TROUBLESHOOTING

Separation Artifacts I: Sample Overload and Injection-Solvent Problems

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In a series of articles beginning this month, focus will be given to the chromatogram itself. This month the topic of separation artifacts will be introduced, and sample overload and problems created when the wrong injection solvent is used will be addressed (1).

SEPARATION ARTIFACTS

Many LC problems are physical or mechanical in nature and are fairly easy to correct. Often, the problems can be isolated and then instructions in the service manual can be followed to fix them. Problems with the chromatogram, however, often originate in the chemistry of the system. Because chemistry is still largely an experimental science, these kinds of problems are usually harder to solve. Most chromatographers, however, have a good background in chemistry or biochemistry, and all that is required in most cases is to put this training to use.

During either method development or routine operation, a variety of separation artifacts can arise. These almost always degrade the quality of the final separation and its use for analysis or sample purification. For example, bands in the chromatogram may be asymmetric or exhibit other peculiarities in peak shape. In other cases, the bands in the chromatogram seem abnormally wide and adjacent peaks may be poorly separated. Sometimes extra peaks are present in the chromatogram, peaks that cannot be traced to any compound present in the sample. Occasionally, negative peaks may be observed. Retention times for a given compound also may change from sample to sample without there being anything wrong with the equipment or the way the procedure is carried out. Finally, the sample may react chemically during the separation, resulting in peak distortions and low recovery of eluted material.

Anyone who has injected a lot of samples into an LC system has experienced problems such as these. Most chromatographers will recall cases in which they never really determined the cause of the problem. They tried a lot of different things and eventually the difficulty went away — or else they got tired of waiting for the results. We need to get away from haphazard remedies and, insofar as it is possible, develop a systematic approach to these separation or chemical problems. Fortunately, a lot of work has been done and results published in recent years that can help us in these situations.

Strange-shaped bands represent one of the most vexing problems that can arise in the LC laboratory, and this situation can have serious analytical consequences. There are also about a dozen different causes of band tailing alone; so, discovering why bands tail — and then fixing the problem — can be a difficult undertaking. Fortunately, there is a systematic approach based on logical analysis and practical fixes that have now been documented in numerous laboratories.

First, why are tailing or misshapen bands bad? One reason is that such bands can be hard to quantify. Some data systems have difficulty measuring peak size accurately when the band tails or its shape is otherwise peculiar. As a result, the precision and/or reliability of assay methods involving misshapen peaks is often poor as compared to good chromatography. A second reason for avoiding band tailing is that resolution suffers. Good resolution is the foundation on which to build good HPLC procedures. Anything that compromises resolution will adversely affect the final result. This is especially important when a large band in the chromatogram tails, with resulting overlap of the tail onto smaller bands that elute later. A third negative feature of tailing bands is that columns cannot be coupled in series to give additive plate numbers. A fourth problem with tailing bands is that they often are a symptom of multiple contributions to the retention process as opposed to a single retention mechanism. This in turn often means that relative retention on one column will not be the same for another, identical column. If we can eliminate tailing bands in this situation, we will generally reduce column-to-column variability. Gaussian peak shapes are pleasing to the eye. What detracts from the aesthetics of liquid chromatography should be discouraged.

We try to make the bands as symmetrical as possible, but most elution bands will exhibit some deviation from perfect Gaussian shapes. Here it is helpful to use the band asymmetry factor *As*, defined as the ratio of peak half-widths at 10% of peak height (2). Contributions to band asymmetry include the column, the equipment, the injection procedure, and/or the mobile phase–sample combination.

Consider two extremes in band asymmetry. First, the case of a new column plus the manufacturer's test system represents an ideal situation. Good columns (plus good equipment) should result in bands with *As* values between 0.9 and 1.1. Some commercial columns may show *As* values as large as 1.2 in this situation, but the closer the *As* value is to 1.00, the better. This is particularly important in avoiding problems that arise when connecting columns is series (3).

A second situation is the assay procedure for actual samples. Here it is often necessary to relax the tolerances on band symmetry. For real samples, as opposed to test compounds, we often must accept *As* values of 1.3 or even greater. We are asking for trouble, however, when bands show asymmetry factors greater than 1.5. A serious effort at reducing band tailing is called for in this case before proceeding with routine analyses.

How do we solve the problem of tailing or distorted bands? The remainder of this column, as well as others in this series, cover different aspects of this problem.

SAMPLE OVERLOAD

When one or more bands within the chromatogram tail, and these bands are larger than normal, the column may be overloaded. In other words, if the total mass of solute is

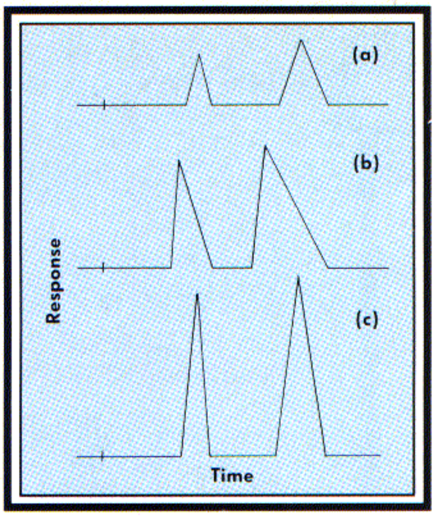

FIGURE 1: Sample overload and band tailing for hypothetical sample. (a) Normal sample, (b) tailing sample, and (c) sample of (b) diluted fourfold and rerun with detector attenuation decreased fourfold.

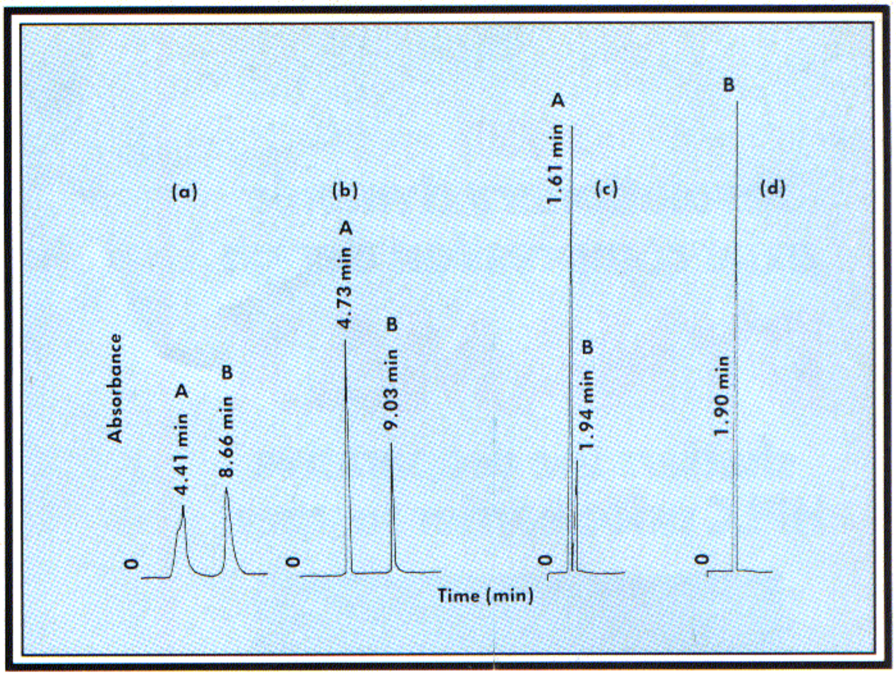

FIGURE 2: Examples of combinations of sample and solvent in which the solvent is too strong. (a) A 30-μL injection of caffeine (peak A) and salicylamide (peak B) in pure acetonitrile with reversed-phase separation using an 18% acetonitrile–water mobile phase. (b) Same as (a), but the sample was dissolved in mobile phase when injected. (c) A 30-μL injection of decamethrin (peak B) in methylene chloride with normal-phase separation on a silica column using an 8% methylene chloride–hexane mobile phase. (d) Same as (c), but the sample was dissolved in mobile phase when injected.

large enough, the linear capacity of the column is exceeded. The result is a decrease in retention time and a change in band shape. This means that at any given point in the column, the stationary phase has only a certain capacity for sample. When this capacity is exceeded, excess sample flows through the column (without interacting with the stationary phase) until it finds stationary phase that is not overloaded. The net result is that part of the sample travels through the column in the normal manner, but excess sample travels at a faster rate, and thus the center of mass of the peak comes out earlier than it should. Therefore, the peak maximum is pushed ahead, and the normally retained portion appears as a tail. This situation is illustrated by the hypothetical example of Figure 1b, which can be compared with a normal chromatogram shown in Figure 1a. In this case, the simplest check for sample overload is to dilute the sample fourfold and reinject it — after the detector attenuation is decreased fourfold. The result is illustrated in Figure 1c. Now the originally tailing peaks of run (b) are more symmetrical, and their retention times have increased to the values observed in the chromatogram of normal sample presented in Figure 1a. We have confirmed that the chromatogram of Figure 1b involves sample overload.

Normally, sample overload should not be a problem in a routine LC method because an important part of method development is to test for the effects of increasing sample size or concentration. When sample overload is seen (broader and/or tailing bands), maximum limits can be set on reported concentration values. For example, it might be found that for a 50-μL sample, concentrations of compound X that are greater than 1 mg/mL cause the band for X to broaden, tail, and/or change its retention time significantly. The method should therefore specify that any samples having apparent concentrations of X greater than 0.5 mg/mL must be diluted and rerun.

Just as the column can be overloaded, it is also possible to overload the detector. UV detectors should be linear to ~1 AU, but this is not always the case — particularly when measuring on the side of an absorption band and at lower wavelengths. Then, the nonlinear response of the detector can lead to band distortion (usually a "fat" peak) and to other problems. When detector overloading is suspected, a calibration curve should be plotted for a range of sample concentrations that overlaps that of the problem sample. Detector overloading will then be evident as nonlinearity of the peak height/concentration plot in the concentration region of the problem sample.

WRONG SOLVENT FOR SAMPLE

Good chromatographic results require that the volume and kind of solvent used to dissolve the sample (before injection) fall within certain limits. Ideally, a small volume of sample solution (dissolved in the mobile phase) is injected. In many cases, a larger volume of sample dissolved in a weaker solvent can be used. Hence, 100–500 μL of sample dissolved in water is often injected for reversed-phase separations. When injecting a relatively large volume of a solvent that is stronger than the mobile phase, however, usually you will observe severe degradation in the quality of the chromatogram. This is illustrated in the chromatograms of Figure 2 (4). In Figures 2a and 2b, 30 μL of the same sample (caffeine and salicylamide) was injected onto a reversed-phase column. In Figure 2a, the sample was dissolved in a strong solvent (pure acetonitrile). Figure 2b illustrates a case in which the sample was injected as a solution in the mobile phase. Peak distortion and increased band broadening are evident in Figure 2a, whereas the peaks in Figure 2b are quite normal. Figures 2c and 2d show a normal-phase separation of a single compound (deca-

TABLE I: CHOOSING A SOLVENT FOR DISSOLVING SAMPLE

Ideal:
The best option is to dissolve the sample in the mobile phase and inject 10–50 µL.

Practical:
Alternatively, a larger volume of weaker solvent can be injected — such as 100–500 µL (or more) of sample dissolved in water (reversed-phase chromatography). The main disadvantage is greater baseline upset at the beginning of the chromatogram, but this occurs only in some cases. It is acceptable practice to dissolve larger volumes (100–500 µL) of sample in the mobile phase, but resolution may suffer for early-eluting bands and/or smaller-volume columns.

When necessary:
When convenient or required, 10–25 µL of a stronger solvent can be injected, such as a 25-µL methanol solution for reversed-phase chromatography.

methrin): 30 µL of the sample is injected in a solution of strong solvent (methylene chloride) (Figure 2c). in Figure 2d the sample is dissolved in the mobile phase. Peak splitting is seen in Figure 2c for the injection of the strong-solvent solution.

Injection-solvent problems should be suspected whenever a large volume of a solvent other than the mobile phase is injected — in particular, when the injection solvent is obviously stronger than the mobile phase. You should question, for example, the injection of 100 µL of sample dissolved in methanol for a reversed-phase separation on a C8 column with 30:70 methanol–water as mobile phase. The general rule is that *up to 25 µL of strong solvent can be injected without problems for columns of the usual size* (15–25 cm × 0.46 cm); smaller-volume columns require proportionally smaller sample volumes. Whenever a stronger solvent is used to dissolve the sample, however, the effect of sample volume on the chromatogram should be tested. If it is suspected that a 25-µL volume of methanol is causing problems, try one of the following alternatives:

Reinject a smaller volume: Reinject 5–10 µL of sample and look for changes in the chromatogram (make sure you change detector attenuation to maintain similar peak heights). If, for example, the two chromatograms (of 5- and 25-µL injections) are similar, then injection-solvent effects are absent.

Dilute the sample with a weaker solvent: Dilute the sample (already dissolved in strong solvent) fourfold with a weaker solvent, then inject a fivefold greater sample volume. Again, examine the chromatogram for any differences that could be attributed to injection-solvent effects. For the preceding example (30:70 methanol–water mobile phase and 25-µL methanol solution of sample), this would require injecting 125 µL of a sample dissolved in 20% methanol–water (instead of 25 µL of sample in pure methanol).

Depending upon the outcome of the above experiments, an alternative means of injecting the sample can usually be found, and you can therefore avoid injection-solvent problems. Table I summarizes the preferred approach to selecting the kind and volume of solvent used for injected samples.

REFERENCES

(1) This article is based on portions of *Troubleshooting HPLC Systems*, by J.W. Dolan and L.R. Snyder (Humana Press, Clifton, New Jersey), in press.
(2) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd Ed. (Wiley-Interscience, New York, 1979).
(3) L.R. Snyder, J.W. Dolan, and Sj. van der Wal, *J. Chromatogr.* **203**, 3 (1981).
(4) T.-L. Ng and S. Ng, *J. Chromatogr.* **329**, 13 (1985).

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