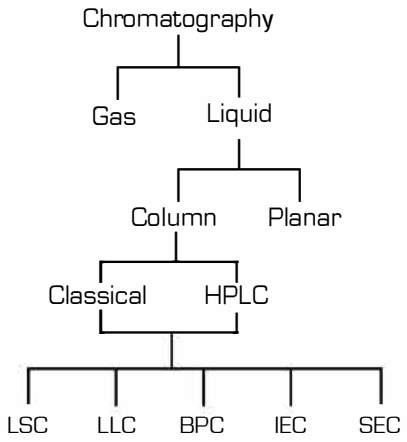
601: GENERAL INFORMATION

In recent years, high performance liquid chromatography (HPLC) has grown in popularity as a determinative step for residue analysis, until today it is accepted as complementary to the more traditional gas liquid chromatography (GLC). HPLC provides capabilities not possible with GLC, most importantly the ability to separate and quantitate residues of polar, nonvolatile, and heat-labile chemicals. These characteristics make HPLC the determinative step of choice for many residues previously beyond the applicability of multiresidue methodology.

601 A: PRINCIPLES

Figure 601-a
Chromatographic Separation Techniques



Chromatography comprises a family of separation techniques (Figure 601-a), all of which share common characteristics. A narrow initial zone of mixture is applied to a sorptive stationary phase having a large surface area. Development with mobile phase causes components of a mixture to move through the stationary phase at different rates and to separate from one another. Differential migration occurs because of differences in distribution between the two phases. The mobile phase can be a gas or a liquid. Liquid chromatography is divided into two main types, planar (thin layer and paper chromatography) and column. Column liquid chromatography, both the classical (low pressure) version and the high performance version discussed here, is further subdivided according to the mechanism of separation into five major types: liquid-solid (adsorption) chromatography, LSC; liquid-liquid (partition) chromatography, LLC; bonded phase chromatography, BPC; ion exchange chromatography, IEC; and size exclusion chromatography, SEC.

HPLC developed steadily during the late 1960s as high efficiency, small particle packings and improved instrumentation were produced. In contrast to classical column liquid chromatography, HPLC uses high pressure pumps; short, narrow columns packed with microparticulate phases; and a detector that continuously records the concentration of the sample.

HPLC systems use the principles of classical column chromatography in an analytical instrument. Development of HPLC has been directly related to availability of suitable hardware (columns, pumps, inlet systems, low dead volume fittings, *etc.*) that allows precise flow control under the elevated pressures needed, as well as the ability to manufacture a wide variety of column packing materials in particle sizes of exacting micron (µm) dimensions.

In contrast to GLC, where the gas mobile phase is inert and does not affect separation of analytes from one another, the HPLC mobile phase is critical to this

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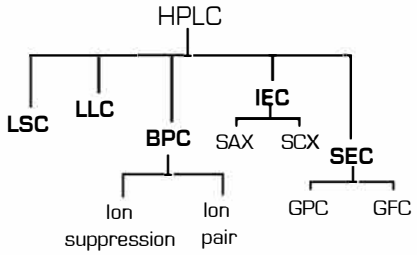
resolution. Choice of mobile phase is second only to the choice of operating mode in determining the suitability of the system to produce the desired separations.

HPLC had limited use for routine trace multiresidue analysis in the absence of sensitive element-selective detectors. Early development work relied primarily on refractive index (RI) or fixed wavelength UV absorbance detectors. Neither detector demonstrated sufficient sensitivity or selectivity for use in trace residue analysis. In the mid-1970s, the fluorescence detector was shown to provide the needed sensitivity and specificity for pesticides that are naturally fluorescent or can be chemically labeled with a fluorophore. This resulted in the first practical application of HPLC to multiresidue pesticide determination (see method for N-methylcarbamates, Section 401).

More recently, scientists have investigated photoconductivity and electrochemical detectors and certain applications of the newer multiwavelength UV detectors. This research indicates that these detectors can also fulfill the sensitivity and selectivity requirements for determination of certain pesticides at residue levels.

601 B: MODES OF OPERATION

Figure 601-b
HPLC Modes of Operation



Separations by HPLC are achieved using the five basic operational modes (Figure 601-b). The mode chosen for a particular application will depend on the properties of the analyte(s) to be separated and determined. For residue determination, as for HPLC analyses in general, BPC is the most widely used.

There are two variations within the five operational modes of HPLC operation; these distinctions are based on the relative polarities of stationary and mobile phases:

1) **normal phase (NP)** chromatography: stationary phase is more polar than the mobile phase; the least polar analytes elute first; analyte retention is increased by decreasing mobile phase polarity.

2) **reverse phase (RP)** chromatography: stationary phase is less polar than the mobile phase; the most polar analytes elute first; analyte retention is increased by increasing mobile phase polarity.

Liquid-Solid Chromatography

LSC, also called adsorption chromatography, uses an adsorbent, usually uncoated silica gel. The basis for separation is the selective adsorption of polar compounds, presumably by hydrogen bonding, to active silanol (Si●H) groups by orientation and on the surface of the silica gel. Analytes that are more polar will be attracted more strongly to the active silica gel sites. The solvent strength of the mobile phase determines the rate at which adsorbed analytes are desorbed and eluted.

LSC is useful for separation of isomers and classes of compounds differing in polarity and number of functional groups. It works best with compounds that have relatively low or intermediate polarity. Highly polar compounds may irreversibly adsorb on the column. Poor LSC separations are usually obtained for chemicals containing only nonpolar aliphatic substituents.

Liquid-Liquid Chromatography

LLC, also called partition chromatography, involves a solid support, usually silica gel or kieselguhr, mechanically coated with a film of an organic liquid. A typical system for NP LLC is a column coated with ß,ß'-oxy dipropionitrile and a nonpolar solvent like hexane as the mobile phase. Analytes are separated by partitioning between the two phases as in solvent extraction. Components more soluble in the stationary liquid move more slowly and elute later. LLC has now been replaced by BPC for most applications.

Bonded Phase Chromatography

BPC uses a stationary phase that is chemically bonded to silica gel by reaction of silanol groups with a substituted organosilane. Unlike LLC, the stationary phase is not altered by mobile phase development or temperature change. All solvents can be used, presaturation of the mobile phase with the stationary phase is not required, and gradient elution can be used to improve resolution.

Specialized applications of BPC have been developed for ionized compounds, which are highly water soluble and generally not well retained on RP BPC columns. Retention and separation can be increased by adding an appropriate pH buffer to suppress ionization (ion suppression chromatography) or by forming a lipophilic ion pair (ion pair chromatography) between the analyte and a counter ion of opposite charge. The resultant nonionic species are separated by the same column techniques used for naturally nonionic organic molecules.

Ion suppression is the preferred method for separation of weak acids and bases, for which the pH of the mobile phase can be adjusted to eliminate analyte ionization while remaining within the pH 2-8 stability range of bonded silica phases. The analyte is chromatographed by RP HPLC, usually on a C-18 column, using methanol or acetonitrile plus a buffer as the mobile phase. The technique is often preferred over IEC (see below) because C-18 columns have higher efficiency, equilibrate faster, and are generally easier to use reproducibly compared to ion exchange phases. Strong acids and bases are usually separated on an ion exchange column or by ion pair chromatography.

Ion pair chromatography is used to separate weak or strong acids or bases as well as other types of organic ionic compounds. The method involves use of a C-18 column and a mobile phase buffered to a pH value at which the analyte is completely ionized (acid pH for bases, basic pH for acids) and containing an appropriate ion pairing reagent of opposite charge. Trialkylammonium salts are commonly used for complex acidic analytes and alkylsulfonic acids for basic analytes. The ion pairs separate as if they are neutral polar molecules, but the exact mechanism of ion pair chromatography is unclear. Retention and selectivity are affected by the chain length and concentration of the pairing reagent, the concentration of organic solvent in the mobile phase, and its pH. Retention increases up to a point as the chain length of the pairing reagent or its concentration increases, then decreases or levels off [1].

Compounds not ionized at the operative pH will not pair with the reagent, but they may still be strongly retained by a C-18 column depending on their alkyl structure. In this case, however, retention will not increase with the addition of an ion pairing reagent, and some decrease in retention may occur, probably due to reagent competition for the stationary phase [1].

Ion Exchange Chromatography

IEC is used to separate ionic compounds. Microparticulate insoluble organic polymer resin or silica gel is used as the support. Negatively charged sulfonic acid groups chemically bound to the support produce strong acid cation exchange (SCX) phases. Positively charged quaternary ammonium ions bound to the support produce strong base anion exchange (SAX) phases. The most widely used resin support is cross-linked copolymer prepared from styrene and divinylbenzene. Mobile phases are aqueous buffers.

Separations in IEC result from competition between the analytes and mobile phase ions for sites of opposite charge on the stationary phase. Important factors controlling retention and selectivity include the size and charge of the analyte ions, the type and concentration of other ions in the buffer system, pH, temperature, and the presence of organic solvents.

Ion chromatography, a subcategory of IEC, has been used primarily for separations of inorganic cations or anions. Because a conductivity detector is usually employed, some means is required to reduce the ionic concentration and, hence, the background conductance of the mobile phase. A second ion exchange suppressor column to convert mobile phase ions to a nonconducting compound may be used. Alternatively, a stationary phase with very low exchange capacity may be used with a dilute, low conductance mobile phase containing ions that interact strongly with the column.

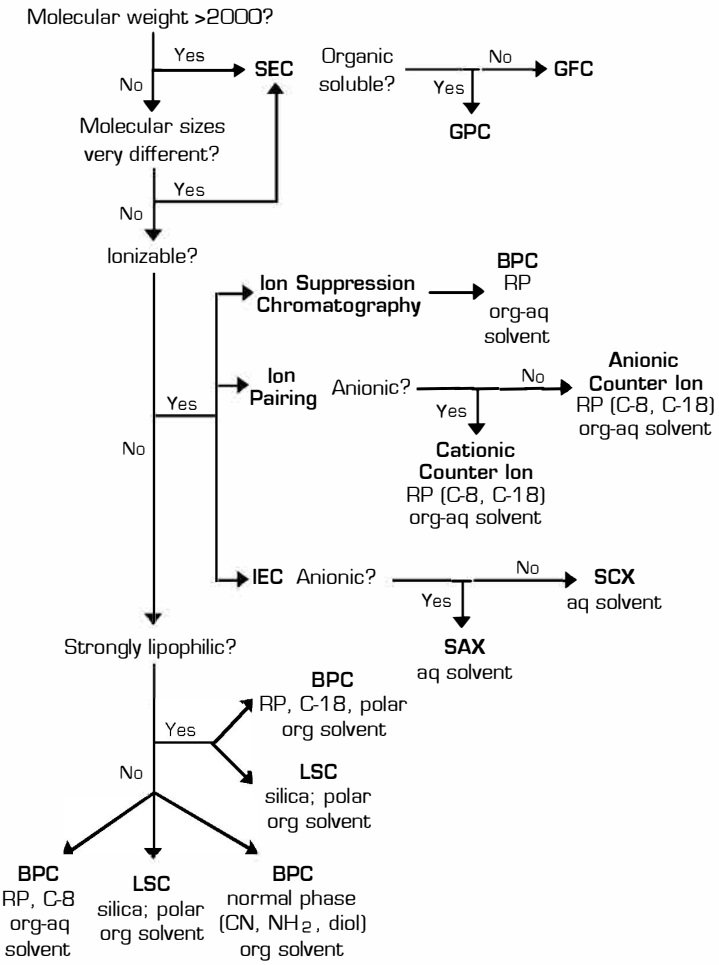
Size Exclusion Chromatography

SEC separates molecules based on differences in their size and shape in solution. SEC cannot separate isomers. SEC is carried out on silica gel or polymer packings having open structures with solvent-filled pores of limited size range. Small analyte molecules can enter the pores and spend a longer amount of time passing through the column than large molecules, which are excluded from the pores. Ideally, there should be no interaction between the analytes and the surface of the stationary phase.

Two important subdivisions of SEC are gel permeation chromatography (GPC) and gel filtration chromatography (GFC). GPC uses organic solvents for organic polymers and other analytes in organic solvents. GFC uses aqueous systems to separate and characterize biopolymers such as proteins and nucleic acids.

The chemist developing an HPLC method must first consider the properties of the analytes of interest and choose an HPLC separation method that best takes advantage of those properties. Many of the references in the bibliography (Section 608) offer guidance to making these choices. A general, simplified guide for selecting an HPLC mode according to the properties of the analyte(s) is illustrated in Figure 601-c; the guide is based on the principles of Snyder and Kirkland [2].

Figure 601-c
Guide to Selection of HPLC Mode
(based on analyte characteristics)



This scheme categorizes analytes as either ionic/ionizable (and therefore water soluble) or nonionic/nonionizable (not water soluble). Based on these distinctions, and on the polarity of the analytes, the diagram provides general rules for choosing an HPLC mode of operation likely to separate the analytes.

601 C: INSTRUMENTATION AND APPARATUS

Basic Components

The following basic components are typically included in an HPLC system (Figure 601-d): solvent reservoir(s); optional gradient-forming device; one or more precision solvent delivery pumps; injector; analytical column and optional precolumn and guard column; column oven; detector; recorder, integrator, or computerized digital signal processing device; and associated plumbing and wiring.

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