LC TROUBLESHOOTING

Problems in Size-Exclusion Chromatography

John W. Dolan

Size-exclusion chromatography is perhaps the simplest LC method to understand, but the problems associated with this technique may not be so simple.

Size-exclusion chromatography is often called by different names. When nonaqueous mobile phases are used, the technique is called gel permeation chromatography (GPC). With aqueous mobile phases, gel filtration chromatography (GFC) is the name of choice. Here, we'll use size-exclusion chromatography (SEC) to encompass both of these techniques. This month's "LC Troubleshooting" briefly reviews the principles of SEC separation and addresses some potential problems and their solutions.

HOW SEC WORKS

Size-exclusion chromatography separates sample molecules in the mobile phase by their effective size. (In most cases, molecular size can be correlated directly to molecular weight, so the two terms will be used interchangeably here.) The column is filled with particles of the same pore diameter (or a very narrow pore-diameter range), and mobile phase conditions are selected so that the sample is soluble and does not interact chemically with the column packing. Any sample molecules that are smaller than the packing's pores can diffuse into and out of the pores, whereas sample molecules larger than the pores cannot enter. As a result, the larger molecules pass quickly through the column, and the smaller molecules lag behind, as shown in Figure 1 (1). The illustration shows a single pore and two sample molecules of different sizes. The smaller molecule can penetrate the pore more fully (its path is represented by the dotted line next to the pore wall), whereas the larger molecule is somewhat restricted in its pore access (dotted line in center of pore). Because the smaller particle spends more time (on the average) in the pore than the larger one, the larger molecule passes through the column first. Theoretically, a single pore size should separate compounds differing by about $10^{1.5}$ Da, but in practice, commercial columns cover a minimum range of 10^2–$10^{2.5}$ Da.

Any molecules that are larger than the pores elute first and at the same time; the smallest molecule that cannot penetrate the pores defines the *exclusion limit* of the column. Molecules smaller than a certain size have equal access to the pores, so they elute together at the *total permeation volume*, which is the same as the column dead time, t_0. Molecules between these extremes elute in order of their molecular size. This principle is illustrated in Figure 2 for a hypothetical column whose packing has a single pore size. We can see that peak A is the first peak and consists of all the (excluded) sample molecules of molecular weight $> 10^5$ Da. Next comes band B, which is smaller, then band C. Finally, band D consists of all the molecules that have total access to the pores (MW $< 10^3$ Da). Note that when the SEC column is behaving properly, all the sample molecules elute in the well-defined region between the exclusion volume and the total permeation volume. Because retention depends on molecular size in relation to pore size, SEC columns can be used for the determination of the molecular weight of sample molecules if the columns have been calibrated as in Figure 2.

EXTENDING THE RANGE

Traditionally, the use of SEC columns has been limited to compounds > 2000 Da, but more recently, smaller-pore columns have become available for effective separations below 1000 Da. The molecular-weight range of a single-pore-size column (for example, 10^2 Da) may be too small for use with some samples. To solve this problem, two columns of complementary pore sizes can be used. For example, a column with a 10^3–10^5 Da range can be coupled with a 10^5–10^7 Da column to cover the range of 10^3–10^7 Da; this is referred to as a bimodal column set. Although bimodal column sets can conveniently be made by connecting individual columns in series, some manufacturers sell bimodal and trimodal columns that are packed with a mixture of particles having two (or three for trimodal) distinct pore sizes. For further discussion of this subject, see reference 2 or literature from SEC column manufacturers.

PACKING MATERIALS AND SOLVENTS

Two types of packing materials are commonly used for SEC columns. Polymeric packing materials (usually based on styrene-divinylbenzene polymers) can now be made sufficiently rigid to make robust columns of broad utility. Other columns are prepared with porous silica beads, which are silanized for GPC or unsilanized for GFC. Both types of packing materials can be compatible with a wide range of mobile phase solvents: tetrahydrofuran (THF), toluene, chloroform, dichlorobenzene, aqueous mobile phases, and other solvents. But before using a solvent for the first time, it is best to consult the column manufacturer's literature to be sure that the solvent is compatible with the column. SEC columns typically cost twice as much as standard analytical columns and can be somewhat less rugged — it would be a shame to ruin a column by inadvertently using an incompatible solvent.

In contrast to solvents used in other LC methods, solvents for SEC are chosen primarily for sample solubility and low column interaction. This is because the solvent composition does not affect retention in SEC;

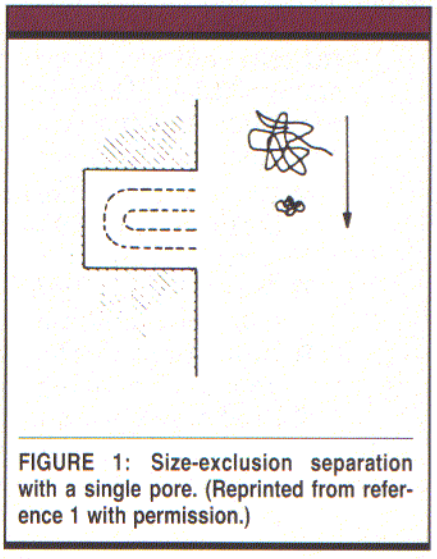

FIGURE 1: Size-exclusion separation with a single pore. (Reprinted from reference 1 with permission.)