T R O U B L E S H O O T I N G

Separation Artifacts II: Extracolumn Effects, Tailing, and Strong Retention Sites

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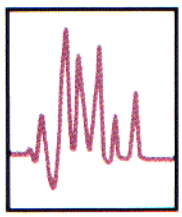

Last month, the topic of separation artifacts and some of their causes was introduced. This month, three additional problems that create separation artifacts will be discussed (1). Extracolumn effects are physical phenomena that are not related to the column packing, whereas strong retention sites are formed by specific interactions between the sample and the stationary phase. Tailing bands can result from physical or chemical factors.

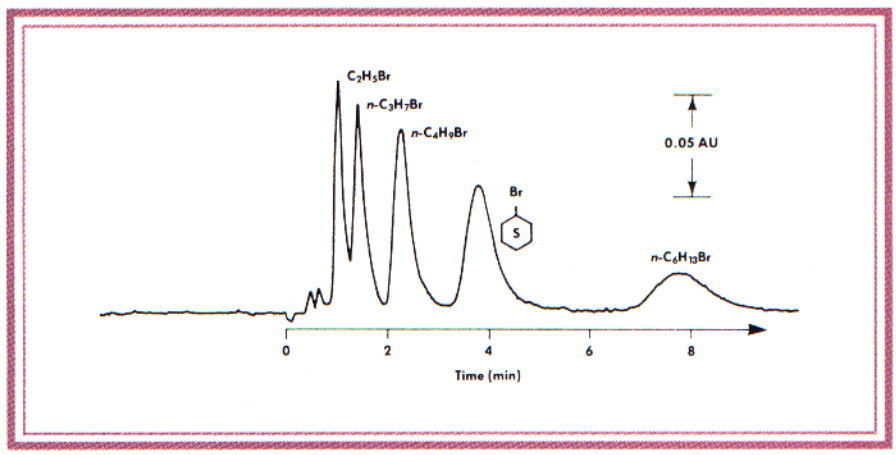

FIGURE 1: Band tailing caused by extracolumn band broadening. Reversed-phase chromatography using 35% methanol–water as mobile phase. Peaks: mixture of alkyl bromides.

EXTRACOLUMN EFFECTS

With the growing use of short, small-particle HPLC columns (such as 3-cm columns with 3-μm particles) (2), extracolumn effects are playing an increasingly important role in the overall efficiency of the HPLC system. Some of the LC equipment now in use was designed to handle columns that were first introduced a decade ago — that is, 30 cm × 0.46 cm columns packed with 10-μm particles. Since that time, columns of smaller dimensions containing smaller particles have gained in popularity. A ramification of this trend is that now extracolumn band broadening often contributes significantly to the plate number and shape of early bands in the chromatogram. Peak tailing is then seen for bands with small k' values, with asymmetry factors decreasing as k' increases (Figure 1). In general, this is not a serious problem because we attempt to adjust k' values for bands of interest so that $1 < k' < 10$, and band asymmetry is not often a problem for $k' > 1$. That is, the (common) tailing of bands near $k' = 0$ can be ignored.

For the case in which extracolumn band broadening significantly affects the shapes of peaks of interest, either another LC system (having less extracolumn volume) can be used or the system should be replumbed to reduce its extracolumn volume. For well-designed LC equipment, the major contribution to extracolumn volume is usually the detector flow cell. Some detectors allow the substitution of smaller-volume flow cells (≤2 μL), which reduces extracolumn effects — but usually also reduces detector sensitivity. In some cases, the connecting tubing may be too long or have an internal diameter that is too large. Minimal lengths of 0.007- or 0.010-in. i.d. tubing can be substituted in these cases. In extreme cases, 0.005-in. i.d. tubing can be used, but be aware that it can become easily blocked by particulate matter in the system. Selection of connecting tubing was discussed in an earlier Troubleshooting article (3).

A major contributor to extracolumn volume is an excessively long connecting tube between the autosampler and the column, which can result in band spreading in the tubing because of laminar flow. The effect of the tubing is the same as that observed when a very large volume of sample in mobile phase is used as an injection solvent. The result is broad bands in the chromatogram. In this case, it may be necessary to replumb the autosampler using 0.010-in. i.d. tubing of minimal lengths.

BAND FRONTING

Fronting bands, in which the "tail" appears on the leading edge of the peak, are observed less frequently in HPLC than are tailing bands, but they can be readily distinguished from other band-shape problems. Fronting bands are found most often in ion-pair chromatography (IPC), especially when the separation is run at ambient temperature. It is generally good practice to run IPC separations under thermostated conditions because relative retention tends to vary with temperature for this chromatographic mode. Temperatures of 40 °–50 °C are also generally favored for IPC because narrower bands and better separations result.

Another source of fronting bands in IPC is the use of a sample solvent other than the mobile phase. For a variety of reasons, in IPC the sample should be injected as a solution in the mobile phase only; if possible, no more than 25–50 μL of sample should be injected. Failure to follow these recommendations can lead to fronting bands.

Whereas tailing bands, which are far more common than fronting bands, suggest that sample retention decreases with increasing sample size or concentration, fronting bands suggest just the opposite: increasing retention for larger samples. In both cases, a decrease in sample size may eliminate peak distortion; however, this is often not practical because some minimum sample size is required for good detectability. In the case of band tailing, it is believed that peak distortion often arises because a sample that is too large will use up some part of the stationary phase. For fronting bands, some other chemical phenomenon must be invoked. For example, in the case of anionic sample molecules separated with higher-pH mobile phases on silica-based packings, the packing becomes increasingly negatively charged as pH increases (4). This results in repulsion of anionic sample molecules from the packing pores. With larger

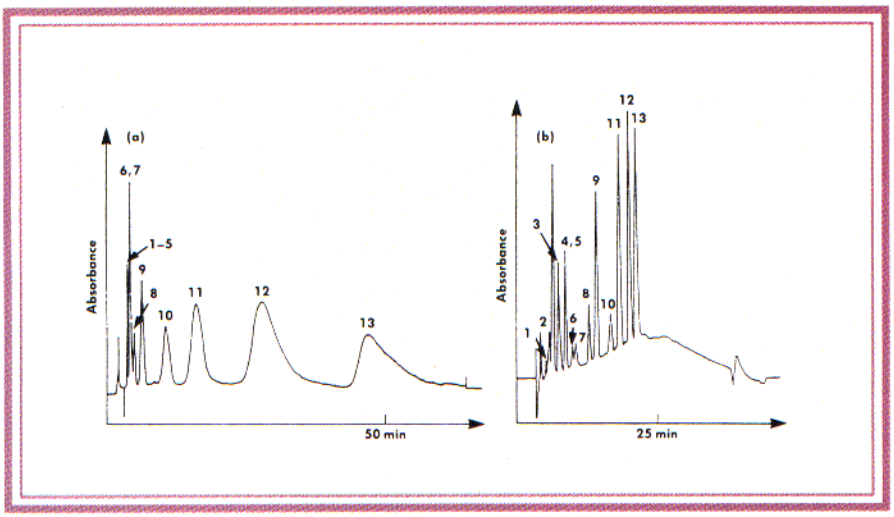

FIGURE 2: Elimination of tailing in late-eluting bands from isocratic anion-exchange separation of carboxylic acids using gradient elution: (a) 55 m*M* sodium nitrate and (b) linear gradient from 10 m*M* to 100 m*M* nitrate in 20 min. Reprinted with permission from reference 5.

sample sizes, however, this effect is overcome by the relative increase in ionic strength arising from the sample. In this case, the logical solution to the problem is to increase the ionic strength of the the mobile phase by increasing the mobile phase buffer concentration so as to fall in the 25–100 m*M* range. (Note that ionic or ionizable samples should never be separated with unbuffered mobile phases.)

STRONG RETENTION SITES

Separations obtained by normal-phase (adsorption) or ion-exchange chromatography involve binding of sample molecules to specific sites on the surface of the column packing [for example, the silanol (–Si–OH) groups on silica or the sulfonate ($-SO_3^-$) groups on a cation exchanger]. Often these sites are not all equivalent; for example, some sites are favorably situated to facilitate particularly strong interactions with sample molecules. In other words, some of these strong retention sites will be preferred by sample molecules and will be used up first. Because these strong sites are often present in low concentrations (that is, they constitute only a small fraction of total retention sites on the packing), they are quickly depleted so that only weaker sites remain available for further retention of sample molecules. This means that such packings (for normal-phase or ion-exchange chromatography) may become overloaded more quickly than those used in other modes (such as size-exclusion chromatography, IPC, or reversed-phase chromatography).

Another feature of strong retention sites is that they generally attract sample molecules having larger k' values. In other words, strong sites interact strongly with strongly retained molecules. This means that premature column overloading will occur mainly for later-eluting bands in the chromatogram — usually those with $k' > 10$. Consequently, it is the last bands in the chromatogram that overload first; this is observed as band tailing. In ion-exchange or normal-phase chromatography, band tailing that is caused by strong retention sites can sometimes be reduced by injecting less sample, but this compromises detection sensitivity. A more effective approach would be to reduce the strength of the mobile phase — that is, reduce the k' value of the last (tailing) band in the chromatogram to a value far less than 10. If lowering k' results in unacceptable loss in resolution at the beginning of the chromatogram, then the only alternative is the use of gradient elution. This is illustrated by the ion-exchange separations of Figure 2. Here bands 12 and 13 tail in the isocratic separation shown in Figure 2a, but tailing is eliminated in the gradient separation of Figure 2b.

For normal-phase separations using silica columns, strong retention sites can be suppressed by adding water to the mobile phase. Water deactivation of the column in this way can in turn improve the symmetry of late-eluting bands.

REFERENCES

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