LC Troubleshooting

Retaining Polar Compounds

Some compounds just won't stick to the column.

John W. Dolan
LC Troubleshooting Editor

Many workers use gradient elution scouting runs to screen the retention characteristics of compounds to be separated by reversed-phase liquid chromatography (LC). For example, they might use a 20-min gradient of 5–100% acetonitrile–water with a 150 mm × 4.6 mm, 5-µm d_p C8 or C18 column. The volume of this column is approximately 1.5 mL, so the column dead volume (t_o) is roughly 1 min at a flow rate of 1.5 mL/min. Under conditions such as these, most compounds will generate retention times in the 2–20 min range, which shows that chromatographic retention is occurring. When retention times are at or very close to t_o, little useful chromatography occurs. These poorly retained compounds are quite polar and can present a challenge for method development using reversed-phase LC.

This installment of "LC Troubleshooting" will examine techniques that you can use to increase retention of polar compounds. The options discussed below need not be followed in order — individual experience or samples may lead you to reject some options without trying them.

pH Adjustment

The scouting run described above used acetonitrile–water as the mobile phase. This combination will be suitable for nonpolar molecules, but acidic or basic compounds usually require a buffer to obtain significant and reproducible retention. I generally begin method development with scouting runs that use a low-pH aqueous phase rather than water because our laboratory works primarily with pharmaceutical compounds that rarely are neutral. Low-pH conditions will suppress the ionization of acids and make them neutral and more apt to be retained in a reversed-phase system. Low-pH conditions also suppress the silanol activity on the column to decrease the unwanted interactions between basic molecules and the acidic silanols. A simple substitution of 0.1% trifluoroacetic acid or 0.1% formic acid for the water component of a mobile phase is adequate for screening retention. A good alternative is 10–20 mM phosphate buffer adjusted to pH 2.5. If chromatographers obtain adequate retention under these conditions, they can continue to refine the mobile-phase conditions for their samples.

If low-pH conditions do not aid in the retention of the compound of interest, it may be basic in nature. To suppress the charge on most basic compounds, analysts generally must use a mobile phase with pH greater than pH 8. Historically, pH levels greater than pH 8 have not been recommended for silica-based columns because the silica backbone of these columns is soluble at high pH levels. Thus, although retention might be obtained at high pH, the lifetime of the column could be reduced severely. However, recent advances in column technology and a better understanding of the role of the buffer have led to the development of columns that have increased stability at high pH levels.

Column manufacturers have used several approaches. One approach, based on Agilent's Zorbax Extend column (Agilent Technologies, Wilmington, Delaware), uses a stationary phase that is bonded to the silica particles at two points instead of one as in normal packings. This double attachment stabilizes the bonded phase in high-pH conditions. Waters Corp. (Milford, Massachusetts) took a different approach with its XTerra columns. These columns have a modified silica, in which some of the silicon atoms have been replaced with carbon. This substitution greatly reduces the solubility of the base silica at high pH, yet leaves sufficient silanol activity to enable the use of traditional bonding chemistry to attach the stationary phase. Base-resistant silica packings are much more tolerant of high-pH buffers than traditional silica-based packings. However, it is best to avoid inorganic buffers such as phosphate, which tend to dissolve the silica much more readily than organic buffers such as glycine or carbonate. If you work at high pH with one of these modified silicas, be sure to follow the manufacturer's instructions for selecting the buffer.

Some other columns have the stationary phase bonded to a nonsilica base material. For example, ZirChrom Separations (Anoka, Minnesota) has zirconia-based packings with C18-like bonded phases. Several vendors make packings that use a polymeric bead instead of silica with C18 or C18-like bonded phases. In general, these nonsilica columns can tolerate nearly any buffer at nearly any pH. Of course, you should consult the manufacturers' recommendations when selecting a buffer.

If you are fortunate, a high-pH buffer with a suitably stable column will generate acceptable retention for your analytes.

A More Retentive Column

To obtain the maximum retention under reversed-phase conditions, you should use the most nonpolar column available. You should use a C18 bonded phase instead of a C8 phase. Several manufacturers supply high-carbon content or polymeric silica-based packings that use special chemistry techniques to increase the bonded-phase density. This increase in carbon content can increase the retentivity of a column. Sometimes, however, a truly polymeric phase can restrict diffusion and cause lower column plate numbers than traditional monofunctional C18 columns. An added benefit of the high-carbon-content columns is that the more bulky bonded phase tends to shield the silica and, thus, protect the surface from attack by basic mobile phases.

Use Less Water

If the compound of interest is eluted at t_o with 5% organic solvent in the mobile phase under acidic, basic, or neutral conditions, it may be possible to obtain enough retention by reducing the organic content of the mobile phase. Generally, I advise against using more than 95% water in the mobile phase because the bonded phase can collapse if too much water is present, thus changing the retention characteristics of the column. This collapse occurs in the 97–98% aqueous region. With care, chromatographers can obtain reliable separations with as little as 2–3% organic solvent in the mobile phase, but this concentration still may provide insufficient retention.

To use less than 2% organic solvent in the mobile phase, you should select a column designed for this purpose. The embedded polar phase class of columns allows the use of 100% water without collapse. These columns use a C8 or C18 bonded phase with a polar functional group bonded near the base of the phase. Some examples of these columns are Agilent's Zorbax Bonus RP, Waters' Symmetry Shield, and Supelco's Discovery amide (Supelco, Bellefonte, Pennsylvania) columns. Other manufacturers also supply phases that will tolerate 100% water. These columns often have *AQ* as part of their names, such as YMC AQ (YMC Inc., Wilmington, Delaware), or have some other description that indicates that they will tolerate 100% aqueous conditions.

Ion Pairing

If you try 100% water (or buffer) with one of the water-tolerant columns and still cannot obtain any significant retention for your compound of interest, it may be time to try ion pairing. A traditional C8 or C18 column is used in ion-pairing chromatography. The ion-pairing reagent is a molecule with a charge at one end and a nonpolar tail. An ion-pairing reagent is added to the mobile phase. The nonpolar end of the reagent sticks to the nonpolar stationary phase, and the charged end extends into the mobile phase. The result is a hybrid between an ion-exchange column and a reversed-phase column. If you are trying to retain bases, you should use a negatively charged ion-pairing reagent such as hexane sulfonate. For acids, you should select a positively charged reagent such as tetramethylammonium bromide.

Ion pairing is useful for obtaining satisfactory retention of mixtures of acids, bases, and neutral compounds. The retention is controlled by adjusting the percentage of organic solvent in the mobile phase, the ion-pairing reagent concentration, the nature of the ion-pairing reagent, the temperature, and other variables. This technique is complex and fraught with potential problems, but it is a very effective tool for retention of charged polar molecules.

Don't Forget Normal-Phase Chromatography

In an analytical world where most LC separations are achieved with reversed-phase conditions, it is easy to forget about normal-phase chromatography. Normal-phase chromatography often is the best separation technique for very polar molecules. Instead of relying upon hydrophobic, nonpolar interactions between the sample molecules and the stationary phase, normal-phase separations depend upon polar adsorptive interactions. Because the stationary phase is polar and the mobile phase is nonpolar, polar molecules will be more strongly retained than nonpolar ones in normal-phase chromatography.

A separation is classified as normal or reversed phase based upon the change in retention with a change in organic solvent concentration in the mobile phase, rather than strictly by the column packing. When retention increases with increased water (polar) content, reversed-phase retention is happening. When retention decreases with increases in the polar component of the mobile phase, normal-phase retention is occurring.

Bare silica is the traditional stationary phase for normal-phase separations and is widely used, especially for preparative purposes. Silica earned a bad reputation throughout the years because of the difficulty analysts had obtaining reproducible separations and good peak shape unless the water content of the mobile phase (for example, at 0.1%) was controlled very carefully. This problem is somewhat mitigated by using alcohol or acetonitrile (for example, at 1–2%) instead of water to control the humidity of the mobile phase. In recent years, the improvement of silica gel with the introduction of Type B silica has led to fewer reproducibility problems.

An alternative to bare silica is one of the polar bonded-phase columns. A cyano bonded phase, for example, can be used in the normal-phase mode, although it is not recommended to switch one column back and forth between reversed and normal phase. Diol columns are another example. In these columns, a short-chain hydrocarbon phase terminates in a diol functional group and gives the stationary phase a polar nature. Polar bonded-phase columns use a traditional normal-phase solvent system such as isopropanol–hexane. An advantage of the bonded phase columns is that gradient elution can be used for scouting, whereas gradients are not recommended with bare silica. Remember that hexane is the weak solvent, so the gradient should run from a high hexane content to a low one.

Other specialty phases allow you to use traditional reversed-phase solvents and obtain normal-phase separations. The hydrophilic interaction chromatography columns — often called HILIC columns — from PolyLC (Columbia, Maryland) are an example of these phases. These columns are based upon a polyaspartamide stationary phase that has a polar surface exposed to the mobile phase. Chromatogra-

phers can use mobile phases such as acetonitrile–water with these columns, but they will observe increased retention with increasing acetonitrile content in the mobile phase.

Specialty Columns

Specialty columns that don't fall into the reversed- or normal-phase categories abound. One of these phases might be just what you're seeking for specific separations problems. Search the Internet or look through column manufacturers' literature to see what is available that might be useful for your application. Sometimes these columns are available from a single vendor, whereas others are available from many sources.

Getting More Help

If you need help developing a separation using one of the techniques discussed above, a good place to start is *Practical HPLC Method Development* (1). This classic reference provides practical guidelines for each mode of chromatography to get you on your way to developing a successful separation with any of these techniques.

Search the scientific literature for work published by others about separating your analytes or similar ones. In many cases, you may find some useful hints that will help you obtain the separation you need.

Column manufacturers have published hundreds of application notes over the years. With the search capabilities of the Internet available and copies of the application notes on manufacturers' web sites, you can quickly locate information about specific columns and the separation of specific sample types. A good place to start for information about columns and phases is *LCGC*'s annual Buyers' Guide (2), now available on-line at http://www.chromatographyonline.com.

Another source of good advice about tough separations is the "Chromatography Forum" web site (http://www.chromforum.com), jointly sponsored by *LCGC* and LC Resources. Separations experts monitor this popular discussion group, and hundreds of chromatographers actively contribute to it. Just post your question and wait for an insightful answer.

Summary

This discussion is meant to highlight some options available when chromatographers are trying to separate polar molecules. It is not meant as a step-by-step guide to separations. I've surveyed several different techniques for improving the retention of polar compounds. No single approach is the best for the problem. Your experience, knowledge of the molecule, and available resources all will influence your strategy.

My personal preference is to start with traditional reversed-phase techniques and use low-pH conditions and solvent strength to effect the desired changes. I find that the embedded polar phases are very effective in many applications, so my laboratory usually checks a column of this type if a traditional reversed-phase column doesn't work. Next, I'll usually try high-pH conditions with a stable column to see if it will help. I tend to avoid ion pairing until I must use it, because it has many experimental problems and the reagents seldom allow detection at low-UV wavelengths (for example, less than 220 nm). However, when it works, ion pairing is great.

As do most other workers, I leave normal-phase separations to the end. Part of the reason for this choice is in response to experimental problems and the inconvenience of changing an LC system to normal-phase solvents and part is because I find that tuning and controlling the separation with normal-phase chromatography is much less intuitive than reversed-phase or ion-pairing techniques.

References

(1) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997).
(2) *LCGC* **19**(8), 749–928 (2001).

John W. Dolan
"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LCGC*'s editorial advisory board. Direct correspondence about this column to "LC Troubleshooting,"* LCGC, *859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@LCResources.com.*

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