Innovators in Chromatography

A continuing series of guest editorials contributed by collaborators and internationally recognized leaders in chromatography.

The Role of Selectivity in Liquid Chromatography Method Development

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The name of the game in chromatography is the separation of chemical compounds. The resolution of one analyte from another in a chromatographic separation is determined by three main factors: efficiency, selectivity, and retention. The interplay of these is described by the master resolution equation,

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right) \quad (1)$$

where N is the number of theoretical plates (a measure of efficiency), α is selectivity, and k'_2 is the capacity factor (or retention factor) for the later eluting peak of the analyte pair of interest. Incidentally, in some forms of the master resolution equation, an average capacity factor k'_{avg}, calculated from the retention of both analytes, is used in the third term. As we are largely considering a pair of closely eluting analytes, the difference between k'_2 and k'_{avg} would be minimal. The magnitude of contributions of each of the three terms in Equation 1 to resolution varies, but the maximization of each term (without the complete disregard of the other two) will help yield the separation of analytes of interest ($R_s \geq 1.5$ is the target value for baseline separation).

Here, we focus on the selectivity term. Selectivity is defined in Equation 2 as

$$\alpha = \frac{k'_2}{k'_1} \quad (2)$$

It is the ratio of capacity factors for two chromatographic peaks. Conceptually, a capacity factor is the ratio of the amount of time an analyte spends in the stationary phase to the amount of time it spends in the mobile phase. Since all analytes spend the same amount of time in the mobile phase (equal to the dead time t_0), selectivity is the ratio of the amount of time the later eluting analyte spends in the stationary phase relative to that of the earlier eluting analyte. While the mobile phase composition in liquid chromatography can be varied to encourage an overall greater or lesser retention, the primary factor controlling selectivity is the ability of the stationary phase to differentially interact with each analyte. The primary means to alter selectivity in a chromatographic separation is to change the stationary phase or the mode by which analytes interact with the stationary phase.

While different separation modes (e.g., reversed phase [RP], hydrophilic interaction [HILIC], aqueous normal phase [ANP], normal phase [NP], etc.) can be used to affect the ways that analytes interact with a given stationary phase, we confine ourselves here to discussions on RP separations. Virtually every chemistry student has experience in RP separations—most likely focused on generic separations using an octadecylsilyl (C18-bonded silica gel) bonded phase. The first thing to note is that all C18 phases are not created equal. Changes in the underlying support chemistry, the way bonded groups are attached to the support, and the ways potentially deleterious interactions with residual silanol groups are shielded, significantly affect the retention of different analytes. For example, amine-containing compounds often exhibit significant tailing in chromatograms if they can interact with silanol groups. The strategy is to induce a uniform dominant interaction mode between the analyte and the stationary phase so that nicely symmetrical peaks are observed. For a typical C18 phase, the dominant interaction is induced by the hydrophobic effect. Significant differences in the hydrophobic content in chemical structures allow the C18 phase to exert selective interactions with each analyte and, assuming adequate retention and good efficiency are maintained, chromatographic resolution will result.

Complex mixtures will contain a multitude of chemical compounds that possess variable physicochemical properties. Oftentimes, the chromatographer is concerned with the qualitative and quantitative speciation of multiple analytes from a single class (e.g., polyphenols, drugs and their metabolites, steroids, etc.). If each compound has a different molecular weight, one might be able to bypass the need for chromatographic resolution of all components of interest by using a selective detector, such as a mass spectrometer. However, a mass spectrometer cannot directly differentiate compounds that have the same mass, and many analytes in a class of compounds may simply be isomers, which have the same elemental formula. While it is possible to use some tandem mass spectrometry approaches to differentiate coeluting isobaric compounds, the most reliable means by which to differentiate them for speciation would be to chromatographically resolve them prior to detection. A generic C18 phase may not provide sufficient selectivity to accomplish this task.

Those who move beyond college course-based laboratory exercises will quickly learn that there are other stationary phases available to impart additional selectivity in reversed-phase separations. Recent moves to alter support chemistries, including the use of superficially porous particles, have a major impact on efficiency of separations. However, to impact changes in selectivity, more important are changes in the chemistry of moieties bonded to these supports. Different manufacturers offer a milieu of alternatives that can range from the incorporation of polar units imbedded in the C18 chain or the bonding of different functional units all together. A favorite question I ask my senior-level instrumental analysis class is, "How can a cyano-bonded phase be used in both NP and RP separation modes?" The cyano phase is ideal for NP separations where a polar stationary phase is paired with a nonpolar mobile phase. However, in reversed-phase mode, this polar phase can impart vastly different retention interactions to more polar analytes compared to a C18 phase. This can cause large changes in elution order for a mixture of analytes because the cyano group provides a vastly different selectivity, and it is still effective for use in RP mode with a polar mobile phase. Similarly, use of phases that incorporate polar groups embedded somewhere along a C18 chain enable hydrogen-bonding interactions to assist in selective retention of different compound classes. Care should still be taken that these interactions are uniform and do not impart poor peak shape due to non-uniformity of chromatographic separations (similar to silanol effects), but for certain classes these additional interaction sites can be the difference between separation or coelution. Available now are also biphenyl phases which, in the presence of the right mobile phase, exert pi-interactions that can improve selectivity and retention for aromatic analytes. Interestingly, a biphenyl phase will exert these interactions in the presence of an aqueous methanolic mobile phase, but in the presence of acetonitrile, which itself has a strong pi-character, the phase will behave more like a C18. The change in selectivity can be quite dramatic.

The chromatographer's toolbox is ever expanding. Sometimes this can be overwhelming. Manufacturers have given different generic (and sometimes difficult to interpret) names to the different stationary phase supports and bonded phases they use to create their products. Luckily, they also spend a great deal of time and effort providing educational materials to guide the choice of the proper phase for different applications. Even so, one should always go back to the master resolution equation to reason the underlying fundamentals that will eventually yield separation of target compounds of interest. Chemists and biochemists will never stop creating new chemical compounds, and we are still figuring out the chemical diversity provided by nature. Thus, analytical chemists will always have a job in characterizing new analytes or determining their presence in various systems. It is a good thing that there are a lot of choices in the tools that one can use to accomplish these tasks.

This editorial was previously published in The Restek® Advantage 2015.1.