# TROUBLESHOOTING

## Peak Shape

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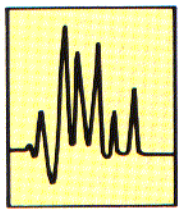

The shape of a chromatographic peak or band can provide a great deal of information about the proper operation of a liquid chromatography system. During methods development, abnormal peak shape can suggest that changes need to be made in the mobile or stationary phases to ensure a reliable assay. Sudden or gradual changes in peak shape over time can alert you to problems that need to be addressed. This article discusses some common peak-shape problems (Figure 1), suggests further steps that should be taken to confirm whether or not a problem exists, and gives possible solutions.

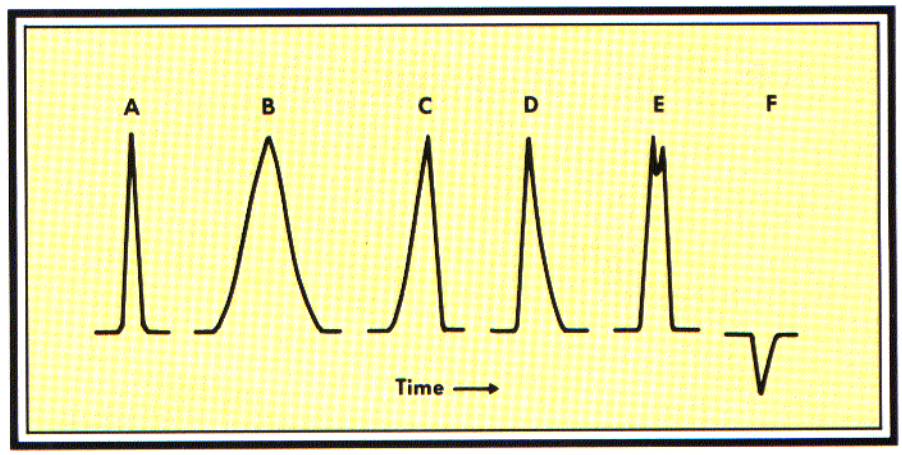


**FIGURE 1: Peak shapes commonly encountered during LC runs. Peaks: A = normal, B = broadened, C = fronting, D = tailing, E = doubled, F = negative.**

### BROAD PEAKS

Correcting broadened peaks in a chromatogram can be a time-consuming and frustrating problem if troubleshooting is not undertaken in a systematic manner. First, determine if broad peaks are the result of column deterioration or of late elution from a previous injection. If all peaks in the chromatogram are broadened, with early peaks showing more pronounced broadening, column deterioration is the problem. Measure the column efficiency or plate number ($N$) for the sample compound or for the evaluation standards recommended by the column manufacturer and compare the results to the record kept in your logbook. A review of the standard runs in your logbook will generally show a gradual reduction in efficiency over time if the column is deteriorating. Resolution requirements for each assay dictate when a reduction in plate number renders a column unusable; sometimes the assay is invalid if a 25% decrease in efficiency occurs, whereas other assays can tolerate a loss of 50% or more of the original column efficiency. Column replacement usually solves this problem. Be aware of other system problems that affect efficiency for a given separation, such as deteriorating guard columns and extra-column effects. If the mobile phase and system settings have not been changed, retention time should not vary significantly when efficiency drops.

A late-eluting peak from a previous sample may be suspected whenever a single broad peak appears in a chromatogram with otherwise narrow bands. This band may not appear in all runs and may not be noticed immediately, especially in samples with complex background peaks. The late-eluting peak is like a weed — there is nothing intrinsically wrong with it except that it appears where it is not wanted. It is simply a band with a high capacity factor ($k'$) from an earlier injection. Tracing the peak's origin is straightforward, though time-consuming because of its high capacity factor. One approach is to inject a sample and allow the chromatogram to run for several times the normal run time, while decreasing attenuation to amplify peak size. If you can spot the late-eluting peak, then you can address the problem. Sometimes, however, this peak does not occur in every sample, and much time can be wasted waiting for a peak that was not in the sample in the first place. A much more fruitful approach to tracing a late-eluting peak is to calculate its true retention time first; this approach allows you to identify the particular sample injection with which the peak is associated. First measure the plate number of a known peak in the chromatogram. As an example, we will use a peak with a retention time of 8 min and a width at half-height of 0.2 min. This gives a plate number of 8860 using Equation 1:

$$N = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \qquad [1]$$

where

$N$ = column plate number
$t_R$ = retention time
$w_{0.5}$ = band width at half-height.

We can solve Equation 1 for $t_R$ as shown in Equation 2:

$$t_R = 0.425\,(w_{0.5})\sqrt{N} \qquad [2]$$

Now we can predict $t_R$ for the late-eluting peak. Measure the width at half-height; in this example we will use 0.6 min for the problem peak. Using this $w_{0.5}$ value and the column plate number of the known band, which is equal to 8860, we calculate the retention time to be 24 min. This means that the peak is associated with an injection made 24 min previously. We confirm this by reinjecting the suspect sample. It is often not possible to eliminate these late-eluting peaks; hence, instead of modifying the separation conditions or waiting until the peak elutes before injecting the next sample, it is usually easier to adjust the run time so that the late-eluting peak falls in an unimportant region of a later chromatogram.

Two other causes of broadened peaks are injection problems and high sample molecular weight. Samples injected in too large a

volume of too strong a solvent will give broadened peaks. The strong solvent tends to spread the band out at the top of the column because band migration occurs momentarily under stronger than normal mobile phase conditions. A good rule of thumb, therefore, is to limit injection volumes to about 20 µl when an injection solvent as strong as the mobile phase is used. For more reliable results, use an injection solvent of approximately half the mobile-phase strength or less.

Reduced plate numbers, and thus broader peaks, are normal for high-molecular-weight compounds such as proteins and polymers because of their slow diffusion as compared with the rates of diffusion of smaller compounds. High-viscosity mobile phases will also give broader peaks.

## FRONTING PEAKS

A peak that is broadened only on its leading edge is said to be *fronting*. This corresponds to asymmetry (*As*) values of less than 0.9. The primary cause of peak fronting is column overload. During overload, the excess sample causes all active sites at the head of the column to be occupied, and the capacity of the column is exceeded. Hence, sample remaining in the mobile phase moves along the column without interacting with the stationary phase until it finds free stationary phase. Reduced retention times are also observed in the overload condition.

In analytical separations, overload is not desired because it reduces precision; however, overload is used intentionally in preparative separations to increase sample throughput. To test for overload, dilute the sample 10- or 100-fold (or reduce the injection size correspondingly) and reinject. A reduction of peak fronting and an increase in retention time are indications that an overload problem exists. To establish the amount of injected sample at which overload occurs, construct a linearity curve starting at dilute sample levels. The point at which a doubling of the injected sample mass produces less than a doubling of peak *height* (peak area should not change) is the overload condition. For reliable analytical separations, it is good to operate well below this overload point.

Peak fronting can also occur if too large a volume of a strong solvent is injected with the sample. As was discussed in the preceding section, a pulse of strong solvent can cause all or part of the band momentarily to travel more quickly through the column, resulting in distorted bands. The retention time may or may not be reduced, and the effect is more dramatic for early-eluting peaks than for those with higher values of $k'$. As was discussed for broad bands, dilute the sample or reduce injection volumes to solve this problem.

Another possible cause of peak fronting is the presence of a small band eluting just before the band of interest. This can be checked quickly by changing the detection wavelength or by increasing resolution by connecting two columns in series. If a second band is present, the chromatographic method may need to be modified to eliminate the problem.

Band broadening can also be caused by the use of too large a detector or recorder time constant. Many detectors have selectable time constants to increase their flexibility. These time constants are often selected by means of a toggle switch on the rear panel of the detector, which may get bumped into the wrong position when a signal cable is changed. Time constants of 0.1 sec or 0.5 sec are usually satisfactory for separations on 15- or 25-cm analytical columns. Columns that produce much narrower peaks, such as microbore or 3–5 cm minicolumns, require smaller time constants. Incorrect detector-cell volumes will also broaden peaks when narrow peaks are produced by the column.

## TAILING PEAKS

Tailing peaks are the most common form of peak-shape distortion observed in liquid chromatography. A band is classified as *tailing* if its asymmetry is greater than 1.2, although peaks with *As* values as large as 1.5 are acceptable for many assays. The primary cause of peak tailing is the occurrence of more than one mechanism of retention for the solute. In reversed-phase liquid chromatography, the primary mechanism is hydrophobic retention, but polar interactions with unreacted silanol groups on the particulate silica support are also common. After the initial bonding, many columns are reacted with a trimethylsilyl functional group to reduce the number of unreacted silanol groups. This "end-capping" reaction (or other proprietary silica treatments) will reduce tailing of polar molecules, but it is estimated that even with the use of these procedures about 50% of surface silanols are unreacted.

Compounds containing amine and other basic functional groups interact strongly with the residual silanols and produce tailing peaks. Adding triethylamine to the mobile phase at about the 5-m*M* level will reduce or even eliminate tailing of these compounds. It is also important to buffer the mobile phase to control the ionization of charged molecules, because both acids and bases give tailing peaks in their ionized forms. Another trick — which doesn't really address the mechanism of tailing — is to reduce $k'$ for the peak of interest by adjusting the mobile-phase strength. Sometimes a peak that tails badly at $k' = 5$–$10$ will be satisfactory for quantification at $k' = 1$, even with a tail. Any peak tailing not reduced to an acceptable level by silanol suppression or mobile-phase buffering may indicate that another LC method should be tried. Ion-pair chromatography may solve the tailing problem for acidic or basic solutes; ion-exchange chromatography is another choice for ionic analytes.

Tailing peaks can also result from a small peak eluting just after the peak of interest. The possible presence of an interfering compound is the major reason why peak tailing should not be ignored, even if quantification is possible. As was the case with fronting peaks, the best way to check for the presence of interference is to change the detection wavelength or the resolution. If a tail is observed at one detection wavelength but not at another, there is probably a second compound present. Sometimes, using a wavelength at which the interferent does not respond may be satisfactory for an acceptable assay. At other times, especially if the peak of interest is to be collected, the two bands must be separated more completely.

## NEGATIVE PEAKS

Most negative peaks are caused by factors other than the interaction between the sample compound and the column. Negative peaks will be observed if the recorder signal wires are reversed or if the detector-polarity switch is in the wrong position. Negative peaks are normal with refractive index detectors. You should be aware that most data processors do not accurately integrate peaks that are significantly below the recorder zero unless special software is used.

Negative peaks also appear if the absorbance of the sample is less than that of the mobile phase. This technique is used intentionally to enhance detection with nonsuppressed ion chromatography, where a highly UV-absorbing compound such as phthalic acid is added to the mobile phase; compounds that have an absorbance less than phthalic acid will produce negative peaks. If strongly absorbing materials build up in the mobile phase over time, a similar situation can occur when the mobile phase is recycled. If you do use mobile-phase recycling and you begin to see negative peaks after extended use of the mobile phase, wash the column with strong solvent and reequilibrate with freshly prepared mobile phase. If the negative-peak problem disappears, contaminated mobile phase is indicated.

A small negative peak is normally observed at the column dead-volume time in many LC systems. This is a result of an equilibrium disturbance when the injection solvent passes through the column.

## DOUBLE PEAKS

Whereas negative peaks in a chromatogram are often normal, the presence of double peaks is always a reason for concern. If all peaks in the chromatogram are doubled, the column probably has a void or channel at the top. Column substitution will quickly identify this problem. If a bad column is indicated, remove the end fitting and check the column (before discarding it). If a void is not observed, replace the frit and try the column

again to be sure that the problem was not caused by a partially blocked frit.

An interfering peak is suspected if only one peak in a chromatogram is doubled. To confirm the presence of an interferent, change the detection wavelength or increase resolution by doubling the column length. Separating compounds that are very similar, such as isomers or enantiomers, will often produce chromatograms with closely eluting peaks for the pair of interest. In these cases, highly efficient columns and special techniques, such as the use of chiral phases, are often necessary to separate the peaks adequately.

## GHOST PEAKS

Ghost peaks are peaks that appear even when no sample is injected. There are two common causes of ghost peaks. First, late-eluting material from a previous chromatogram may appear, seemingly from nowhere. This is usually a broad peak and can be traced using the retention-time-calculation method discussed earlier. A second cause of ghost peaks is dirty mobile phase. Under isocratic conditions the peak may be distinct, or you may observe a baseline shift only after the chromatograph has been in operation for several hours. Under gradient-elution conditions the peak(s) usually occurs at the same time in the chromatogram and appears in a blank gradient as well as in a sample run. In either case, these ghost peaks usually can be traced to a contaminated mobile phase. The best way to trace the contaminant is to use freshly prepared mobile phase containing only HPLC-grade solvents, HPLC-grade water, and high-purity additives. If use of a clean and carefully prepared mobile phase still results in ghost peaks, eliminate one ingredient of the mobile phase at a time until the problem is isolated.

## CONCLUSIONS

Several chromatographic problems that result in peak-shape changes have been discussed. Some of these are sample-related and some are caused by the equipment. In all cases, however, the problem is much more readily isolated and eliminated if careful records have been kept in a logbook. The logbook helps to trace chromatographic performance over time and to identify when changes in equipment, sample preparation, and reagents were made. These problems are solved with minimum downtime if a logical troubleshooting procedure is followed. Be sure to make a note of the solution so that the problem can be solved more quickly if it occurs again.

Readers are invited to contribute their troubleshooting tips to this column or to submit topics or questions for discussion in future articles. Write to: The Editor, *LC Magazine*, P.O. Box 50, Springfield, OR 97477.

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