Introduction to CHROMATOGRAPHY

Second Edition

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Chapter 1

INTRODUCTION AND THEORETICAL CONCEPTS

INTRODUCTION

The various methods of chromatography provide the most powerful separations techniques in the chemistry laboratory. The basic ideas are simple to grasp; the techniques vary from simplicity itself to fairly complex operations and instrumentation; and the methods are applicable to every type of substance. Although the word **chromatography*** implies color, there is no direct connection except that the first compounds separated by the technique were the green pigments of plants.

The chromatographic method, because of its broad utility, is widely used for analytical as well as preparative separations. Almost every chemical mixture from low to high molecular weight can be separated into its components by some chromatographic method. The type of separation, analytical or preparative, is not defined by the sample size, but more likely by the specific need. Normally, analytical chromatography would initially be used on all samples, and preparative chromatography would be carried out only when pure fractions of a mixture are needed.

Chromatographic separations are carried out by straightforward manipulations of certain of the general physical properties of molecules. The major properties involved are: (1) the tendency of a molecule to dissolve in a liquid **(solubility),** (2) the tendency for a molecule to attach itself to the surface of a finely divided solid **(adsorption),** and (3) the tendency for a molecule to evaporate or enter the vapor state **(volatility).** In a chromatographic system, the mixture to be separated is placed in a situation such that its components must exhibit *two* of these properties. This may involve two different properties such as adsorption and solubility, or it may involve one property in two environments such as solubility in two immiscible liquids.

Although chromatography is a *dynamic* interplay between properties, it can best be approached by first considering some *static* situations. For example, if one places a compound in a separatory funnel with two liquids

*The **bold type** will be used the first time an important term is mentioned. Other especially important concepts or ideas will be given in *italics*.

having a limited mutual solubility (such as ether and water), the compound or **solute** will tend to distribute itself or to **partition** between two liquids or **phases** depending upon its solubility properties (Figure 1.1a). This technique is, of course, the basis of simple extraction procedures. Such a partition represents a competition between solubility in two liquids. When one places a solute in a flask with a liquid and a finely divided solid (such as charcoal), the solute will distribute between the liquid wherein it exhibits a solubility property and the solid surface on which it will exhibit an adsorption property (Figure 1.1b). Finally, when one places a solute in a flask containing a small amount of a non-volatile liquid, the solute will distribute between the vapor state and solubility in the liquid, thus exhibiting solubility and volatility properties (Figure 1.1c). These systems can all be described as two phases in close contact and in equilibrium with one another and with a solute that is distributed between them.

It is highly unlikely that two compounds will exhibit *exactly* the same behavior with respect to the *two* phases. In a chromatographic system, it is possible to capitalize on such differences, even when they are very small, and to make them the basis for separations.

The basic idea in chromatography is to convert a static distribution situation as described above (and in Figure 1.1) into a flowing, dynamic equilibrium system. This is brought about by causing one phase (the **moving phase** or more correctly the **mobile phase**) to move mechanically with respect to the other phase (the **stationary phase**) while remaining in equilibrium with it. This is shown schematically in Figure 1.2a. (Although a

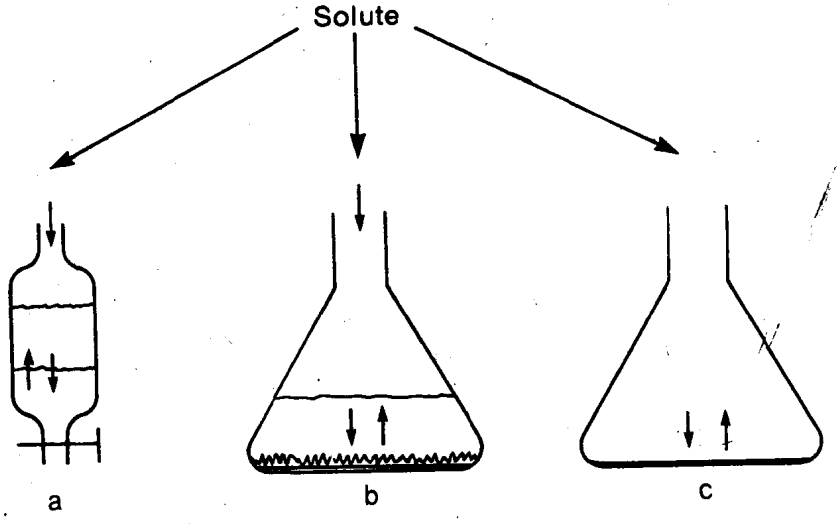


Figure 1.1 Static equilibrations of solute between two phases.

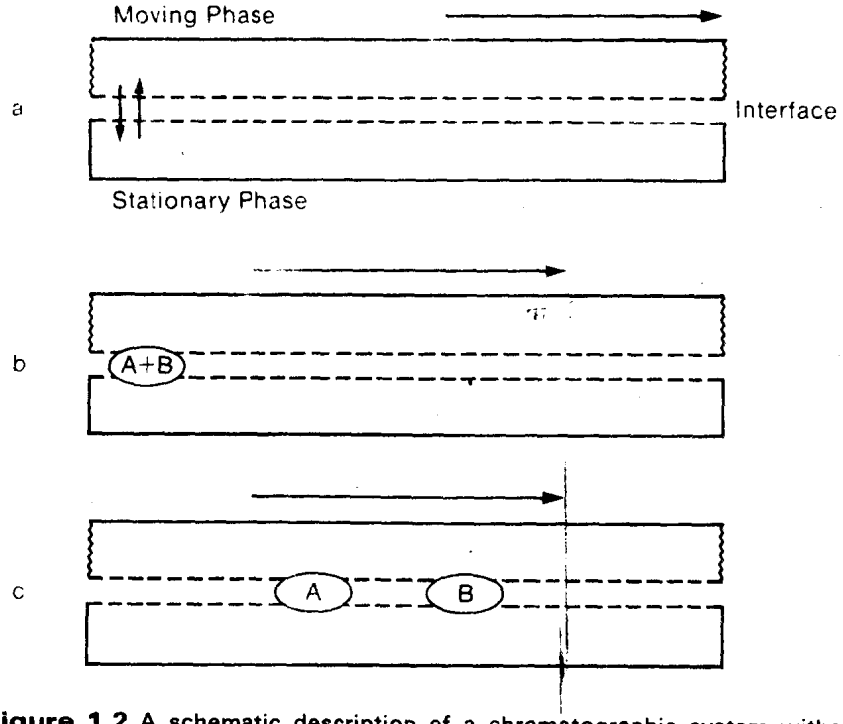


Figure 1.2 A schematic description of a chromatographic system without any solute (a), with two solutes at the beginning of a separation (b), and with two solutes after separation (c).

small space is shown between the phases in the figure for the sake of clarity, it should be understood that they are in direct contact with one another.) The mobile phase may be a liquid or a gas, and the stationary phase may be a liquid film on some type of support or a solid surface functioning as such. If the mixture to be separated (A plus B) is then introduced into the system (Figure 1.2b), the two compounds will distribute themselves between the two phases according to their respective properties. Since one of the phases is moving, the substances in the mixture must also move. The compound having the greater affinity for the mobile phase (or lesser affinity for the stationary phase) will move faster than the compound with the opposite properties.

After a period of time, the situation can be depicted as shown in Figure 1.2c, where compound B has moved at a faster rate than compound A. If A and B have very different properties, one may flow with the mobile phase and the other remain in the introductory position. In this case, the separation is easy. More usually, A and B have similar properties and both migrate, but at different rates. This difference in migration rates is the

		Moving Phase: Liquid	Moving Phase: Gas
Stationary Phase	Solid	Liquid-Solid Chromatography or LSC	Gas-Solid Chromatography or GSC
	Liquid	Liquid-Liquid Chromatography or LLC	Gas-Liquid Chromatography or GLC

Figure 1.3 Types of chromatography showing how the name is derived from the nature of the mobile and stationary phases.

basis for all chromatographic separations, and the search for conditions that will produce the greatest difference between the rates represents the challenge of chromatography.

There are a confusingly large number of different types, variations, and techniques of chromatography. However, it is possible to make some general divisions and perhaps view the field in a reasonably systematic way. On the basis of the mobile phase, which may be a liquid or a gas, it is possible to divide chromatography into **liquid chromatography (LC) and gas chromatography (GC).** On the basis of the stationary phase, which may be a liquid or a solid, it is possible to divide chromatography into **partition chromatography** or **adsorption chromatography.** In an obvious blend of these two concepts, one may then have, for example, liquid-solid chromatography, where the mobile phase is liquid and the stationary phase is solid. Other combinations are shown in Figure 1.3. We will use the terms in the figure.

The general division of the field into liquid chromatography and gas chromatography appears to be the more useful division and will be the one stressed in this book. A number of other types of chromatography have been devised that involve phenomena other than simple partition, adsorption and volatility. These types of chromatography will be briefly described in a Glossary of such methods in the back of this book. The Glossary also contains other names for the general types of chromatography.

1.1 DEFINITION OF TERMS

The introduction above has required the definition of a number of terms, but several more need to be defined for a general understanding of chromatography.

The solid phase, which serves as the stationary phase for liquid-solid or the less used gas-solid chromatography, is called the **adsorbent,** whereas the material that holds a stationary phase in liquid-liquid or gas-liquid chromatography is called the **support.** When the mobile phase is caused to flow over the stationary phase, to effect the chromatographic separation, the

process is known as **development.** After the substances have been separated by development, the results are **detected** or **visualized.** When the substances being separated are actually washed out of the system, they have been **eluted,** or **elution** has taken place. The substances being separated are normally termed the **solutes,** or, collectively, the **sample.** The total result is then called a **chromatogram.**

1.2 LIQUID CHROMATOGRAPHY

A number of techniques have evolved in which the mobile phase is a liquid. These differ from one another in several ways. First, some involve a solid stationary phase (liquid-solid) and some involve a liquid stationary phase (liquid-liquid). These are often called **adsorption** and **partition chromatography,** respectively. Second, the techniques of LC differ in the shape or conformation of the stationary phase. The phase may be in a thin layer on some type of support as in **thin layer chromatography (TLC)** or as such in **paper chromatography (PC),** or it may be in a column that is held in place by a glass, metal, or plastic tube as in **column chromatography.** Finally, the techniques of LC differ in the rates at which the mobile phase is moved and in the methods by which the separations are detected. When the mobile phase is allowed to flow down through a column by a gravity flow, the method is called simply column chromatography. When the mobile phase is moved rapidly under pressure and the results are detected instrumentally, the process is called **high performance liquid chromatography, HPLC.**

A liquid chromatogram may be developed with a pure solvent, a mixture of pure solvents (both sometimes called **isocratic systems),** or, more often, a constantly changing mixture of solvents, usually two or three. When a changing mixture of solvents is being used, it is said that a **gradient development** or **gradient elution** is taking place.

Thin Layer and Paper Chromatography

These two techniques are similar in that the stationary phase is a thin layer and the mobile phase is allowed to flow through by capillary action. They differ in the nature and function of the stationary phase. In paper chromatography, the stationary phase is a liquid, usually water, suspended on the fibers of a piece of high-grade filter paper, thus giving rise to liquid-liquid chromatography. In TLC, the stationary phase is a thin layer (0.1-2 mm thick) of some solid material deposited on a flat supporting surface that is usually glass, but may be a polymer film or a metal foil. The layer is held in place by a binding agent of some type, usually plaster of Paris or starch.

In TLC the layer generally functions as a solid adsorbing surface (liquid-solid, see Figure 1.3), although it can also be used as a liquid support, giving rise to liquid-liquid chromatography. Of the two techniques, PC is the older, but probably TLC is much more commonly used at present. While it is possible to carry out a gradient development of a paper or thin layer chromatogram, the apparatus is complex, and simple development with a pure solvent or an unchanging mixture of solvents is more common.

The same series of operations is involved in PC and TLC, but they will be illustrated using a TLC system. The mixture to be separated is dissolved in any suitable solvent, preferably the developing solvent or one similar to it in polarity (see Chapter 3) and applied as a spot (1-5 mm in diameter) to the layer a short distance (ca. 2 cm) from one end. Such an application is generally made with a glass capillary (Figure 1.4), but can be made with a syringe or an automated device. The application solvent is allowed to evaporate or is removed in a stream of dry air or nitrogen. The layer is then placed in a developing chamber containing a layer of solvent about 1 cm deep which will act as the mobile phase. This is done in such a manner that the solvent is in contact with the layer on the end nearest the sample spot, but, of course, below it (Figure 1.5). The chamber is then closed securely, and the solvent is allowed to ascend the layer by capillary action to a point 10-15 cm above the sample spot (Figure 1.6).

Paper chromatography can be carried out in much the same manner except that the paper must be suspended from some type of hook in the chamber since it does not have a firm backing. When the mobile phase and the stationary phase have been properly selected, the original sample spot will have been resolved into a series of spots, each hopefully representing a single component of the mixture (Figure 1.7). The chromatography is usually carried out in a chamber that has been saturated as completely as possible with the mobile phase. This is the purpose of the piece of filter paper that partially lines the chamber in Figures 1.4 through 1.7. When the spots are not colored, they must be visualized by spraying them with a suitable color-developing reagent or by placing them under an ultraviolet light.

An almost infinite number of variations of this simple procedure have been published since PC and TLC were first described. Some of these are important; some are trivial; and some are complex beyond the scope of this introductory book. The important ones will be described in Chapter 4.

Several terms are used in TLC and PC in addition to those given earlier. The point at which the mixture is deposited at the beginning of the chromatogram is called the **origin,** and the technique of placing it there is known as **spotting.** The **solvent front** is the top of the mobile phase or **solvent** as it moves through the layer and, after the development is completed, represents the maximum height achieved by the solvent. The behavior of a specific compound in a specific chromatographic system is described

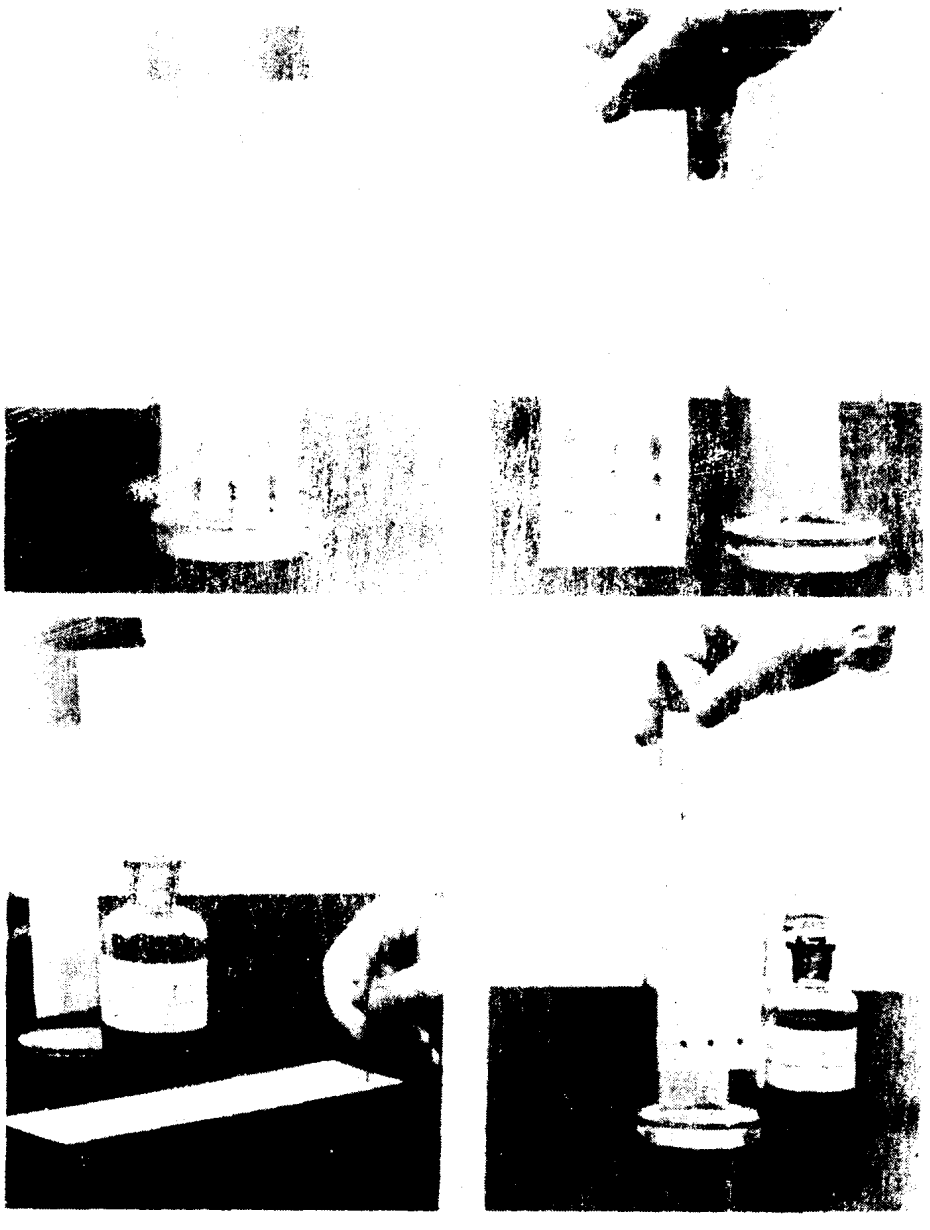


Figure 1.4–1.7 A mixture of three dyes in benzene is spotted on a silica gel G layer in three concentrations (1.4), placed in a saturated chamber containing benzene as a developer (1.5), developed (1.6), and removed and dried (1.7).

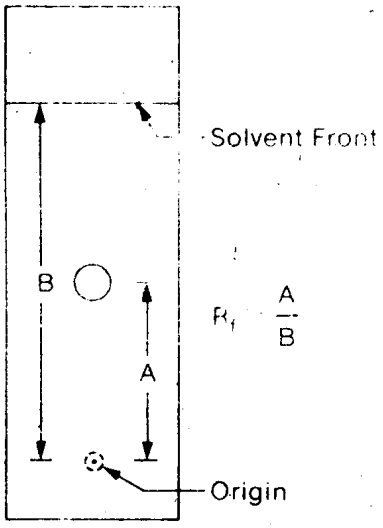
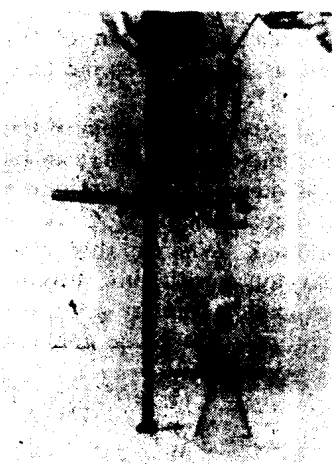


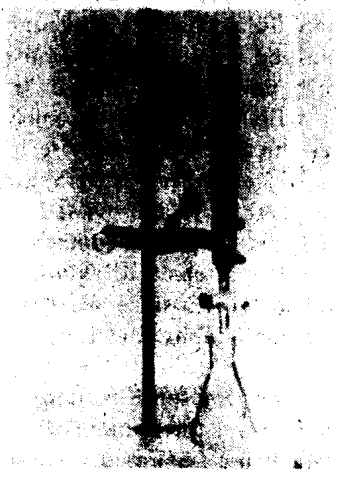
Figure 1.8 An idealized thin layer chromatogram showing how an **Rf** value is measured and calculated.

by the ***Rf* value.** This number is obtained by dividing the distance moved by the solvent front into the distance moved by the solute spot. Both distances are measured from the origin, and ***Rf*** values vary from 0 to 1. This is shown in Figure 1.8.

Column Chromatography

Classic column chromatography is the oldest of all of the many chromatographic methods and, as it has been traditionally practiced, is a form of liquid chromatography. The stationary phase, either an adsorbing material (LSC) or a supported liquid film (LLC), is placed in a glass cylindrical tube closed at the bottom by a valve or stopcock, and the mobile phase is allowed to flow down through it by gravity flow. The **chromatographic column,** Figure 1.9, is usually prepared by pouring a slurry of the stationary phase in a suitable solvent into the column and allowing it to settle out. Next, the level of the solvent is lowered to the top of the adsorbent, and the sample mixture, dissolved in a suitable solvent, is placed on the top of the column and allowed to flow into the top layer of adsorbent or support (Figure 1.10). The mobile phase solvent is then placed on top of the column and allowed to flow through and develop the chromatogram (Figure 1.11). Under well-chosen conditions, the solute components of the mixture





Figures 1.9–1.12 A slurry of silica gel in benzene is poured into a glass column (1.9) and the solvent is allowed to flow out until the level is just over the adsorbent. A small circle of filter paper is placed on the top of the column and the dye mixture (see Figure 1.4) is added with a dropper (1.10). Additional benzene, as a developer, is allowed to flow through the column so that the colored bands are resolved (1.11) and finally until one band emerges from the bottom (1.12). The time involved in this particular experiment was just about 30 min.

proceed down the column in **bands** at different rates and are thus resolved. The solutes are generally isolated by allowing them to flow out of the column (Figure 1.12) and collecting them as fractions, often with a mechanical fraction collector.

In the case of colorless compounds, the effluent from the bottom of the column must be monitored to find out where the solutes are. This can be done continuously with a suitable detector or by dividing the effluent into a number of sequential samples and analyzing them, generally by TLC, or by weighing the fractions after the solvent is evaporated. Whereas thin layer and paper chromatograms are more frequently developed with pure solvents or unchanging mixtures of solvents, columm chromatograms are generally developed with constantly changing mixtures of solvents by a gradient technique.

High Performance Liquid Chromatography

Theoretically, the best chromatographic separations will be produced when the stationary phase has the largest possible surface area, thus ensuring a good equilibrium between the phases. A second requirement for good separation is to have the mobile phase flowing rapidly to ensure a minimum diffusion situation. A large stationary phase surface area means, in most chromatographic situations, a finely divided adsorbent or support. In order to force a rapidly moving mobile phase through a finely divided stationary phase, high pressures must be used. These requirements have given rise to the newest and most powerful of the techniques of liquid chromatography. At first it was called **high pressure liquid chromatography,** abbreviated as **HPLC.** This name was modified to **high performance liquid chromatography,** still **HPLC,** and is sometimes, and incorrectly, referred to as **liquid chromatography** or **LC.** We will consistently use HPLC since we have defined LC in the more general sense as any chromatography that involves a liquid mobile phase.

Still another unique aspect of HPLC is the use of very sensitive **detectors** of one sort or another to analyze the effluent from the column when colorless or very low concentrations of solutes are being separated. These detectors may involve a continuous monitoring of the ultraviolet absorption, the refractive index, or some other physical constant of the effluent that will change sufficiently as the solutes emerge from the column. In short, when some of the advances that were developed for gas chromatography were applied to classical chromatography, the technique of HPLC was born.

HPLC is carried out either as an liquid-solid method or as a liquid-liquid method. The LLC technique uses either a stationary phase *chemically bonded* to the support or, less often, adsorbed to the support. Both chromatographic methods use a packing or support that is very finely divided (3-20 μm). For analytical purposes, such a packing is put into a

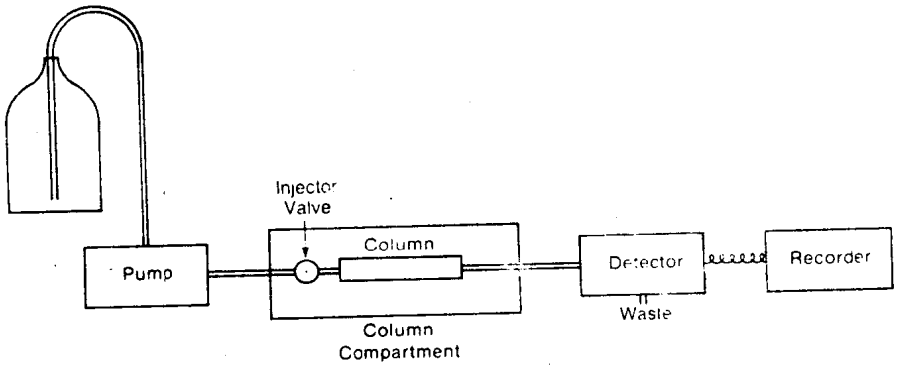


Figure 1.13 Schematic diagram of an apparatus used for HPLC.

stainless steel tube of small diameter (2-6 mm) and moderate length (5-30 cm). The mobile phase is pushed through the column at pressures ranging from 20-10,000 psi. For preparative separations, larger column diameters are used.

Figure 1.13 is a diagram of an apparatus used for HPLC. About half of the parts have some similarity to those used in gas chromatography. The various parts are: an inert, gas-vented storage container for the liquid phase, a high pressure solvent delivery unit, an injection system or valve, an oven (rarely thermostated), the column itself, a detector and associated electronics, and a recording device. All of the connecting tubing and valve systems are designed as small and short as possible to minimize **extra column volume.** Gradient elution or solvent programming techniques are often used in HPLC. That, and the need for a very consistent and surgeless solvent flow, is the reason for the complex solvent delivery unit.

The chromatographic system is operated in the following manner (see Figure 1.14). The mobile phase is forced through the column under the desired pressure and at the desired rate. After the system has reached equilibrium, the sample dissolved in a suitable solvent is injected into the system, generally through a valve. The solutes are carried into the column, separated, and pass out in the effluent through the detector. The detector results are plotted on chart paper, and the resulting graphs can be used to obtain analytical data or to find out where the various mixture components are in the effluent. Note that the sample components are in the form of a **band** or **plug** when they are first placed in the system and that they broaden as they flow through the system.

The separation of a particular mixture of solutes by HPLC under a given set of conditions is quite repeatable, and it is possible to assign numbers to the solutes that describe their behavior. These numbers are

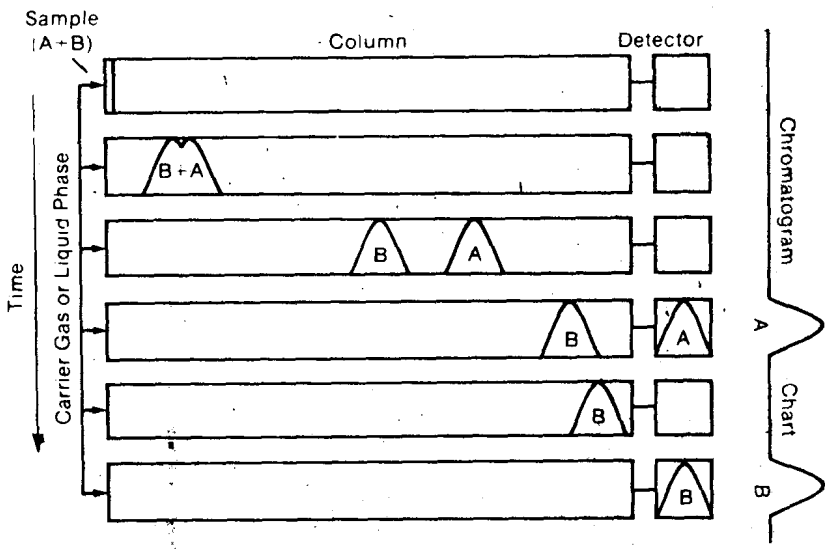


Figure 1.14 A more detailed schematic diagram of GC and HPLC. Solutes A and B can be seen to separate as they pass through the column and the results are recorded on a chart as the components emerge from the column.

called **retention volumes** and are a measure of the volume of solvent needed to move a specific compound through a given system. The number is, in a way, analogous to the *Rf* value as it is used in TLC and PC. The retention volume concept will be discussed in greater detail and more carefully defined later.

1.3 GAS CHROMATOGRAPHY

In gas chromatography, or GC, the mobile phase is an inert gas such as helium, nitrogen, argon, or even hydrogen which is passed under pressure through tubing containing the stationary phase. Although gas-solid chromatography, wherein the stationary phase is a solid surface, is well known, it is much less common than gas-liquid chromatography, where the stationary phase is a liquid film. For chromatographic separations, the liquid stationary phase is present as a thin coating adsorbed or chemically bonded to a solid support which is, in turn, packed in a small diameter (2-8 mm) metal, glass, or plastic tube of moderate length (1-10 m). This is called a **packed column.** In an alternate system called a **capillary** or **open-tubular column,**

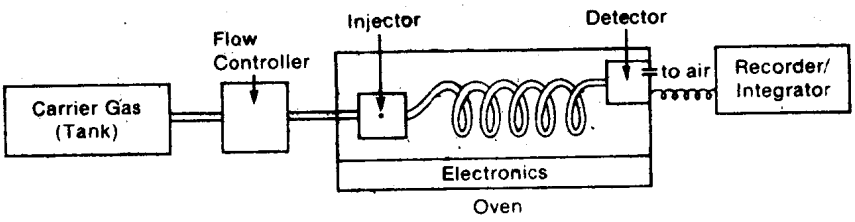


Figure 1.15 A schematic GC apparatus. The sample is injected into the carrier gas stream and allowed to flow into the column where it can be partitioned between the gas phase and the liquid-coated support. The sample is separated and emerges from the column into the detector.

the stationary phase is present as a thin film (0.1-2 μm) on the inside wall of a glass or metal capillary of very small diameter (0.2-1 mm) and very long length (10-100 m).

The column is placed in a thermally controlled oven that can be cooled or heated. Since one of the two key properties exhibited by solutes in GC is solubility, and solubility (or volatility) is closely related to temperature, a precise control of temperature is mandatory. We stated earlier that a major advantage of LC was that the constitution of the mobile phase could be continuously changed (gradient elution) in order to facilitate separations. In an analogous fashion, the temperature can be continuously changed during GC to increase, systematically, the volatility of the solutes and thus enhance separation. In such an operation, the temperature is said to be programmed and the result is **temperature programming** as compared to **gradient elution** in HPLC.

Figure 1.15 shows, schematically, a GC apparatus consisting of a few basic parts, many of which are analogous to those shown in Figure 1.14 for HPLC. The components are a high pressure purified gas supply with a pressure regulator, a sample inlet system or **injector,** a thermostated oven, a column with a suitable packing, a detector and associated electronics, and a recording device for the detector. As in HPLC, it is desirable to keep the non-volume as low as possible. The system is operated in a manner directly analogous to the operation of HPLC as described above and illustrated schematically in Figure 1.14.

The behavior of a specific compound under a given set of circumstances (column, flow rate, temperature) is quite characteristic. Thus, the compound will appear at the detector at a given time after it is **injected.** This is usually called the **retention time** and is the same as the retention volume defined above in HPLC. In actual fact, many use retention times to define both GC and HPLC. As long as the HPLC is done at a constant flow rate, the values represent the same thing.