LC TROUBLESHOOTING

Peak Tailing

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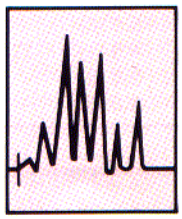

Bonded-phase column packings for liquid chromatography (LC) are by far the most popular column packing materials in use today (1). Workers in analytical-scale LC often use a C8 or C18 column as their first choice. The most commonly reported problem with these columns is inadequate resolution (1). Generally, discussions of resolution problems concentrate on the influence of capacity factor (k'), selectivity (α), and column plate number (N) because these factors can be controlled to a large extent by changing well-understood operating parameters. The problem with discussions of this nature is that peaks are assumed to be Gaussian (symmetric). In many cases, however, tailing peaks can cancel out any gains made in selectivity, retention, or bandwidth. That is, the separation of a band pair with baseline resolution and perfect peak shape may be useless if the first peak tails badly. Although experts say that you should never get serious about developing an LC method until the asymmetry factor (A_s) is <1.5 (2), many workers would be ecstatic if they could reduce the peak tail to $A_s = 1.5$. A great deal is known about the causes of peak tailing and how you can change the separation conditions by making the right choices when selecting the column and formulating the mobile phase. This month we will look at some of this information and see how it can help us get better peak shapes in the lab.

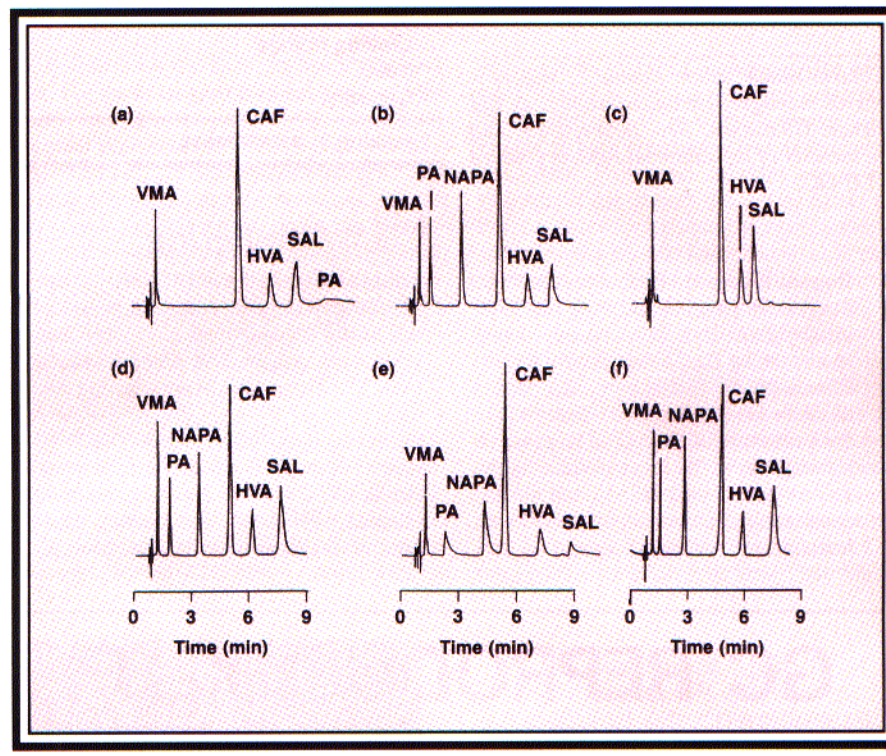

FIGURE 1: Effect of mobile phase additives on acid and base tailing. Solutes are neutral: caffeine (CAF); acids: homovanillylmandelic acid (HVA), vanillylmandelic acid (VMA), salicylic acid (SAL); and bases: procainamide (PA) and *N*-acetyl procainamide (NAPA). Columns: (a–d) Supelcosil LC-18, (e,f) Supelcosil LC-18 DB. Mobile phase: 7 vol % acetonitrile/water, pH 3.5; 10 mM triethylamine added to (b,d,f), 1% acetate added to (c,d,f). (Courtesy of Supelco Inc.)

BONDING THE SILICA SURFACE

Standard bonded-phase columns are packed with silica particles that have an organic moiety bonded to the surface by the reaction:

$$\text{Si–OH} + \text{Cl–Si–R}_3 \rightarrow \text{Si–O–Si–R}_3$$

where, for the C18 phase, the three R-groups include one octadecyl and two methyl groups. Because steric hindrance prevents bonding C18 groups to every silanol, most manufacturers perform an endcapping reaction, in which the surface is reacted again with trimethylchlorosilane. These smaller molecules can get between the C18 chains to further react with the surface. Even when this reaction is completed, however, about half of the silanol groups remain unreacted. Thus, the surface consists of hydrophobic (C18) regions and hydrophilic (SiOH) regions.

This bifunctional surface gives the column packing a split personality. Nonpolar molecules (such as hydrocarbons) interact primarily with the C18 functions and elute as well-shaped bands. Sample molecules that are attracted to the SiOH groups (for example, amines) interact with both the C18 and the SiOH functions. Typically, this gives tailing peaks. Most efforts to suppress peak tailing are aimed at minimizing these silanol interactions, which often are referred to as *secondary retention*. To completely eliminate silanol interactions, we have to use polymer-based reversed-phase columns, which do not have silanol groups.

Also note that the silica-based bonded phase is a chemical surface that can be degraded by pH extremes. Generally accepted guidelines suggest operation between pH 2.5 and pH 7 (3). When the column is operated outside these limits, loss of bonded phase or dissolution of the silica particles should be expected. This degradation will accelerate at pH extremes. When the mobile phase pH exceeds these guidelines, changes in retention and peak shape should be expected. As bonded phase is lost, retention times generally drop; but because the proportion of unbonded silanol groups increases, band tailing often will increase as well. It is wise to use conditions under which the column does not change. Polymeric reversed-phase columns, which are stable at high and low pH, often can be acceptable substitutes when pH extremes must be used.

TWO TYPES OF SILANOLS

The problem of residual silanol groups is further complicated by the fact that some silanol groups are acidic in nature and some are basic (see Figure 1). The sample of Figure 1 contains three acids (homovanillylmandelic acid [HVA], vanillylmandelic acid [VMA], and salicylic acid [SAL]), a neutral compound

(caffeine [CAF]), and two bases (procainamide [PA] and *N*-acetyl procainamide [NAPA]). Under the conditions of Figure 1a, the neutral CAF peak is sharp, and the acids tail a little, but the bases are broad and strongly retained (NAPA elutes after PA). When triethylamine (TEA) is added to the mobile phase (Figure 1b), the basic peaks are sharper and much less strongly retained than previously. Similarly, acetate can be added to sharpen the acidic peaks (Figure 1c) without affecting the basic peaks. These chromatograms show us that there are two types of silanol interactions — one that is specific for bases, and one for acids.

WHAT ABOUT DIFFERENT COLUMNS?

I usually recommend against changing from one brand of column to another to solve a separation problem. This is because most users are looking for dramatic changes in selectivity (band spacing) when they change columns, and such changes can be more readily achieved by changing the mobile phase (for example, from acetonitrile to tetrahydrofuran) or the LC mode (for example, from reversed-phase to ion-pair). There are times, however, when changing the column is advantageous to effect changes in band shape. In the preceding section, we saw that there are acidic and basic silanol interactions. Workers who have examined different commercial products for these interactions have found that some brands exhibit less tailing with bases than others (4–6). Often, a column that is poor for bases will be better for acids. This has to do with the chemistry of the manufacturing process. Figure 1e illustrates this with a column that has been manufactured to minimize basic interactions. Notice the improved peak shape for PA and NAPA when compared with Figure 1a. The flip side is that the acids HVA and SAL show worse tailing on this column.

Table I lists some commercial columns in order of their ability to produce good peak shape for basic compounds. We can see how the columns of Figure 1 fit into the list: Supelcosil DB (Figure 1e) ranks above Supelcosil (Figures 1a–d) in terms of peak shape for basic compounds. The first step in methods development is to select a column that will minimize unwanted acidic or basic interactions with the sample. This doesn't necessarily mean that you need to rush out and change column manufacturers or that you should buy one of every type of column. The column that you have may be fine, but be aware that there are logical choices to make in order to minimize these unwanted interactions. At the same time, however, you should expect some change in band spacing when you change column brand.

TABLE I: COLUMN RANKING IN DESCENDING ORDER OF PEAK-SHAPE QUALITY FOR BASIC SOLUTES*

Zorbax Rx
Vydac
Rsil
Nucleosil
μ-Bondapak
Supelcosil DB
Spherisorb 2
LiChrosorb
Chrompack
Hypersil
Perkin-Elmer
Supelcosil
Zorbax
MicroPak

*reproduced from reference 2 with permission; differences between successive listings may not be significant

THERE'S ANOTHER WAY

As suggested by the comparision of Figures 1a and 1e, you can improve peak shape dramatically by selecting the proper column, but this probably will not solve all the tailing problems. In fact, whereas the column of Figure 1e dramatically improves the peak shape of the bases (PA, NAPA), it shows more tailing for the acids (HVA, SAL) when compared with the column of Figure 1a. We can use mobile phase additives, as shown in Figures 1b and 1c, to suppress unwanted silanol interactions with any column. And if both acidic and basic sample components are present, the

additives can be combined, as in Figure 1d, to give a cumulative effect. You can see that the TEA in the chromatogram of Figure 1d causes the basic peaks to sharpen, just as it did in Figure 1b; at the same time, the acetate sharpens the acidic peaks, as was the case for Figure 1c. This implies that the acidic and basic sites act independently on the sample. It is interesting to note that when both additives are used with the standard column (Figure 1d), the separation is almost identical to that provided by the deactivated column when both additives are used (Figure 1f). At least in this case, much, if not all, of the gain that can be made by selecting the proper column can be made also through the use of mobile phase additives.

WHICH ADDITIVE TO USE

If acids and bases can be used to suppress tailing, how do we go about selecting the appropriate compound to use? The answer is twofold. One part concerns the chemistry of suppressing the silanols, and the other concerns compatibility with the assay. First, the suppressing agent works because it has a stronger polar interaction with the silanol functional groups at the silanol surface than the sample compounds have. Smaller molecules are favored because they diffuse to the surface and equilibrate rapidly in the column. In addition to the polar group, it is good to have a nonpolar organic tail attached to the molecule. This nonpolar end sticks away from the surface — looking, to the sample molecules, much like the C8 or C18 bonded phase. This effectively increases the hydrophobic nature of the column. Hydrophobic interactions between the tail and the bonded phase also help to hold the additive in place.

Triethylamine usually is the additive of choice for suppressing base tailing; concentrations of 10–50 mM generally are sufficient. When TEA is inadequate — as it is for some tertiary amine samples — dimethylhexyl- or dimethyloctylamine may improve peak shape (7,8). Some workers have found that bulkier amines are more effective, but these take much longer to equilibrate with the column, and, in some cases, they can be impossible to wash off the column.

When tailing of acidic sample components must be blocked, acetic acid is a good choice. Similar rules apply for equilibrating and washing off longer-chain acids. Adding 1% acetate, as in Figure 1, should be adequate in most cases.

Finally, when you select mobile phase additives, consider their compatibility with the assay. If you are using the method for quantitative or qualitative analysis, generally it makes little difference which acid or base you use, as long as no unwanted sample interactions occur. When the sample is to be collected, however, the choice may be more limited. If the sample is a protein or peptide in which biological activity must be maintained, you'll want an additive that does not denature the sample. Similarly, if the sample is to be collected for purification, the additive should be volatile enough to easily separate from the sample when the solvent is evaporated.

BUFFER STRENGTH

Another way for tailing to occur is through the ion-exchange interactions of protonated sample bases with ionized silanols (9,10). These ion-exchange effects generally can be suppressed by increasing the ionic strength of the buffer. If you suspect this problem, try increasing the buffer strength to see if peak shape improves. Buffer concentrations are quite sample-dependent. A 10–25 mM buffer is a good starting point, but concentrations of 100 mM or higher may be required in some assays.

OTHER POSSIBILITIES

The preceding discussion has concentrated on how we can change the column so that it does not interact in unwanted ways with the sample. You also can do many things to the sample so that it is less susceptible to secondary retention problems. Thorough treatment of these topics will be saved for future columns, but let's look briefly at some of the possibilities. Perhaps the most obvious way to control sample behavior is to control the pH for ionizable samples. If the pH is adjusted so that the sample molecules are in a neutral form, acidic and basic silanol interactions are much less likely to occur. Another alternative is to switch to ion-pair chromatography. By adding an ion-pairing agent to the mobile phase, we can take advantage of the polar in-

teractions and often obtain separations that are not possible with reversed-phase alone. Another possibility is to derivatize the sample to block a reactive site on the sample molecule before injecting it onto the column.

Changing from a silica-based to a polymeric reversed-phase column may provide a good solution for the analysis of basic compounds that do not respond to the treatments suggested here. When switching to these columns, however, many users are surprised at how important the silanol interactions really are. They find that a separation that worked, even though it produced tailing bands, won't work on a polymeric column. In cases like these, the silanol interactions may be providing most of the selectivity for the assay, and you may need to seek other ways to improve the separation.

SUMMARY

We have seen that "fully bonded" reversed-phase columns still have a high proportion of unreacted silanols available for secondary retention of our sample compounds. Some of these silanol groups affect the peak shape of bases, whereas others cause acidic compounds to tail. By carefully selecting the column and the mobile phase additives, you can eliminate much, if not all, of the unwanted peak tailing. Once you have found conditions that produce reasonable peak shapes, you can expect to obtain separation methods that are much better-behaved and more reproducible.

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