Chromatography

Introduction

Chromatography (from Greek Chroma, color and graph in to write is the collective term for a set of laboratory techniques for the separation mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analyte in a mixture. The two are not mutually exclusive.

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.

Discovery of Chromatography

Chromatography is a physicochemical method for separation of complex mixtures was discovered at the very beginning of the twentieth century by Russian–Italian botanist M.S. Tswett. “On the new form of adsorption phenomena and its application in biochemical analysis” presented on March 21, 1903 at the regular meeting of the biology section of the Warsaw Society of Natural Sciences, Tswett gave a very detailed description of the newly discovered phenomena of adsorption-based separation of complex mixtures, which he later called “chromatography” as a transliteration from Greek “color writing”. Serendipitously, the meaning of the Russian word “tswett” actually means color. Although in all his publications Tswett mentioned that the origin of the name for his new method was based on the colorful picture of his first separation of plant pigments, he involuntarily incorporated his own name in the name of the method he invented. The chromatographic method was not appreciated among the scientists at the time of the discovery, as well as after almost 10 years when L. S. Palmer in the United States and C. Dhere in Europe independently published the description of a similar separation processes.

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds.

During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times.

The history of chromatography begins during the mid-19th century. Chromatography, literally "color writing", was used and named in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll. New types of chromatography developed during the 1930s and 1940s made the technique useful for many types of separation process. Some related techniques were developed during the 19th century (and even before), but the first true chromatography is usually attributed to Russian botanist M. Tswett, who used columns of calcium carbonate for separating plant pigments during the first decade of the 20th century during his research of chlorophyll.

Analytical Chemistry and Chromatography

The types of analysis can be distinguished in two ways:

1- Qualitative Analysis: To refer identity of product, i.e., it yields useful clues from which the molecular or atomic species, the structural features, or the functional groups in the sample can be identified.

2- Quantitative Analysis: To refer the purity of the product, i.e., the results are in the form of numerical data corresponding to the concentration of analyte.

Types of Analytical Methods:

The various methods of analysis can be grouped into two categories. They are:

1. Chemical methods.
2. Instrumental methods.

Chemical Methods:

In these methods, volume and mass are used as means of detection.

1- Titrimetrical methods like acid-base, oxidation-reduction, non-aqueous, complexometric and precipitation titrations.

2. Gravimetric and thermo gravimetric methods.

3. Instrumental Methods: Based on principles different instrumental methods are available. These methods are based on the measurement of specific and nonspecific physical properties of a substance.

Chromatography terms:

Analyte: is the substance to be separated during chromatography.

Analytical chromatography: is used to determine the existence and possibly also the concentration of analyte(s) in a sample.

A bonded phase: is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tuping.

A chromatogram is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture. Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a spectrophotometer, mass spectrometer or a variety of other detectors) corresponding to the response created by the analytes exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analyte separated

A chromatograph: is equipment that enables a sophisticated separation e.g gas chromatographic or liquid chromatographic separation.

Chromatography: is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary which is stationary phase while the other (the mobile phase) moves in a definite direction.

The eluent: is the mobile phase leaving the column.

An eluotropic series: is a list of solvents ranked according to their eluting power.

The mobile phase: is the phase which moves in a definite direction. It may be a liquid (LC), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated

Preparative chromatography: is used to purify sufficient quantities of a substance for further use, rather than analysis

Retention time: is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

Sample: is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.

Solute refers to the sample components in partition chromatography. The solvent refers to any substance capable of solubilizing other substance, and especially the liquid mobile phase in LC.

Stationary phase is the substance which is fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography

Types of Chromatography

Chromatography can be classified by various ways:

(I) On the basis of interaction of solute to the stationary phase

(II) On the basis of chromatographic bed shape

(III) Techniques by physical state of mobile phase

Techniques by chromatographic bed shape:

1- Column chromatography

2- Planar chromatography

i- Paper chromatography

ii- Thin layer chromatography

iii- Displacement chromatography

Techniques by physical state of mobile phase:

1. Gas chromatography
2. Liquid chromatography
3. Affinity chromatography
4. Supercritical fluid chromatography

Techniques by separation mechanism:

1. Ion exchange chromatography
2. Size exclusion chromatography8

Special techniques:

1. Reversed-phase chromatography
2. Two-dimensional chromatography
3. Simulated moving-bed chromatography
4. Pyrolysis gas chromatography

5. Fast protein liquid chromatography

6. Countercurrent chromatography9

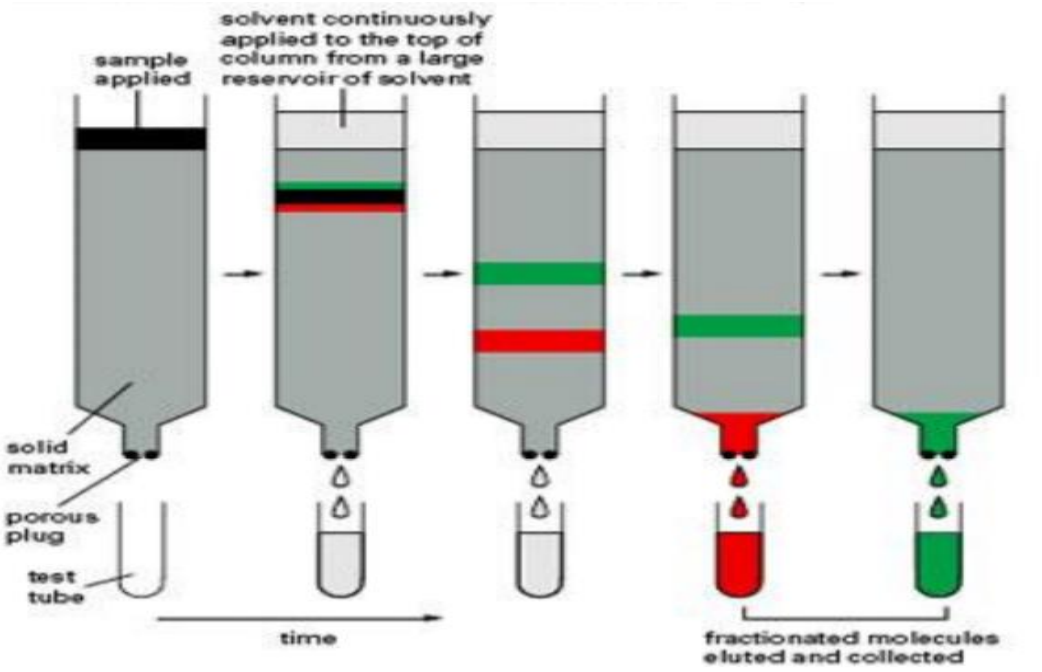
7. Chiral Chromatography.

Chromatographic bed shape

1. Column Chromatography

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample.

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.



Column

2. Planer Chromatography

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retention factor (Rf) of each chemical can be used to aid in the identification of an unknown substance.

2.1. Paper Chromatography

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

2.2. Thin layer Chromatography

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or 11cellulose on aflat,inert substrate. Compared to paper, it has theadvantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used.

2.3 Displacement Chromatography

The basic principle of displacement chromatography is, “A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser

affinities".

There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings.

Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks". Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

Physical state of mobile phase

1. Gas Chromatography

Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary". Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium).

The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry.

2. Liquid Chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC).

In the HPLC technique, the sample is forced through a column that is

packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more

3. Affinity chromatography

Affinity chromatography is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.). Columns are often manually prepared. Traditional affinity columns are used as a preparative step to flush out unwanted biomolecules. However, HPLC techniques exist that do utilize affinity chromatography properties. Immobilized

Metal Affinity Chromatography (IMAC) is useful to separate aforementioned molecules based on the relative affinity for the metal (I.e. Dionex IMAC). Often these columns canbe loaded with different metals to create a column with a targeted affinity.

4. Supercritical fluid chromatography

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

High Pressure Liquid Chromatography

High Pressure Liquid Chromatography (HPLC) called High Performance liquid chromatography is a separation that can be used for the analysis of organic molecules and ions. HPLC is based on mechanisms adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved. The techniques of HPLC are so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation. HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationaryphase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on interactions between the stationary phase, the molecules being analyzed and the

solvent(s) used.

It offers following advantages:

1- Speed (many analysis can be accomplished in 20 minutes or less)

2- Greater sensitivity (various detectors can be employed)

3- Reusable columns (expensive columns but can be used form any analysis)

4- Ideal for the substances of low volatility.

5- Easy sample recovery, handling and maintenance.

6- Instrumentation leads itself to automation and quantitation.

7- Precise and reproducible.

8- Calculations are done by integrator itself.

9- Suitable for preparative liquid chromatography on a much larger scale.

Based on the mode of separation

1- Normal phase chromatography:

It was one of the first kinds of HPLC that chemists developed. In this type stationary phase used is polar in nature and the mobile phase used is non-polar and nonaqueous in nature. If the affinity between the stationary phase and the analyte increases the selection time (RT) of the analyte also increases and vice versa. The interaction strength depends not only on the functional groups in the

analyte molecule but also on steric factors. The effect of steric on interaction strength allows this method to resolve (separate) structural isomers.

2. Reverse phase chromatography

In reverse phase technique, a non-polar stationary phase is used and the mobile phase is polar in nature. Hence polar components get eluted first and nonpolar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, columns used in the mode of chromatogram are ODS (Octadecyl silane) or C18, C8, C4, etc.

3. Partition chromatography

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. Partition chromatography uses a retained solvent, on the surface or within the grains or fibers of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional columbic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent separate analytes based on the polar differences is known as Hydrophilic interaction chromatography (HILIC).

Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. However, HILIC has the advantage of separating acidic, basic and neutral solutes a single chromatogram.

Based on principle of separation:

1. Adsorption chromatography:

When a mixture of compounds (adsorbate) dissolved in the mobile phase (eluent) moves through a column of stationary phase (adsorbent) they travel according to their relative affinities. The compound which has more affinity towards stationary phase travels slower, if less affinity towards stationary phase travels faster.

2. Ion exchange chromatography:

It is the process by which a mixture of similar charged ions can be separated using ion exchange resin. There is a reversible exchange of ions between the ions present in the column. And those present in the ion exchange resin. For cations, cation exchange resin and for anions, an anion exchange resin is used.

3. Size exclusion chromatography

It is the process by which mixture of compounds with molecular sizes are separated by using gels. The gel used acts as molecular sieve. It can be separated by steric and diffusion effects of pores in the gels. The compound can separate according to the molecular sizes and the stationary phase is a porous matrix. Eg: separation of proteins and polysaccharides.