). For example, if the mobile phase were 55% acetonitrile–water and a peak had a retention time of 5 min, a 35% acetonitrile–water mobile phase would increase t_R to approximately 45 min (3 × 3 × 5 min). If we used 35% acetonitrile–water instead as the injection solvent in the 55% acetonitrile–water mobile phase, the sample molecules would migrate much more slowly through the column until the injection solvent gets fully diluted with mobile phase.

Analytes that travel faster than the mobile phase (as when a strong injection solvent is used) tend to produce distorted peaks, but bands that move more slowly than the mobile phase tend to compress. You can visualize this effect by thinking of the molecules spread out on top of the column in the injection solvent where they are being diluted with mobile phase that is traveling through the column after the solvent. As mobile phase reaches the sample molecules, they will begin to move down the column, catching up with those farther down the column that are in a weaker solvent. (This band compression is one factor that contributes to the narrowness of peaks in gradient runs as compared with isocratic ones.)

As the difference between the solvent strength of the injection solvent and the mobile phase increases, you can use larger injection volumes. In the extreme, samples in pure water can be pumped onto the column, and they become concentrated at the head of the column before elution with a stronger mobile phase. This technique is used in environmental analysis for large volumes of dilute samples (for example, pumping 1 L or more of river water onto a C18 column for the measurement of trace quantities of pesticides). Biochemists use on-column concentration with dilute protein and peptide solutions before chromatographic analysis. This method is not always advantageous, however. Problems can arise in gradient elution when impurities in the A solvent get concentrated during equilibration and then are released as interfering peaks during the gradient.

EXCEPTIONS

The guidelines for selecting injection solvents and volumes summarized in Table I may need to be modified for specific cases or even combined to get the result you want. For example, a client once came to my company with a method used in a cleaning validation that had started to produce split peaks.

The procedure called for washing a production reactor with methanol and then injecting a 50-µL aliquot of the wash solvent onto a C18 column with a 50:50 (v/v) methanol–buffer mobile phase. The method had been used for several years with no problems, but suddenly the client observed split peaks. The peak-shape problem was not cured with normal corrective measures such as making a new batch of mobile phase or replacing the column.

One look at the method should show us that the analyst was injecting too large a volume (50 µL) of a solvent that was too strong (100% methanol). A smaller injection volume or a diluted solvent would solve the problem, but the analyst relied on the injection volume to place a large enough mass of sample on the column to accommodate minimum levels of quantitation.

The solution to this problem was simple. I advised her to dilute the sample fourfold with buffer to 25% methanol–buffer and to increase the injection volume fourfold to 200 µL. Then the same mass was injected in a larger volume of a significantly weaker solvent. The result was a separation with well-shaped peaks and satisfactory detection limits. In many cases, you can correct injection problems by injecting a larger volume of a diluted sample.

ION PAIRING — A SPECIAL CASE

When performing an ion-pairing method, you should use mobile phase as the injection solvent to avoid the appearance of ghost peaks and baseline disturbances. A delicate equilibrium between the ion-pairing reagent in the mobile phase and the stationary phase exists in ion pairing. When you make an injection in a solvent that lacks the ion-pairing reagent, the equilibrium shifts momentarily toward the mobile phase, depleting the column of reagent; eventually fresh mobile phase reaches the column, and the equilibrium must be reestablished. This process can cause positive–negative peak pairs.

The retention of these peaks depends on the chromatographic conditions, but the disturbance can occur early, late, or in the middle of the chromatogram. To avoid equilibrium-shift problems, inject the sample in mobile phase. Because the sample initially may not dissolve in mobile phase, you may need to be creative in formulating the injection matrix. One trick is to make a double-strength mobile phase to use for dilution. For example, if you are using 43% methanol–water with an overall concentration of 50 mM ion-pairing reagent, instead make a solution of 86% methanol–water with 100 mM ion-pairing reagent. Then use this double-strength mobile phase to dilute an aqueous sample 1:1, which leaves the sample in an injection solvent that is equivalent to the mobile phase.

Although the problem of disturbed equilibria is common with ion-pairing methods, it is by no means peculiar to ion-pairing methods. Whenever the mobile phase contains trace additives, a mismatch of the injection solvent has the potential for problems. For example, normal-phase separations on bare silica tend to be very sensitive to trace levels of water in the mobile phase. Injecting sample in dry solvent or in solvent with excess water can upset the water balance on the column and cause retention shifts or peak-shape problems.

CONCLUSION

You will minimize the risk of encountering peak-distortion problems if you inject small volumes of samples that are diluted in injection solvent that is the same as the mobile phase. When these limitations are not practical, the guidelines of Table I can help you choose alternative conditions. These guidelines are general in nature, and as such need to be tested to verify that they will give satisfactory results for your samples. In chromatograms with injection-related problems, the peaks eluted early usually are compromised most readily, so compare the shapes of peaks eluted early and late.

When you have the option, it is best to err on the conservative side when choosing injection solvents and volumes. Otherwise, you may develop a marginal method such as in the cleaning validation example in which the chromatography appears to be normal until some unknown stress creates unexpected problems.

REFERENCE

(1) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (Humana Press, Clifton, New Jersey, 1989), p. 397.

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