CHROMATOGRAPHY

1. Introduction

Chromatography is a technique used in many areas of science and engineering: petroleum chemistry, environmental studies, foods and flavorings, pharmaceuticals, forensics, and analysis of art objects (see BIOPOLYMERS, ANALYTICAL TECHNIQUES; FINE ART EXAMINATION AND CONSERVATION; FORENSIC CHEMISTRY), for separating and quantifying the constituents of a mixture. Since most chemical processes result in mixtures, separation techniques are essential for a successful characterization of chemical reactions. Most chemical laboratories employ one or more chromatographs for routine chemical analysis (1), and many processes involve the preparative use of chromatography for obtaining pure materials.

The first scientific reports demonstrating chromatographic phenomena appeared in the 1890s. However, the era of analytical chromatography began in 1903 when a paper described the separation and identification of the components of a mixture of structurally similar yellow and green chloroplast pigments in leaf extracts in carbon disulfide passed through a column packed with chalk (2). The technique was seen as potentially valuable for identifying compounds other than by color. In 1906 the term chromatography was coined for these processes, from the combination of two Greek roots "chroma", meaning color, and "graphe", meaning writing (3). Since that time, a wide variety of chromatographic techniques have been developed to provide separation of many mixtures, the components of which differ only subtly from each other, as in the separation of isomers.

Chromatography relies on differential interactions of the components of a mixture with the phases of a chromatographic system to produce separation, e.g. adsorption on the stationary phase. Thus, in addition to providing separation, the study of chromatographic parameters provides a means to determine fundamental quantities describing the interactions between the phases and

the components, such as stability constants, vapor pressures, and other thermodynamic data.

The importance of chromatographic processes to science can be gauged in many ways. For example, the 1952 Nobel Prize in chemistry was awarded to A. J. P. Martin and R. L. M. Synge for the development of liquid–liquid chromatography, (4) which led not only to an understanding of the fact that both liquid phases need not move simultaneously to effect separation, but also to development of gas–liquid chromatographic techniques (glc) techniques. A second measure of the importance of chromatography is the number of chromatographic instruments in chemical and biological laboratories (1). By whatever measure, the impact of chromatography on chemistry practice has been immense.

A primary use of chromatography is the analysis of mixtures by passage through a column in which the differential interactions cause the components to pass through at different rates. The measurement of the rate of each material is a means of identification of the material, which is analytical chromatography.

Analysis in the laboratory is only one use of chromatography. On-line chromatographic devices are used as analyzers in many chemical processes in industrial applications. Preparative chromatography, in which the goal is recovery of large amounts of materials after purification by separation, is a major tool for process separations. All forms of chromatography are used for preparative purposes, but liquid chromatography has been of highest value, especially in biological and pharmaceutical applications. In preparative chromatography, the column capacity (the amount of material passed through it) is usually stretched to the limit to allow the greatest quantity of sample to be added to the mobile phase. Since chromatography requires a detection of the material, the detector for preparative chromatography is normally nondestructive.

2. Principles

The principle of chromatographic separation is straightforward. A mixture is allowed to come into contact with two phases, a stationary phase and a mobile phase. The stationary phase is contained in a column or sheet through which the mobile phase moves in a controlled manner, carrying with it any material that may prefer to mix with it. Because of differences in the interactions of the mixture's constituents with stationary and mobile phases (the relative affinity of the constituents), the constituents are swept along with the mobile phase at different rates, so that they arrive at the end of the column at different times. This selective interaction is known as partitioning, and the different components are retained on the column for different times. To determine the retention time of substances on the column, a detector measures either the time required to travel to the end of the column or, as for thin-layer chromatography (tlc), the distance traveled in a fixed time. The detector may be as simple as the human nose or the human eye or as complex as a microsensor. A plot of detector response versus time of travel for a fixed distance is called a chromatogram.

In adsorption chromatography the constituents in the dissolved sample compete with the mobile phase for the active sites on the stationary phase. Retention is determined by how effectively the constituents interact with the

active sites. To remove constituents adsorbed on the stationary phase in adsorption chromatrography, the mobile phase's affinity for the stationary phase is increased by changes in its composition, which affects the retention of the analyte.

In preparative chromatography a device may be attached to the end of a column to collect the separated components of a mixture. One must be able to detect the material, so this collection device is in series with the detection device.

The nature of the stationary and mobile phases in a chromatographic experiment determines the efficacy of component separation in a particular mixture. A wide variety of stationary and mobile phases is used. A classification based on the nature of the phases is shown in Figure 1. The stationary phase may be a solid or a liquid supported on a solid. The mobile phase may be a gas, a liquid, or a material such as a supercritical fluid (see SUPERCRITICAL FLUIDS). One names a specific chromatographic technique by naming the mobile and the stationary phase, in that order. Thus, gas–liquid chromatography (glc) uses a gaseous mobile phase in contact with a film of liquid stationary phase.

2.1. Development of the Chromatogram. The term "development" describes the process of performing achromatographic separation. Because the processes that determine retention depend on the nature of the stationary and mobile phases, there are several ways in which separation may be made to occur, and several different ways for development of chromatograms.

Chromatographic techniques may be characterized as frontal, displacement, or elution chromatography. Elution is, by far, the most common, and most people think of elution chromatography upon hearing the word "chromatography".

Frontal chromatography (or frontal analysis) is a technique in which the sample is introduced onto a column continuously. In essence, the sample collected at the end of the column is free of materials that adsorb/absorb on the stationary phase. Once the bed, i.e. the stationary phase, is saturated and no longer removes the adsorbing component, the effluent at the column's end contains

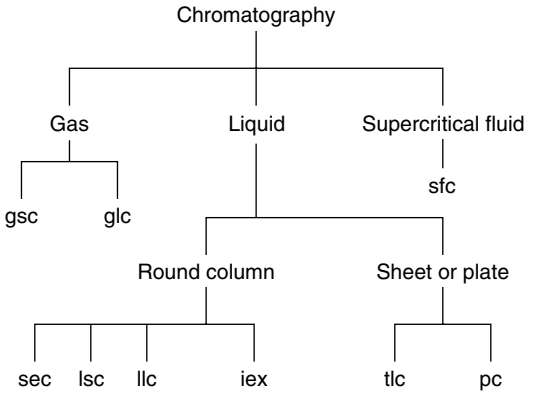

Fig. 1. Classification of chromatographic systems. gsc, gas–solid chromatography; glc, gas–liquid chromatography; sec, size-exclusion chromatography; lsc, liquid–solid chromatography; llc, liquid–liquid chromatography; iec, ion-exchange chromatography; tlc, thin-layer chromatography; pc, paper chromatography; sfc supercritical-fluid chromatography.

these materials. By using an appropriate detector, the condition at which this transition occurs can be determined, as determined by the capacity of the column. Figure 2**a** shows an example of an integral chromatogram from a frontal analysis, in which three adsorbing components gradually saturate the column.

In displacement chromatography a small sample on the column is displaced by a much more strongly held mobile phase. The sample is gradually pushed through the column as the mobile phase advances. As this happens, the components are dispersed into bands that can either be excised to obtain the pure material or displaced from the column. Such techniques are useful for the generation of quantities of pure material. Figure 2**b** shows an integral chromatogram obtained by displacement analysis.

Both frontal and displacement chromatographies suffer a significant disadvantage in that once a column has been used, part of the sample remains on the column, which means it is contaminated for further use. The column must be regenerated before reuse. In elution chromatography all of the sample material is usually removed from the column during the chromatographic process, allowing reuse of the column without regeneration. Most analytical applications of chromatography employ elution methods, in which a small sample is put onto the column, at the column head as a plug or a band. The sample is applied, sometimes by injection, while the mobile phase is moving through the column. Because of the difference in affinities of the sample's components for the stationary phase and the mobile phase, constituents travel through the column at different rates and elute at different times. Figure 2**c** shows a typical differential elution chromatogram.

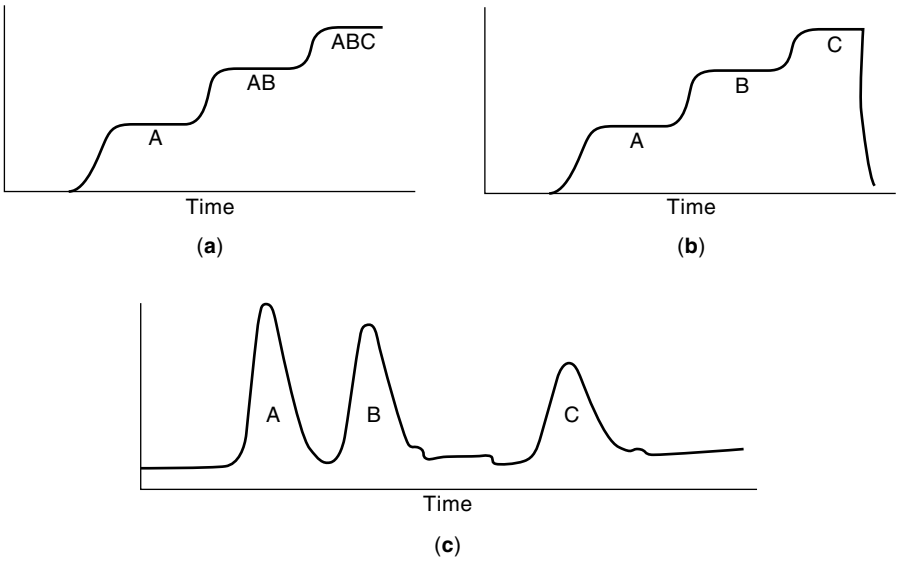

Fig. 2. Chromatograms of a mixture containing three components A, B, and C, where A is less sorbed than B, and B is less sorbed than C; (**a**) frontal analysis; (**b**) displacement analysis; and (**c**) differential elution chromatogram.

3. Gas Chromatography

The most frequently used chromatographic technique is gc, for which instrumentation was first offered commercially in the mid-1950s. Gas chromatographs were the most frequently mentioned analytical instrumentation planned for purchase in laboratory surveys in 1990, and growth in sales has been ~6%/year (1,5).

While solids and liquids are both used as stationary phases in gc, the most commonly used method is glc. Separation in a glc arises from differential partitioning of the sample's components between the stationary liquid phase bound on a porous solid, and the gas phase. In the other variant, gsc, preferential adsorption on the solid or, sometimes, exclusion of materials by size are the means of differentiating between components.

3.1. Packed Columns. The chromatographic column is often described as the heart of the chromatographic system, because it is the single part of the system that must be present to effect a separation. Columns come in a wide variety of sizes and shapes. A schematic drawing of a packed column is shown in Figure 3. They are frequently tubes made of various materials. In these tube is the stationary phase, either coated or bonded chemically to the packing. In general, the larger the diameter of the column, the poorer the separation of the components. Large (> 2-mm internal diameter) columns are often used in preparative chromatography, whereas medium (diameter between 1 and 2 mm) columns are used for analytical chromatography. In glc a packing is coated with a liquid phase, either by chemical bonding or physical coating on porous particles presumed to be inert. In gsc, the packing is usually the stationary phase. The packing is the dominant part of the column, as its interaction with the material determines the retention that underlies the separation process. We discuss this aspect in more detail below.

3.2. Capillary Chromatography. Capillary, or wall-coated open tubular (WCOT), columns are fine tubes having internal diameters in the range of 0.25–0.53 mm. Chromatography with these columns (usually with no packing) gives exceedingly high resolution gc. As the name implies, these columns' walls are often coated with some substance that is, in effect, the stationary phase. Capillary columns are so efficient, they require very little sample, so the system for introduction of a sample onto this sort of column requires the use of a sample splitter, a device to allow a representative aliquot of the sample

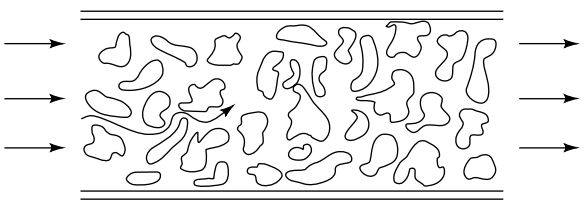

Fig. 3. A packed chromatographic column showing the thin column walls and the irregularly shaped solid support coated with a liquid phase. The arrows indicate the movement of the mobile phase.

onto the column, because the sample capacity of a capillary column is not very great. Wide-bore open-tubular columns (those with column diameters of 0.53 mm) are particularly useful for coupled tandem techniques in which a greater quantity of material is needed for detection: gas chromatography–mass spectrometry (gc–ms), gas chromatography–Fourier transform infrared spectroscopy (gc–ftir), and gas chromatography–nuclear magnetic resonance spectroscopy (gc–nmr), (see ANALYTICAL METHODS, HYPHENATED INSTRUMENTS).

3.3. The Stationary Phase. The stationary phase in an open-tubular column is generally coated or chemically bonded to the wall of the capillary column. In a packed column, it is attached to the packing (or support). For capillary columns, the greater the thickness of the liquid-phase (film) coating, the greater the retarding potential of the column for components attracted to the stationary phase. Chemically bonded stationary phases are usually more resilient than nonbonded phases, tending not to wash out as large amounts of solvent pass through the column, and having much better thermal stability than do nonbonded (coated) phases. In some cases, increased stability of the stationary phase can be achieved by acting on it chemically. The phase can be cross-linked to give more mechanical, chemical, and thermal stability. Cross-linked phases are very stable and can withstand solvent washing to clean the interior of the chromatographic column. Several hundred types of liquid stationary phases are commercially available. These have been used individually or in combination with other liquid phases, inorganic salts, acids, or bases. The selection of stationary phases for a particular application is beyond the scope of this article, however, it is one of the most important chromatographic tasks. Stationary phase selection is discussed at length in books, journal articles, and catalogs from vendors. See *General References* for examples.

3.4. The Support Material. The support is the inert frame onto which the liquid phase is applied. For capillary chromatography, this is the capillary material such as fused silica. The most common support materials for packed-column gc are the diatomaceous earths, the remains of diatoms, single-cell algae. The porous siliceous material has pores $\sim$1 μm in diameter (see DIATOMITE). These materials are typically treated with sodium carbonate to $\sim$900°C. Following this treatment, they are sieved to obtain material of reasonably uniform dimensions. Supports for packed-column chromatography are classified by particle size. Most chromatographic packings are 80/100 mesh (177–149 μm). Supports are washed using acid (HCl) and silanized, i.e. treated with dimethyldichlorosilane, DMCS, to reduce the polarity. Silanizing replaces adjacent SiOH groups with nonpolar CH_3 caps. In addition to diatomaceous earths, packed-column supports of carbon, halocarbons, Teflon®, and glass beads are in use by various chromatographers.

3.5. Separation by Molecular Size. The most common solid-phase packing in gsc is a molecular sieve. Molecular sieves are zeolites or carbon sieves that have a regular pore structure and are used almost exclusively for the separation of small molecules such as permanent gases like oxygen, nitrogen, carbon monoxide, argon, and nitric oxide, or low carbon-number hydrocarbons.

Porous organic polymers have been used as gsc packings for separating low molecular weight mixtures containing halogenated or sulfur-containing compounds, water, alcohols, glycols, free fatty acids, ketones, esters (see ESTERS,

ORGANIC), and aldehydes. Such porous polymers usually have a maximum operating temperature lower than many common liquid phases.

Silica, alumina, and other metal oxides and salts have been used as the stationary phase in gsc systems. The applicability of these materials is limited by the difficulty of producing a consistent, resilient, reproducible material.

3.6. Column Tubing. A packed column is contained in tubing, the composition of which may have a dramatic effect on the separation process, because the sample components may also interact with the walls of the tube. For sensitive compounds such as certain pharmaceuticals, steroids, and pesticides, the standard practice is to use columns packed in glass tubes. The surface of glass is more nearly inert than are the surfaces of metal tubes. Glass columns also have the advantage of being transparent, giving a means to examine the column for degradation, contamination, packing efficiency, and column voids. Glass, however, is more fragile than metal.

Tubes made of metal such as stainless steel, nickel, copper, or aluminum are much more resistant than glass to damage in handling. Stainless steel is often preferred because it is less active than other metals. If corrosive samples are used, however, it is sometimes necessary to contain the column in tubing made of a material such as Teflon®. Columns made of Teflon® generally are difficult to pack. Additionally, connections to the tubing are difficult to make.

For capillary columns, fused silica is the material of choice for the column container. It has virtually no impurities (<1 ppm metal oxides) and tends to be quite inert. In addition, fused silica is relatively easily processed and manufacture of columns from this material is reproducible. Fused-silica columns are externally coated using a protective polyimide layer to improve strength and durability, and to provide a measure of protection against reaction of the silica with water in the environment. The fused-silica column is an inherently straight wire of material, i.e. its resting state is straight, not coiled. To use the material in a chromatographic oven, it must be wound onto a frame that secures it in the coiled configuration. The process of creating a fused-silica column is complex and requires sophisticated, expensive equipment, a high temperature (2000°C) furnace, and a laser-based system for determining the trueness of diameter of the ultimate product.

In addition to fused-silica capillary columns, there are several designs for glass capillary columns, although few are widely used. One system is the so-called porous-layer-open-tubular (PLOT) column, made from glass pulled to a capillary with an internal layer of solid packing. Subsequently this material is removed by etching to produce a column with many pores at the surface of the capillary. Support-coated-open-tubular (SCOT) columns are glass capillary columns having a coating on the column wall. Micropacked columns are glass capillaries packed using small-mesh particles similar to those in liquid–chromatographic columns. There are several different types of capillary column designs.

3.7. The Mobile Phase. The purpose of the mobile phase, also called the carrier gas, is to transport the sample through the chromatographic column. The selection of carrier gas is often dictated by the type of detector attached to the gas chromatographic system. To achieve the best performance, gases such as nitrogen, carbon dioxide, or argon are used as carriers. If these gases do not permit sufficiently high gas velocities, then a lower molecular weight gas such as helium

or hydrogen is used. The purity of the carrier gas is an important consideration because gas passes through the column and impurities could interfere with chromatographic separation or could contaminate the column. Gases used are generally of 99.995% purity or better. Two particularly troubling contaminants are water and air, which can affect the stability of the liquid phase in a packed column. The best compromise for column performance and safety is helium for capillary columns and either helium or nitrogen for packed columns.

3.8. Detectors. The function of the gc detector is to sense the presence of a constituent of the sample at the outlet of the column. Selectivity is the property that allows the detector to discriminate between constituents. A selective detector responds especially well to compounds of one type, but not to other chemical species. The response is the signal strength generated by a given quantity of material. Sensitivity is a measure of the ability of the detector to register the presence of the component of interest, usually given as the quantity of material that can be detected having a response at twice the noise level of the detector.

By far the most used nondestructive detector is the thermal conductivity detector (TCD). This detector is used for preparative chromatography. Detectors like the TCD are called bulk-property detectors, in that the response is to a property of the overall material flowing through the detector, in this case the thermal conductivity of the stream, which includes the carrier gas (mobile phase) and any material that may be traveling with it. The principle behind a TCD is that a hot body loses heat at a rate that depends on the composition of the material. Most materials have lower thermal conductivities than helium, the typical carrier gas, and most organic materials have similar thermal conductivities, thus the TCD is often used for quantitative analysis of organics with gc. Of course, the thermal conductivities of organic compounds are not exactly the same, so very accurate results require an evaluation of the response factor for each material, essentially an evaluation of the thermal conductivity of each material.

The detector most extensively used in capillary chromatography is the flame-ionization detector (FID). The principle behind its operation is the detection of a current from ions formed when organic materials are burned in a small hydrogen–oxygen flame at the end of the column. Typically a voltage is applied across this region and the small current carried by the ions is detected using a sensitive electrometer. One of the advantages of the FID is that it is, in general, more sensitive than the TCD. In addition, it does not respond to materials such as water, carbon dioxide, carbon monoxide, and most simple sulfur-containing gases. The principal disadvantage of the FID is that it destroys the separated material in the process of detection.

A third detector type is the electron-capture detector (ECD), which is very selective for the detection of highly electronegative compounds in the effluent such as chlorinated hydrocarbons, many pesticides, and polychlorinated biphenyls (see CHLOROHYDROCARBONS). Its principle of action is the interaction of such compounds with electrons emitted from a radioactive source. A detector sensitive to these beta emissions positioned across the stream from a source senses a drop in emissions when the stream contains compounds that capture the emitted electrons. Thus the response to the passage of materials is a loss of signal. The sources generally used in these detectors are nickel-63 or tritium. One disadvantage is the radioactive source, which requires special handling. In addition, the

linear response range is not very great and the detector is subject to high background noise, unless care is taken to eliminate column, carrier-gas, or sample contaminants.

The flame-photometric detector (FPD) is selective for organic compounds containing phosphorus and sulfur. In this detector, chemiluminescent species formed in a flame from these materials are detected through a filter by a photomultiplier. The photometric response is linear in concentration for phosphorus, but it is second order in concentration for sulfur.

The alkali flame-ionization detector (AFID), sometimes called a thermionic (TID) or nitrogen–phosphorus detector (NPD), has as its basis the fact that a phosphorus- or nitrogen-containing organic material, when placed in contact with an alkali salt above a flame, forms ions in excess of thermal ionic formation. The ions are detected as a current. Such a detector at the end of a column reports on the elution of these compounds. The mechanism of the process is not clearly understood, but the enhanced current makes this type of detector popular for trace analysis of materials such as phosphorus-containing pesticides.

The ms is a common adjunct to a chromatographic system (see MASS SPECTROMETRY). The combination of a gas chromatograph for component separation and a mass spectrometer (gc–ms) for detection and identification of the separated components is a powerful tool, particularly when the data are collected using an on-line data-handling system. Qualitative information inherent in the separation can be coupled with the identification of structure and relatively straightforward quantification of a mixture's components.

Infrared (ir) spectrometers are used as detectors for gc systems, particularly because the ftir spectrometer allows spectra of the eluting stream to be gathered quickly. The data are valuable alone and as an adjunct to experiments with gc–ms. Gc–ir is a definitive tool for identification of isomers (see INFRARED AND RAMAN SPECTROSCOPY).

Plasma atomic emission spectrometry is also employed as a detection method for gc (see PLASMA TECHNOLOGY). By monitoring selected emission lines, selective detection of materials based on elemental composition can be achieved (see SPECTROSCOPY).

3.9. Theory. Most theoretical models of gas chromatographic processes are based on analogy to processes such as distillation or countercurrent extraction experiments (6). The separation process is viewed as a type of successive partitioning of the components of a mixture between the stationary and mobile phases similar to the partitioning that occurs in distillation columns. In those experiments an important parameter is the number of theoretical plates of which the column may be considered to be composed; the greater the number of theoretical plates, the greater the efficiency of the column for achieving separations of similar components. In gc, the equivalent measure of efficacy is the height equivalent to theoretical plates (HETP), which measures the ultimate ability of the column to separate like components. This quantity depends on many instrumental parameters such as wall or particle diameter, type of carrier gas, flow rate, liquid-phase thickness, etc. The theoretical expression relating HETP to gas velocity is the van Deemter equation:

$$\mathrm{HETP} = A + B/\mu + C\mu$$

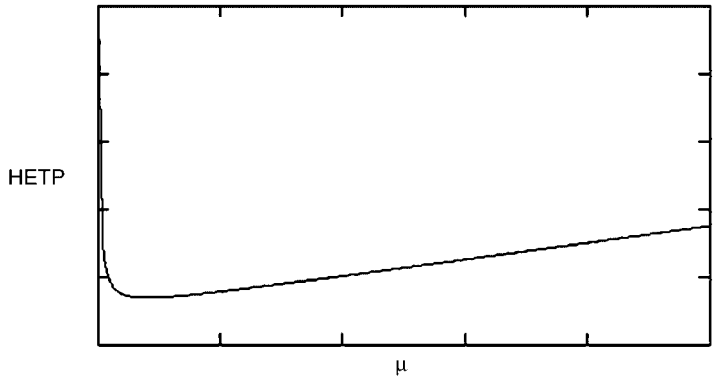

Fig. 4. An example of van Deemter's relation between gas velocity, μ, and HETP number.

where A represents the effects of eddy diffusion, the term containing B represents the effects of molecular diffusion, μ is the linear gas velocity, and C represents the resistance to mass transfer. A plot of HETP versus C gives a hyperbola with a minimum HETP, as shown in Figure 4. This minimum is the optimal gas velocity, μ, where the column operates most efficiently.

3.10. Inlet Systems. The inlet (or injector) is the means by which the sample is introduced onto the gc column. Sample introduction requires one to create a representative aliquot of the sample at the beginning of the column without degradation or without discrimination among the components of the sample. Most inlets operate on the principle that a sample can be vaporized quickly, assuming it is not already a gaseous material, after being injected from a microliter syringe into a small, heated volume, usually ~50°C hotter than the maximum temperature of the column during the experiment. This vaporized material is quickly swept as a narrow band onto the column by the carrier gas. Once on the column, the components interact with the stationary phase and begin to travel along with the carrier gas at differing rates, depending on the strengths of interaction with the stationary phase.

Sample introduction is critical to the proper operation of a gas chromatograph. To obtain reliable data, a representative sample must reach the column in a narrow band so all components begin the chromatographic process simultaneously. Injection with a syringe is the most widely used technique. However, other means such as injection valves, pyrolyzers, headspace samplers, thermal desorbers, and purge-and-trap samplers are found in various applications.

Inlets for syringe sampling are divided into two categories, depending on the column. For packed columns, all injected material is carried by the mobile phase onto the column, and the inlet is usually an open tube. Sometimes, albeit rarely, the inlet itself may be packed, eg, to assure that the first centimeters of the column do not become contaminated with degradation products or nonvolatile materials that may affect the efficacy of the column.

The second category is inlets for capillary columns. Because of the much smaller capacity of the column, injection requires great care and special tech-

niques. There are four different inlet designs for this purpose: direct, split/splitless, programmed-temperature vaporization, and cool on-column injectors. Direct inlets are generally used with capillary columns of larger diameter and work much as direct injectors for packed columns.

Split/splitless injectors operate in two modes. In the split mode, most of the sample introduced into the inlet goes out a vent that has less resistance to flow than the column. Splitters usually exhaust 90–99% of the volume of the material injected, so that only a small percentage of the injected material enters the column. A primary problem with this injection mode is that the material finding its way onto the column is not always representative of the sample. Discrimination resulting from differential boiling points of the components makes it probable that certain components of the mixture are vented, rather than introduced onto the column. In the splitless mode, the vent is turned off and everything injected goes onto the column. After a short period, the vent is opened and any residual solvent is vented. The splitless mode is found particularly in trace analytical schemes (see TRACE AND RESIDUE ANALYSIS). Splitless sample injection is an art that requires practice to ensure reproducible introduction of sample onto the column.

Programmed-temperature vaporizers are flexible sample-introduction devices offering a variety of modes of operation such as split/splitless, cool-sample introduction, and solvent elimination. Usually the sample is introduced onto a cool injection-port liner so that no sample discrimination occurs. After injection, the temperature is increased to vaporize the sample, and the sample finds its way onto the column.

Cool on-column injection is used for trace analysis. The sample is introduced without vaporization by inserting the needle of the syringe at a place where the column has been previously stripped of liquid phase. The injection temperature must be at or below the boiling point of the solvent carrying the sample. Injection must be rapid and of a very small volume. Cool on-column injection is the most accurate and reproducible injection technique for capillary chromatography, but it is the most difficult to automate.

3.11. Temperature Considerations. The inlet, detector, and the oven compartment with the column are usually controlled at different temperatures, because each part serves a different function best performed in a specified temperature range. In practice, the maximum oven temperature in the course of an analysis should only be high enough to achieve the desired result in a minimum time. This temperature should be low enough to minimize the probability of column liquid-phase degradation. The injection port's temperature is usually slightly higher than the maximum oven temperature, but low enough to minimize thermal degradation or thermal rearrangement of sample components. Ideally, the thermal energy in the injection port will cause instantaneous vaporization without causing a loss of separation. The detector temperature is usually 10–30°C higher than the injector, but low enough to avoid thermal degradation of the column's liquid phase in that part of the column near the detector.

For materials with a wide boiling range, temperature programming is often used. The initial temperature, if possible, should be near the boiling point of the most volatile component; the final temperature should be near or, if possible,

slightly higher than the least volatile component. The heating rate from low to high temperature is usually empirically determined to obtain the most efficient separation in the shortest possible analysis time.

4. Liquid Chromatography

Liquid chromatography (lc) refers to any chromatographic process in which the mobile phase is a liquid. Traditional column chromatography, tlc, pc, ce, cec, and high-performance liquid chromatography (hplc) are members of this class. Modern lc techniques originated in the late 1960s and early 1970s. Developments in hplc were driven by improvements in instrumentation, column packings, and theoretical understanding of the various separation processes involved. For example, use of pressurized mobile phases in place of gravity-driven ones in chromatography greatly shortened the time for separation.

Liquid chromatography is complementary to gc because samples that cannot be easily handled in the gas phase, such as nonvolatile compounds or thermally unstable ones, e.g. many natural products, pharmaceuticals, and biomacromolecules, are separable by partitioning between a liquid mobile phase and a stationary phase, often at ambient temperature. Many separations once done exclusively with gc are nowadays conveniently done with lc.

One advantage of lc is that the composition of the mobile phase, and perhaps of the stationary phase, can be varied during the experiment to provide a means of enhancing separation. There are many more combinations of mobile and stationary phases to effect a separation in lc than one would have in a similar gas chromatographic experiment, where the gaseous mobile phase often serves as little more than a convenient carrier for the components of the sample. In classical column chromatography the usual system consists of a polar adsorbent, or stationary phase, and a nonpolar mobile phase such as a hydrocarbon. In many instances, the polarities of the stationary and mobile phases are reversed for the separation, in which case the technique is known as reversed-phase liquid chromatography.

Paper chromatography (pc) originated in the 1940s and tlc came into use in the 1950s. These techniques are similar in the manner of development of the chromatogram. A chamber is used to isolate the column, which is a piece of filter paper in pc and a glass plate coated with an adsorbent such as silica gel in tlc, from the laboratory environment. The chromatogram is developed as a mobile phase migrates through the column, carrying with it materials from the sample deposited above the initial solvent level. Because of differences in solubility in the mobile phase, the components move at different rates up the column. After this process has proceeded sufficiently, the column is removed from the solvent tank and the mobile phase evaporated. The separated components are visualized elsewhere, e.g. under an ultraviolet (uv) lamp in which various fluorescent bands indicate how fluorescent materials are separated by the movement of the solvent (mobile phase). The distance from the origin to the migrated spot at the end of development is used to calculate a retardation factor (r_f), the ratio of the distance a component travels to the distance the solvent front has traveled. Retardation

factors are always ≤ 1 and are used to characterize the partitioning of a component for a particular solvent and stationary phase. An advantage of tlc is that a fresh plate is used for each analysis, avoiding "carry-over", contamination from previous experiments. Another important advantage is that many visualizing techniques are available that are not available using lc detectors. In addition, the spots or, more frequently, the band in prep tlc, can be physically cut from the plate.

Paper and tlc may be further classified as one- or two-dimensional (1D or 2D), and as either analytical or preparative. In the traditional 1D pc or tlc described above, a small spot of sample is applied, usually from a micropipet, at a point near the edge from which solvent enters the paper or plate, as described above. In 2D chromatography, after the plate or paper is removed from one solvent and allowed to dry, it is placed in another tank with a different solvent entering the paper or plate at 90° to the direction of travel of the first solvent. The result is a further resolution of components that may have had similar partitioning in the first solvent.

If large amounts of material can be separated, then one may use tlc as a form of preparative chromatography. One-dimensional tlc plates have a very high capacity, e.g. up to 20 samples and standards applied, as compared to single passes in column chromatography (hplc or gc).

Capillary electrophoresis (ce, also know as "capillary zone electrophoresis", cze) is a separations process that occurs inside a small-diameter capillary tube, based on the differential mobilities of components under the influence of an electric field. Fused silica is usually used for the capillary, since silanol groups can easily form a negatively charged surface, which contributes to electroosmotic flow. The movement of the analyte is determined by the sum of the electrophoretic velocity and the electroosometic velocity.

Capillary electrochromatography (cec) is a form of lc that uses an electric field to drive the eluent through the chromatographic column. Although the mobile phase is a liquid, the column types are similar to a gc column with packed, open-tubular, and continuous bed options.

4.1. Columns. As for gc, the column is the heart of the lc system. Columns for modern lc can be packed with a variety of materials: inert particles bonded to a liquid phase (llc); a porous gel as for sec or gpc; an ion-exchange resin as for iec; or an affinity adsorbent as for affinity chromatography (ac). Columns are generally $\sim$5 cm in length. An important consideration is uniformity of the particles, which are generally $\sim$5 μm in diameter for typical lc experiments.

4.2. Packings. Most packings for lc are made of chemically modified silica gel having functional groups covalently attached to the surface of the particles. Zirconia has replaced many of these original stationary phases since zirconia is thermally and chemically stable over the entire pH range from 1–14.

Reversed-phase packings have covalently bonded octadecyl groups (a C-18 phase) or octyl groups (a C-8 phase) at the surface to provide a nonpolar environment. Sometimes the further reaction of these materials with other reagents to attach trimethylsilyl groups (endcapping) is attempted. This treatment is generally supposed to cover the regions of the surface that are not covered by the first treatment, eliminating interactions that may degrade the efficiency of the column. In addition to the C-18 and C-8 column packings, other species used

for chemically binding to the support particles include phenyl, nitro, and amino groups.

For sec, porous polymers such as polystyrene–divinylbenzene are sometimes used, as are the usual treated and untreated silicas.

4.3. The Mobile Phase. The great power of lc to separate the components of a mixture lies in the differential solubility of the components in the mobile liquid phase and the stationary phase. In isocratic lc, the composition of the mobile phase remains constant throughout the course of the experiment. However, the effective separating power of lc can often be enhanced by changing the composition of the mobile phase during the course of the experiment. This process, known as gradient lc, is analogous to programming the column temperature in gc. Frequently the switch is from a weak solvent for a given material to a strong one. The change can be made in a single step or by slowly varying the composition of the mobile phase with time during the separation process. Most processes involve two solvents or solvent mixtures, although there are some cases in which three solvents are used. Obviously, the more solvents used, the more complex the program of mixing. An important consideration in gradient lc is the selection of a detector. The detector must be compatible with all the solvents used in the separation process.

Solvent-delivery systems ensure uniform transfer of the mobile phase to the column. These devices must give reproducibly uniform flow without pulsations, to ensure reproducible retention times and peak areas for analyses. Because of the small diameter of the particles used in modern hplc, there is a high resistance to flow through the column, and high-pressure pumps are required (see PUMPS). To obtain the best signal from a detector, it is important that the detector be insensitive to pump strokes at all flows. The pump materials must not only be able to withstand such pressures, they must not be affected by the solvents in the system. For gradient elution lc, the pump should have a small mixing (hold-up) volume. This minimizes memory effects from solvent changes.

Sample introduction onto lc columns is usually accomplished with a sampling valve. A sample loop of volume between 5 and 50 μL attached to the sampling valve is filled or partly filled with sample solution. At the time of introduction of the sample, the valve is either manually or pneumatically actuated so flow of solvent through the sample loop moves sample onto the column.

4.4. Detectors. Liquid-chromatographic detectors must be compatible with the solvent system (mobile phase) and are optimized for sensitivity, stability, and speed of response. They are designed to retain the quality of the separation. No versatile, universal detector is in use for lc, as the flame-ionization or thermal-conductivity detectors are for gc. Instead, the most common detector found in lc is the uv detector, a selective detector that measures the absorption of radiation at a specified wavelength. These devices may be set at a fixed wavelength or the wavelength may be variable. The detectors are only sensitive to materials that absorb radiation in the range of the detector. The uv detectors are relatively insensitive to temperature or flow changes, but the response can be sensitive to solvent composition, which can effect sample absorption characteristics, as in gec.

The fluorescence detector, perhaps the most sensitive commonly used detectors in lc, is limited in its utility to the detection of materials that fluoresce or

have derivatives that fluoresce. These detectors find particular use in analysis of environmental and food samples, where measurements of trace quantities are required.

Electrochemical detectors sense electroreducible and electrooxidizable compounds at low concentrations. For these detectors to work efficiently, the mobile phase (solvent) must be conductive and not subject to electrochemical decomposition.

Other detectors determine bulk properties of the system of mobile phase plus sample. The most commonly used bulk-property detector is the refractive-index (RI) detector. The RI detector, the closest thing to a universal detector in lc, monitors the difference between the RI of the effluent from the column and the pure solvent. These detectors are not very good for detection of materials at low concentrations. Moreover, they are sensitive to fluctuations in temperature.

Conductivity detectors, commonly employed in ion chromatography, can be used to determine ionic materials at levels of parts per million (ppm) or parts per billion (ppb) in aqueous mobile phases. The ir detector is one that may be used in either nonselective or selective detection. Its most common use has been as a detector in sec, although it is not limited to sec. The detector is limited to use in systems in which the mobile phase is transparent at the wavelength being monitored. It is possible to obtain complete spectra, much as in some gc–ir experiments, if the flow is not very high or can be stopped momentarily.

The ms detection is ideal for qualitiatively and quantitatively determining the constituents of a sample. The lc/ms provides a mass spectrum for each chromatographic peak or can be used in a single-ion-monitoring mode to detect components of similar fragmentation structure. The lc/ms/ms provides an additional dimension to improve the qualitaive aspect of the separation by providing not only primary structural identification but also secondary ion degradation (or the mass spectrum of a mass spectrum). The major drawback to lc/ms and lc/ms/ms is the cost and maintenace of the equipment.

4.5. Affinity Chromatography. This technique, sometimes called bioselective adsoprtion, involves the use of a bioselective stationary phase placed in contact with the material to be purified, the ligate. Because of its rather selective interaction, sometimes called a lock-and-key mechanism, this method is more selective than other lc systems based on differential solubility.

4.6. Chiral Chromatography. Chiral chromatography is used for the analysis of enantiomers, and finds applications in the separations of pharmaceuticals and biochemical compounds (see BIOPOLYMERS, ANALYTICAL TECHNIQUES). There are several types of chiral stationary phases: those that use attractive interactions, metal ligands, inclusion complexes, and protein complexes. The separation of optical isomers has important ramifications, especially in biochemistry and pharmaceutical chemistry, where one form of a compound may be bioactive and the other inactive, inhibitory, or toxic.

4.7. Ion-Exchange Chromatography. In iec, the column contains a stationary phase having ionic groups such as a sulfonate or carboxylate. The charge of these groups is compensated by counterions such as sodium or potassium. The mobile phase is usually an ionic solution, e.g. sodium chloride, having ions similar to the counterions. Ionic samples are introduced into the mobile phase, and retardation in movement results from ion exchange with the

stationary phase. The more the ion interacts with the exchanger, the more strongly it is retained. For cation-exchange chromatography, positively charged ions are separated. In anion-exchange chromatography, negatively charged ions in the sample interact with and bind to cationic stationary phases (see ION EXCHANGE).

Ion chromatography (ic), a variant of ion-exchange chromatography, is a technique in which a weak ion-exchange column is used for separation. After passing through the weak ion-exchange column, the eluent passes through a subsequent column called a stripper column, in which the stream, usually made acidic or basic in the ion-exchange column, is neutralized. This stream gives no conductimetric response in this condition; however, when added ions are present, such as happens when sample is passing through the stripper column, the conductivity of the solution changes and a signal is detected. Ion chromatography is a powerful technique for examining low concentrations of anions and cations. It has the advantage over selective ion-electrode analysis that it simultaneously gives information on many ions in a single experiment (see ELECTROANALYTICAL TECHNIQUES).

Ion-pair chromatography (ipc), another variant of iec, is also sometimes called pic, soap chromatography, extraction chromatography, or chromatography with a liquid ion exchanger. In this technique the mobile phase consists of a solution of an aqueous buffer and an organic cosolvent containing an ion of charge opposite to the charge on the sample ion. The sample ion and the solvated ion form an ionic pair that is soluble in the stationary phase. Thus retention is determined by the ability to form the ion pair as well as the solubility of the complex in the stationary phase.

4.8. Size-Exclusion Chromatography. In sec or gpc, the material with which the column is packed has pores of a certain range of size. Molecules or solvent-molecule complexes too large to pass through these pores pass rapidly through the column, whereas molecules or complexes of suffiently small size are retained and are the last to exit the column. Molecules of intermediate size are partially retained and elute from the chromatographic column at intermediate times. Size-exclusion chromatography is extremely useful as a tool for characterization of polymer materials because the retention mechanism is reproducible enough to give good comparative data and can also give valuable information about the distribution of sizes of molecules in a sample.

5. Supercritical-Fluid Chromatography

Supercritical-fluid chromatography, developed in the late 1960s, was not used extensively until the early 1980s. This technique is the link between gc and lc, because its mobile phase, a supercritical fluid, has physicochemical properties intermediate between a gas and a liquid (see SUPERCRITICAL FLUIDS). The physicochemical properties of the mobile phase are strong factors determining the selectivity, sensitivity toward a component, and efficiency of separation in the chromatographic process. Supercritical fluids, e.g., can be viewed as dense gases that cannot become liquid. The density of a supercritical material increases continuously with pressure at constant temperature and its solvating power increases with pressure, because the solubility of materials in a solvent usually

increases with density and can be used as a powerful means of changing retention. Carbon dioxide is the mobile phase most often used in sfc.

This technique can be performed with either capillary or lc-like packed columns. Carbon dioxide is compatible with chromatographic hardware, is readily available, and is noncorrosive. The most important detector for sfc is the flame-ionization detector because the mobile phase does not give a significant background signal. Most early applications of sfc were in the separation of petroleum products. More recent applications of sfc include separations in fields as diverse as natural products, drugs, foods, pesticides, herbicides, surfactants, and polymers. These are a direct result of the advantages that sfc has over other forms of chromatography because of low operating temperature, selective detection, and sensitivity to molecular weight.

BIBLIOGRAPHY

"Chromotography, Affinity" in *ECT* 3rd ed., Vol. 6, pp. 35–54, by A. H. Nishikawa, Haffman–LaRoche, Inc.; "Chromotography" in *ECT* 4th ed., Vol. 6, pp. 207–228, by Mary A. Kaiser, Dupont Company and Cecil Dybowski, University of Delaware; "Chromotography" in *ECT* (online), posting date: December 4, 2000, by Mary A. Kaiser, Dupont Company and Cecil Dybowski, University of Delaware.

CITED PUBLICATIONS

1. G. Wilkinson, *Today's* Chemist **29** (Dec. 1990).
2. M. S. Tswett, *Proc. Warsaw Soc. Nat. Sci.* (1903).
3. M. S. Tswett, *Ber. Deut. Bot. Ges.* **24**, 384 (1906).
4. A. J. P. Martin and R. L. M. Synge, *Biochem. J. (London)* **35**, 1358 (1941).
5. *Res. Dev.*, 19 (Jan. 1991).
6. R. L. Grob, *Modern Practice of Gas Chromatography*, 3rd ed., John Wiley & Sons, Inc., 3rd ed., New York, 1995.

GENERAL REFERENCES

S. Ahuja, *Chiral Separations by Liquid Chromatography*, ACS Books, Washington, D.C., 1991.

H. J. Cortes, *Multidimensional Chromatography: Techniques and Applications*, Marcel Dekker, New York, 1990.

J. S. Fritz, D. T. Gjerde *Ion Chromatograph*, John Wiley & Sons, Inc., New York, 2000.

N. Grinberg, *Modern Thin-Layer Chromatography*, Marcel Dekker, New York, 1990.

R. L. Grob, *Chromatographic Analysis of the Environment*, 2nd ed., Marcel Dekker, New York, 1983.

C. Horvath and J. Nikelly, *Analytical Biotechnology: Capillary Electrophoresis and Chromatography*, ACS Books, Washington, D.C., 1990.

F. G. Kitson, C. N. McEwen, Barbara S. Larsen, *Gas Chromatography and Mass Spectrometry*, Academic Press San Diego, California, 1996.

M. S. Klee, *GC Inlets—An Introduction*, Hewlett Packard, Avondale, Pa., 1990.

H. M. McNair and J. M. Miller, *Basic Gas Chromatography*, John Wiley & Sons, Inc., New York, 1997.
S. Mori and H. G. Barth, *Size Exclusion Chromatography*, Springer-Verlag, New York, 1999.
T. Provder, *Chromatography of Polymers*, American Chemical Society, Washington, D.C., 1999.
D. Rood, *A Practical Guide to Care, Maintenace, and Troubleshooting of Gas Chromatographic Systems*, John Wiley & Sons, Inc., New York, 1999.
L. R. Snyder, J. J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, John Wiley & Sons, Inc., New York, 1997.
H. F. Walton and R. D. Rocklin, *Ion Exchange in Analytical Chemistry*, CRC Press, Boca Raton, Fla., 1990.

CECIL DYBOWSKI
University of Delaware
MARY A. KAISER
Dupont Company