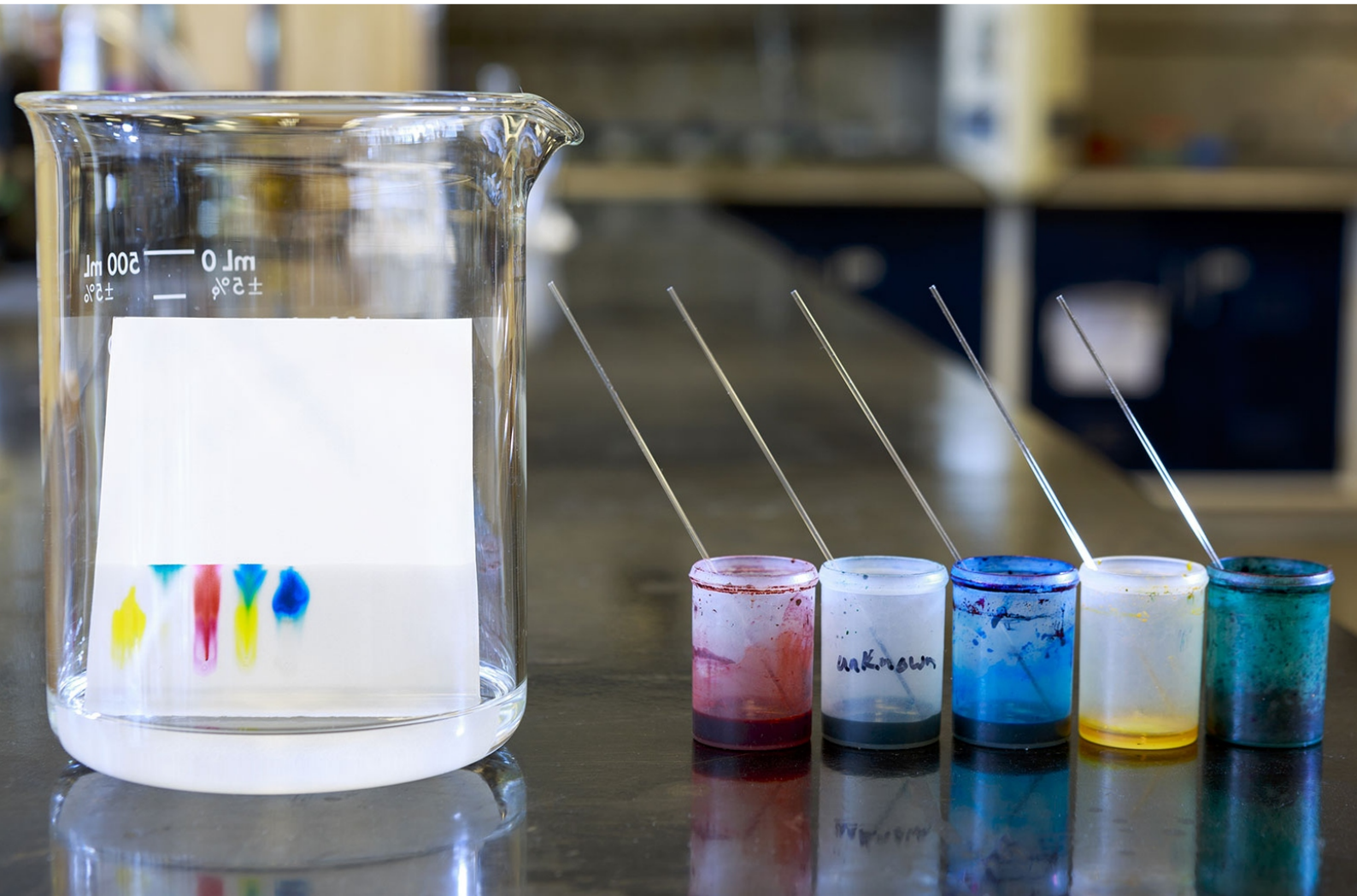
Separation Techniques of Chromatography

Robert Harvey



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by Robert Harvey

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Chapter 1

Introduction

In chemical analysis, **Chromatography** is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid (gas or solvent) called the *mobile phase,* which carries it through a system (a column, a capillary tube, a plate, or a sheet) on which a material called the *stationary phase* is fixed. The different constituents of the mixture have different affinities for the stationary phase.

The different molecules stay longer or shorter on the stationary phase, depending on their interactions with its surface sites. So, they travel at different apparent velocities in the mobile fluid, causing them to separate.

The separation is based on the differential partitioning between the mobile and the stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification.

Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

Etymology and pronunciation

Chromatography, pronounced is derived from Greekχρῶμα*chroma*, which means "color", and γράφειν*graphein*, which means "to write". The combination of these two terms was directly inherited from the invention of the technique first used to separate pigments.

History

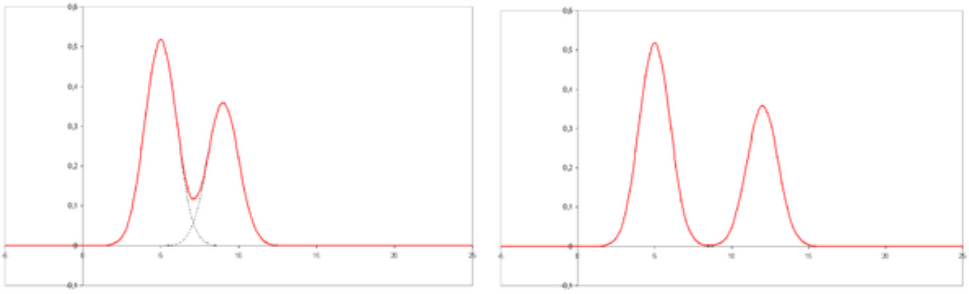
Chromatography was first devised in Russia by the Italian-born scientist Mikhail Tsvet in 1900. He developed the technique, he coined *chromatography*, in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components separate in bands of different colors (green, orange, and yellow, respectively) they directly inspired the name of the technique. New types of chromatography developed during the 1930s and 1940s made the technique useful for many separation processes.

Chromatography technique developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s, for which they won the 1952 Nobel Prize in Chemistry. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods: paper chromatography, gas chromatography, and what would become known as high-performance liquid chromatography. Since then, the technology has advanced rapidly. Researchers found that the main

principles of Tsvet's chromatography could be applied in many different ways, resulting in the different varieties of chromatography described below. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules.

Chromatography terms

- Analyte – the substance to be separated during chromatography. It is also normally what is needed from the mixture.
- Analytical chromatography – the use of chromatography to determine the existence and possibly also the concentration of analyte(s) in a sample.
- Bonded phase – a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.
- Chromatogram – 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.

Chromatograph or **aerograph** – an instrument that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.

- **Chromatography** – a physical method of separation that distributes components to separate between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction.
- **Eluent** (sometimes spelled *eluant*) – the solvent or solvent mixure used in elution chromatography and is synonymous with *mobile phase*.
- **Eluate** – the mixture of *solute* (see Eluite) and *solvent* (see Eluent) exiting the column.
- **Effluent** – the stream flowing out of a chromatographic column. In practise, it is used synonymously with *eluate*, but the term more precisely refers to the stream independent of separation taking place.
- **Eluite** – a more precise term for *solute* or *analyte*. It is a sample component leaving the chromatographic column.
- **Eluotropic series** – a list of solvents ranked according to their eluting power.

- **Immobilized phase** – a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
- **mobile phase** – the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)), 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 a polar solvent such as methanol in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Preparative chromatography** – the use of chromatography to purify sufficient quantities of a substance for further use, rather than analysis.
- **Retention time** – the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions. See also: Kovats' retention index
- **Sample** – 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** – the sample components in partition chromatography.
- **Solvent** – any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.
- **Stationary phase** – the substance fixed in place for the chromatography procedure. Examples include the silica layer in thin-layer chromatography
- **Detector** – the instrument used for qualitative and quantitative detection of analytes after separation.

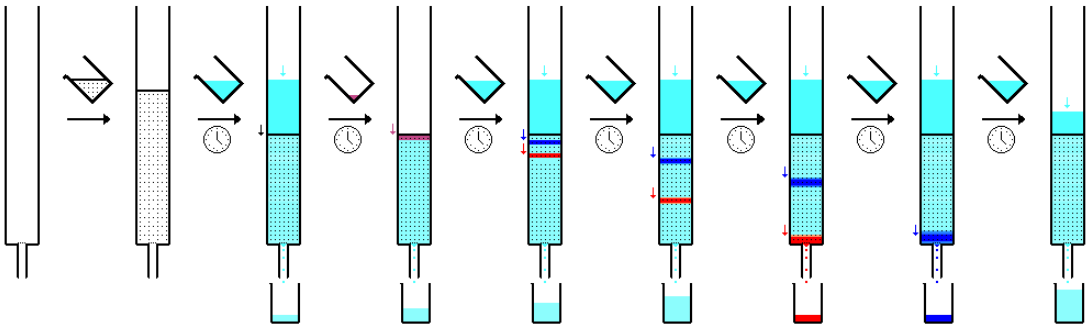
Chromatography is based on the concept of partition coefficient. Any solute partitions between two immiscible solvents.

When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If the matrix support, or stationary phase, is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase.

Techniques by chromatographic bed shape

Column chromatography

- Further information: 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 1978, W. Clark Still introduced a modified version of column chromatography called *flash column chromatography* (flash). The technique is very similar to the traditional column chromatography, except that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

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.

Phosphocellulose chromatography utilizes the binding affinity of many DNA-binding proteins for phosphocellulose. The stronger a protein's interaction with DNA, the higher the salt concentration needed to elute that protein.

Planar 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 (R_f) of each chemical can be used to aid in the identification of an unknown substance.

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 container with 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 further if they are less polar. More polar

substances bond with the cellulose paper more quickly, and therefore do not travel as far.

Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is a widely employed laboratory technique used to separate different biochemicals on the basis of their relative attractions to the stationary and mobile phases. It 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 cellulose on a flat, inert substrate. TLC is very versatile; multiple samples can be separated simultaneously on the same layer, making it very useful for screening applications such as testing drug levels and water purity. Possibility of cross-contamination is low since each separation is performed on a new layer. Compared to paper, it has the advantage of faster runs, better separations, better quantitative analysis, and the choice between different adsorbents. For even better resolution and faster separation that utilizes less solvent, high-performance TLC can be used. An older popular use had been to differentiate chromosomes by observing distance in gel (separation of was a separate step).

Displacement chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) competes effectively for binding sites, and thus displaces 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 for 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.

Techniques by physical state of mobile phase

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 chromatographic separation is

always carried out in a column, which is typically "packed" or "capillary". Packed columns are the routine work horses of gas chromatography, being cheaper and easier to use and often giving adequate performance. Capillary columns generally give far superior resolution and although more expensive are becoming widely used, especially for complex mixtures. Further, capillary columns can be split into three classes: porous layer open tubular (PLOT), wall-coated open tubular (WCOT) and support-coated open tubular (SCOT) columns. PLOT columns are unique in a way that the stationary phase is adsorbed to the column walls, while WCOT columns have a stationary phase that is chemically bonded to the walls. SCOT columns are in a way the combination of the two types mentioned in a way that they have support particles adhered to column walls, but those particles have liquid phase chemically bonded onto them. Both types of column are made from non-adsorbent and chemically inert materials. Stainless steel and glass are the usual materials for packed columns and quartz or fused silica for capillary columns.

Gas chromatography is based on a partition equilibrium of analyte between a solid or viscous liquid 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 (commonly 0.53 – 0.18mm inside diameter) glass or fused-silica 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 denatures them), frequently encountered in

biochemistry, it is well suited for use in the petrochemical, environmental monitoring and remediation, and industrial chemical fields. It is also used extensively in chemistry research.

Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. It 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 to as high-performance liquid chromatography (HPLC).

In HPLC the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase composed of irregularly or spherically shaped particles, a porous monolithic layer, or a porous membrane. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Methods in which the stationary phase is more polar than the mobile phase (e.g., toluene as the mobile phase, silica as the stationary phase) are termed 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 termed reversed phase liquid chromatography (RPLC).

Specific techniques under this broad heading are listed below.

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, 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, liquid chromatography techniques exist that do utilize affinity chromatography properties. Immobilized metal affinity chromatography (IMAC) is useful to separate the aforementioned molecules based on the relative affinity for the metal. Often these columns can be loaded with different metals to create a column with a targeted affinity.

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.

Techniques by separation mechanism

Ion exchange chromatography

Ion exchange chromatography (usually referred to as ion chromatography) uses an ion exchange mechanism to separate analytes based on their respective charges. It is usually performed in columns but can also be useful in planar mode. Ion exchange chromatography uses a charged stationary phase to separate charged compounds including anions, cations, amino acids, peptides, and proteins. In conventional methods the stationary phase is an ion-exchange resin that carries charged functional groups that interact with oppositely charged groups of the compound to retain. There are two types of ion exchange chromatography: Cation-Exchange and Anion-Exchange. In the Cation-Exchange Chromatography the stationary phase has negative charge and the exchangeable ion is a cation, whereas, in the Anion-Exchange Chromatography the stationary phase has positive charge and the exchangeable ion is an anion. Ion exchange chromatography is commonly used to purify proteins using FPLC.

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is also known as *gel permeation chromatography* (GPC) or *gel filtration chromatography* and separates molecules according to their size (or more accurately according to their hydrodynamic diameter or hydrodynamic volume). Smaller molecules are able

to enter the pores of the media and, therefore, molecules are trapped and removed from the flow of the mobile phase. The average residence time in the pores depends upon the effective size of the analyte molecules. However, molecules that are larger than the average pore size of the packing are excluded and thus suffer essentially no retention; such species are the first to be eluted. It is generally a low-resolution chromatography technique and thus it is often reserved for the final, "polishing" step of a purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, especially since it can be carried out under native solution conditions.

Expanded bed adsorption chromatographic separation

An expanded bed chromatographic adsorption (EBA) column for a biochemical separation process comprises a pressure equalization liquid distributor having a self-cleaning function below a porous blocking sieve plate at the bottom of the expanded bed, an upper part nozzle assembly having a backflush cleaning function at the top of the expanded bed, a better distribution of the feedstock liquor added into the expanded bed ensuring that the fluid passed through the expanded bed layer displays a state of piston flow. The expanded bed layer displays a state of piston flow. The expanded bed chromatographic separation column has advantages of increasing the separation efficiency of the expanded bed.

Expanded-bed adsorption (EBA) chromatography is a convenient and effective technique for the capture of proteins directly from unclarified crude sample. In EBA

chromatography, the settled bed is first expanded by upward flow of equilibration buffer. The crude feed, a mixture of soluble proteins, contaminants, cells, and cell debris, is then passed upward through the expanded bed. Target proteins are captured on the adsorbent, while particulates and contaminants pass through. A change to elution buffer while maintaining upward flow results in desorption of the target protein in expanded-bed mode. Alternatively, if the flow is reversed, the adsorbed particles will quickly settle and the proteins can be desorbed by an elution buffer. The mode used for elution (expanded-bed versus settled-bed) depends on the characteristics of the feed. After elution, the adsorbent is cleaned with a predefined cleaning-in-place (CIP) solution, with cleaning followed by either column regeneration (for further use) or storage.

Special techniques

Reversed-phase chromatography

Reversed-phase chromatography (RPC) is any liquid chromatography procedure in which the mobile phase is significantly more polar than the stationary phase. It is so named because in normal-phase liquid chromatography, the mobile phase is significantly less polar than the stationary phase. Hydrophobic molecules in the mobile phase tend to adsorb to the relatively hydrophobic stationary phase. Hydrophilic molecules in the mobile phase will tend to elute first. Separating columns typically comprise a C8 or C18 carbon-chain bonded to a silica particle substrate.

Hydrophobic interaction chromatography

Hydrophobic Interaction Chromatography (HIC) is a purification and analytical technique that separates analytes, such as proteins, based on hydrophobic interactions between that analyte and the chromatographic matrix. It can provide a non-denaturing orthogonal approach to reversed phase separation, preserving native structures and potentially protein activity. In hydrophobic interaction chromatography, the matrix material is lightly substituted with hydrophobic groups. These groups can range from methyl, ethyl, propyl, butyl, octyl, or phenyl groups. At high salt concentrations, non-polar sidechains on the surface on proteins "interact" with the hydrophobic groups; that is, both types of groups are excluded by the polar solvent (hydrophobic effects are augmented by increased ionic strength). Thus, the sample is applied to the column in a buffer which is highly polar, which drives an association of hydrophobic patches on the analyte with the stationary phase. The eluent is typically an aqueous buffer with decreasing salt concentrations, increasing concentrations of detergent (which disrupts hydrophobic interactions), or changes in pH. Of critical importance is the type of salt used, with more kosmotropic salts as defined by the Hofmeister series providing the most water structuring around the molecule and resulting hydrophobic pressure. Ammonium sulfate is frequently used for this purpose. The addition of organic solvents or other less polar constituents may assist in improving resolution.

In general, Hydrophobic Interaction Chromatography (HIC) is advantageous if the sample is sensitive to pH change or harsh solvents typically used in other types of chromatography but

not high salt concentrations. Commonly, it is the amount of salt in the buffer which is varied. In 2012, Müller and Franzreb described the effects of temperature on HIC using Bovine Serum Albumin (BSA) with four different types of hydrophobic resin. The study altered temperature as to effect the binding affinity of BSA onto the matrix. It was concluded that cycling temperature from 50 to 10 degrees would not be adequate to effectively wash all BSA from the matrix but could be very effective if the column would only be used a few times. Using temperature to effect change allows labs to cut costs on buying salt and saves money.

If high salt concentrations along with temperature fluctuations want to be avoided you can use a more hydrophobic to compete with your sample to elute it. [source] This so-called salt independent method of HIC showed a direct isolation of Human Immunoglobulin G (IgG) from serum with satisfactory yield and used Beta-cyclodextrin as a competitor to displace IgG from the matrix. This largely opens up the possibility of using HIC with samples which are salt sensitive as we know high salt concentrations precipitate proteins.

Hydrodynamic chromatography

Hydrodynamic chromatography (HDC) is derived from the observed phenomenon that large droplets move faster than small ones. In a column, this happens because the center of mass of larger droplets is prevented from being as close to the sides of the column as smaller droplets because of their larger overall size. Larger droplets will elute first from the middle of the column while smaller droplets stick to the sides of the column and elute last. This form of chromatography is useful

for separating analytes by molar mass, size, shape, and structure when used in conjunction with light scattering detectors, viscometers, and refractometers. The two main types of HDC are open tube and packed column. Open tube offers rapid separation times for small particles, whereas packed column HDC can increase resolution and is better suited for

particles with an average molecular mass larger than daltons. HDC differs from other types of chromatography because the separation only takes place in the interstitial volume, which is the volume surrounding and in between particles in a packed column.

HDC shares the same order of elution as Size Exclusion Chromatography (SEC) but the two processes still vary in many ways. In a study comparing the two types of separation, Isenberg, Brewer, Côté, and Striegel use both methods for polysaccharide characterization and conclude that HDC coupled with multiangle light scattering (MALS) achieves more accurate molar mass distribution when compared to off-line MALS than SEC in significantly less time. This is largely due to SEC being a more destructive technique because of the pores in the column degrading the analyte during separation, which tends to impact the mass distribution. However, the main disadvantage of HDC is low resolution of analyte peaks, which makes SEC a more viable option when used with chemicals that are not easily degradable and where rapid elution is not important.

HDC plays an especially important role in the field of microfluidics. The first successful apparatus for HDC-on-a-chip system was proposed by Chmela, et al. in 2002. Their design was able to achieve separations using an 80 mm long

channel on the timescale of 3 minutes for particles with diameters ranging from 26 to 110 nm, but the authors expressed a need to improve the retention and dispersion parameters. In a 2010 publication by Jellema, Markesteijn, Westerweel, and Verpoorte, implementing HDC with a recirculating bidirectional flow resulted in high resolution, size based separation with only a 3 mm long channel. Having such a short channel and high resolution was viewed as especially impressive considering that previous studies used channels that were 80 mm in length. For a biological application, in 2007, Huh, et al. proposed a microfluidic sorting device based on HDC and gravity, which was useful for preventing potentially dangerous particles with diameter larger than 6 microns from entering the bloodstream when injecting contrast agents in ultrasounds. This study also made advances for environmental sustainability in microfluidics due to the lack of outside electronics driving the flow, which came as an advantage of using a gravity based device.

Two-dimensional chromatography

In some cases, the selectivity provided by the use of one column can be insufficient to provide resolution of analytes in complex samples. Two-dimensional chromatography aims to increase the resolution of these peaks by using a second column with different physico-chemical (chemical classification) properties. Since the mechanism of retention on this new solid support is different from the first dimensional separation, it can be possible to separate compounds by two-dimensional chromatography that are indistinguishable by one-dimensional chromatography. Furthermore, the separation on the second dimension occurs faster than the first dimension.

An example of a two-dimensional TLC separation is where the sample is spotted at one corner of a square plate, developed, air-dried, then rotated by 90° and usually redeveloped in a second solvent system. Two-dimensional chromatography can be applied to GC or LC separations. This separation method can also be used in a heart-cutting approach, where specific regions of interest on the first dimension are selected for separation by the second dimension, or in a comprehensive approach, where all the analytes from the first dimension undergo the second dimension separation.

Simulated moving-bed chromatography

The simulated moving bed (SMB) technique is a variant of high performance liquid chromatography; it is used to separate particles and/or chemical compounds that would be difficult or impossible to resolve otherwise. This increased separation is brought about by a valve-and-column arrangement that is used to lengthen the stationary phase indefinitely. In the moving bed technique of preparative chromatography the feed entry and the analyte recovery are simultaneous and continuous, but because of practical difficulties with a continuously moving bed, simulated moving bed technique was proposed. In the simulated moving bed technique instead of moving the bed, the sample inlet and the analyte exit positions are moved continuously, giving the impression of a moving bed. True moving bed chromatography (TMBC) is only a theoretical concept. Its simulation, SMBC is achieved by the use of a multiplicity of columns in series and a complex valve arrangement, which provides for sample and solvent feed, and also analyte and waste takeoff at appropriate locations of any column, whereby it allows switching at regular intervals the

sample entry in one direction, the solvent entry in the opposite direction, whilst changing the analyte and waste takeoff positions appropriately as well.

Fast protein liquid chromatography

Fast protein liquid chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins. As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities for two materials, a moving fluid (the "mobile phase") and a porous solid (the stationary phase). In FPLC the mobile phase is an aqueous solution, or "buffer".

The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. FPLC resins are available in a wide range of bead sizes and surface ligands depending on the application.

Countercurrent chromatography

Countercurrent chromatography (CCC) is a type of liquid-liquid chromatography, where both the stationary and mobile phases are liquids and the liquid stationary phase is held stagnant by a strong centrifugal force.

Hydrodynamic countercurrent chromatography (CCC)

The operating principle of CCC instrument requires a column consisting of an open tube coiled around a bobbin. The bobbin is rotated in a double-axis gyratory motion (a cardioid), which causes a variable gravity (G) field to act on the column during each rotation.

This motion causes the column to see one partitioning step per revolution and components of the sample separate in the column due to their partitioning coefficient between the two immiscible liquid phases used. There are many types of CCC available today. These include HSCCC (High Speed CCC) and HPCCC (High Performance CCC). HPCCC is the latest and best-performing version of the instrumentation available currently.

Hydrostatic countercurrent chromatography or centrifugal partition chromatography (CPC)

In the CPC instrument, the column consists of a series of cells interconnected by ducts attached to a rotor. This rotor rotates on its central axis creating the centrifugal field necessary to hold the stationary phase in place.

The separation process in CPC is governed solely by the partitioning of solutes between the stationary and mobile phases, which mechanism can be easily described using the partition coefficients (K_D) of solutes. CPC instruments are commercially available for laboratory, pilot, and industrial-scale separations with different sizes of columns ranging from some 10 milliliters to 10 liters volume.

Periodic counter-current chromatography

In contrast to Counter current chromatography (see above), periodic counter-current chromatography (PCC) uses a solid stationary phase and only a liquid mobile phase. It thus is much more similar to conventional affinity chromatography than to counter current chromatography. PCC uses multiple columns, which during the loading phase are connected in line. This mode allows for overloading the first column in this series without losing product, which already breaks through the column before the resin is fully saturated. The breakthrough product is captured on the subsequent column(s). In a next step the columns are disconnected from one another. The first column is washed and eluted, while the other column(s) are still being loaded. Once the (initially) first column is re-equilibrated, it is re-introduced to the loading stream, but as last column. The process then continues in a cyclic fashion.

Chiral chromatography

Chiral chromatography involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three-dimensional mirror images. Conventional chromatography or other separation processes are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes. Chiral chromatography HPLC columns (with a chiral stationary phase) in both normal and reversed phase are commercially available.

Aqueous normal-phase chromatography

Aqueous normal-phase (ANP) chromatography is characterized by the elution behavior of classical normal phase mode (i.e. where the mobile phase is significantly less polar than the stationary phase) in which water is one of the mobile phase solvent system components. It is distinguished from hydrophilic interaction liquid chromatography (HILIC) in that the retention mechanism is due to adsorption rather than partitioning.

Applications

Chromatography is used in many fields including the pharmaceutical industry, the food and beverage industry, the chemical industry, forensic science, environmentanalysis, and hospitals.

Chapter 2

History of Chromatography

The **history of chromatography** spans from the mid-19th century to the 21st. 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 (which is green) and carotenoids (which are orange and yellow). New forms of chromatography developed in the 1930s and 1940s made the technique useful for a wide range of separation processes and chemical analysis tasks, especially in biochemistry.

Precursors

The earliest use of chromatography—passing a mixture through an inert material to create separation of the solution components based on differential adsorption—is sometimes attributed to German chemist Friedlieb Ferdinand Runge, who in 1855 described the use of paper to analyze dyes. Runge dropped spots of different inorganic chemicals onto circles of filter paper already impregnated with another chemical, and reactions between the different chemicals created unique color patterns. According to historical analysis of L. S. Ettre, however, Runge's work had "nothing to do with chromatography" (and instead should be considered a precursor of chemical spot tests such as the Schiff test).

In the 1860s, Christian Friedrich Schönbein and his student Friedrich Goppelsroeder published the first attempts to study

the different rates at which different substances move through filter paper. Schönbein, who thought capillary action (rather than adsorption) was responsible for the movement, called the technique capillary analysis, and Goppelsroeder spent much of his career using capillary analysis to test the movement rates of a wide variety of substances. Unlike modern paper chromatography, capillary analysis used reservoirs of the substance being analyzed, creating overlapping zones of the solution components rather than separate points or bands.

Work on capillary analysis continued, but without much technical development, well into the 20th century. The first significant advances over Goppelsroeder's methods came with the work of Raphael E. Liesegang: in 1927, he placed filter strips in closed containers with atmospheres saturated by solvents, and in 1943 he began using discrete spots of sample adsorbed to filter paper, dipped in pure solvent to achieve separation.

This method, essentially identical to modern paper chromatography, was published just before the independent—and far more influential—work of Archer Martin and his collaborators that inaugurated the widespread use of paper chromatography.

In 1897, the American chemist David Talbot Day (1859–1915), then serving with the U.S. Geological Survey, observed that crude petroleum generated bands of color as it seeped upwards through finely divided clay or limestone. In 1900, he reported his findings at the First International Petroleum Congress in Paris, where they created a sensation.

Tsvet and column chromatography

The first true chromatography is usually attributed to the Russian-Italian botanist Mikhail Tsvet. Tsvet applied his observations with filter paper extraction to the new methods of column fractionation that had been developed in the 1890s for separating the components of petroleum. He used a liquid-adsorption column containing calcium carbonate to separate yellow, orange, and green plant pigments (what are known today as xanthophylls, carotenes, and chlorophylls, respectively). The method was described on December 30, 1901 at the 11th Congress of Naturalists and Doctors (XI съездестествоиспытателейиврачей) in Saint Petersburg. The first printed description was in 1903, in the Proceedings of the Warsaw Society of Naturalists, section of biology. He first used the term *chromatography* in print in 1906 in his two papers about chlorophyll in the German botanical journal, *Berichte der Deutschen Botanischen Gesellschaft.* In 1907 he demonstrated his chromatograph for the German Botanical Society. Mikhail's surname "Цвет" means "color" in Russian, so there is the possibility that his naming the procedure chromatography (literally "color writing") was a way that he could make sure that he, a commoner in Tsarist Russia, could be immortalized.

In a 1903 lecture (published in 1905), Tsvet also described using filter paper to approximate the properties of living plant fibers in his experiments on plant pigments—a precursor to paper chromatography. He found that he could extract some pigments (such as orange carotenes and yellow xanthophylls) from leaves with non-polar solvents, but others (such as

chlorophyll) required polar solvents. He reasoned that chlorophyll was held to the plant tissue by adsorption, and that stronger solvents were necessary to overcome the adsorption. To test this, he applied dissolved pigments to filter paper, allowed the solvent to evaporate, then applied different solvents to see which could extract the pigments from the filter paper. He found the same pattern as from leaf extractions: carotene could be extracted from filter paper using non-polar solvents, but chlorophyll required polar solvents.

Tsvet's work saw little use until the 1930s.

Martin and Synge and partition chromatography

Chromatography methods changed little after Tsvet's work until the explosion of mid-20th century research in new techniques, particularly thanks to the work of Archer John Porter Martin and Richard Laurence Millington Synge. By "the marrying of two techniques, that of chromatography and that of countercurrent solvent extraction", Martin and Synge developed partition chromatography to separate chemicals with only slight differences in partition coefficients between two liquid solvents. Martin, who had previously been working in vitamin chemistry (including attempts to purify vitamin E), began collaborating with Synge in 1938, brought his experience with equipment design to Synge's project of separating amino acids. After unsuccessful experiments with complex countercurrent extraction machines and liquid-liquid chromatography methods where the liquids move in opposite directions, Martin hit on the idea of using silica gel in columns

to hold water stationary while an organic solvent flows through the column. Martin and Synge demonstrated the potential of the methods by separating amino acids marked in the column by the addition of methyl red. In a series of publications beginning in 1941, they described increasingly powerful methods of separating amino acids and other organic chemicals.

In pursuit of better and easier methods of identifying the amino acid constituents of peptides, Martin and Synge turned to other chromatography media as well.

A short abstract in 1943 followed by a detailed article in 1944 described the use of filter paper as the stationary phase for performing chromatography on amino acids: paper chromatography. By 1947, Martin, Synge and their collaborators had applied this method (along with Fred Sanger's reagent for identifying N-terminal residues) to determine the pentapeptide sequence of Gramicidin S. These and related paper chromatography methods were also foundational to Fred Sanger's effort to determine the amino acid sequence of insulin.

Refining the techniques

Martin, in collaboration with Anthony T. James, went on to develop gas chromatography (GC; the principles of which Martin and Synge had predicted in their landmark 1941 paper) beginning in 1949. In 1952, during his lecture for the Nobel Prize in Chemistry (shared with Synge, for their earlier chromatography work), Martin announced the successful separation of a wide variety of natural compounds by gas

chromatography. Previously, Erika Cremer had laid the theoretical basis of GC in 1944 and Austrian chemist Fritz Prior, under the direction of Erika Cremer, constructed in 1947 the first prototype of a gas chromatograph and achieved separating oxygen and carbon dioxide, in 1947 during his Ph.D. research.

The ease and efficiency of gas chromatography for separating organic chemicals spurred the rapid adoption of the method, as well as the rapid development of new detection methods for analyzing the output.

The thermal conductivity detector, described in 1954 by N. H. Ray, was the foundation for several other methods: the flame ionization detector was described by J. Harley, W. Nel, and V. Pretorius in 1958, and James Lovelock introduced the electron capture detector that year as well. Others introduced mass spectrometers to gas chromatography in the late 1950s.

The work of Martin and Synge also set the stage for high performance liquid chromatography, suggesting that small sorbent particles and pressure could produce fast liquid chromatography techniques. This became widely practical by the late 1960s (and the method was used to separate amino acids as early as 1960).

Thin layer chromatography

The first developments in thin layer chromatography occurred in the 1940s, and techniques advanced rapidly in the 1950s after the introduction of relatively large plates and relatively stable materials for sorbent layers.

Later developments

In 1987 Pedro Cuatrecasas and Meir Wilchek were awarded the Wolf Prize in Medicine for the invention and development of affinity chromatography and its applications to biomedical sciences.

Chapter 3

Gas and Affinity Chromatography

Gas chromatography

Gas chromatography (**GC**) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

Gas chromatography is also sometimes known as **vapor-phase chromatography** (VPC), or **gas–liquid partition chromatography** (GLPC). These alternative names, as well as their respective abbreviations, are frequently used in scientific literature.

Gas chromatography is the process of separating compounds in a mixture by injecting a gaseous or liquid sample into a mobile phase, typically called the carrier gas, and passing the gas through a stationary phase. The mobile phase is usually an inert gas or an unreactive gas such as helium, argon, nitrogen or hydrogen. The stationary phase is a microscopic layer of viscous liquid on a surface of solid particles on an inert solid support inside a piece of glass or metal tubing called a column. The surface of the solid particles may also act as the stationary phase in some columns. The glass or metal column through which the gas phase passes is located in an oven

where the temperature of the gas can be controlled and the eluent coming off the column is monitored by a computerized detector.

History

Chromatography dates to 1903 in the work of the Russian scientist, Mikhail Semenovich Tswett, who separated plant pigments via liquid column chromatography. German physical chemistErika Cremer in 1947 together with Austrian graduate student Fritz Prior developed the theoretical foundations of GC and built the first liquid-gas chromatograph, but her work was deemed irrelevant and was ignored for a long time. English chemists Archer Martin and Richard Synge received a Nobel Prize in 1952 for the invention of partition chromatography in the 1940s, laying the foundations of gas chromatography. The popularity of gas chromatography quickly rose after the development of the flame ionization detector.

GC analysis

A **gas chromatograph** is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph is made up of a narrow flow-through tube, known as the column, through which the sample passes in a gas stream (the carrier gas) at different rates depending on their various chemical and physical properties and their interaction with a specific column lining or filling, called the 'stationary phase'. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different

components, causing each one to exit the column at a different time. Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature.

In a GC analysis, a known volume of gaseous or liquid analyte is injected through a rubber disk and into a hot, temperature controlled, port attached to the column. As the carrier gas transports the analyte molecules through the column, there is adsorption of the analyte molecules either onto the column walls or onto packing materials (stationary phase) in the column to give separation. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time). A detector is used to monitor the time at which each component reaches the outlet and ultimately the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order in which they elute from the column and by the retention time of the analyte in the column.

Physical components

Autosamplers

- The autosampler provides the means to introduce a sample automatically into the inlets. Manual insertion of the sample is possible but is no longer common. Automatic insertion provides better reproducibility and time-optimization.

Different kinds of autosamplers exist. Autosamplers can be classified in relation to sample capacity (auto-injectors vs. autosamplers, where auto-injectors can work a small number of samples), to robotic technologies (XYZ robot vs. rotating robot – the most common), or to analysis:

- Liquid
- Static head-space by syringe technology
- Dynamic head-space by transfer-line technology
- Solid phase microextraction (SPME)

Inlets

The column inlet (or injector) provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the column head.

Common inlet types are:

- S/SL (split/splitless) injector; a sample is introduced into a heated small chamber via a syringe through a septum – the heat facilitates volatilization of the sample and sample matrix. The carrier gas then either sweeps the entirety (splitless mode) or a portion (split mode) of the sample into the column. In split mode, a part of the sample/carrier gas mixture in the injection chamber is exhausted through the split vent. Split injection is preferred when working with samples with high analyte concentrations (>0.1%) whereas splitless injection is best suited for trace analysis with low amounts of analytes (<0.01%). In splitless mode the split valve opens after a pre-set amount of time to purge heavier

elements that would otherwise contaminate the system. This pre-set (splitless) time should be optimized, the shorter time (e.g., 0.2 min) ensures less tailing but loss in response, the longer time (2 min) increases tailing but also signal.

- On-column inlet; the sample is here introduced directly into the column in its entirety without heat, or at a temperature below the boiling point of the solvent. The low temperature condenses the sample into a narrow zone. The column and inlet can then be heated, releasing the sample into the gas phase. This ensures the lowest possible temperature for chromatography and keeps samples from decomposing above their boiling point.
- PTV injector; Temperature-programmed sample introduction was first described by Vogt in 1979. Originally Vogt developed the technique as a method for the introduction of large sample volumes (up to 250 μL) in capillary GC. Vogt introduced the sample into the liner at a controlled injection rate. The temperature of the liner was chosen slightly below the boiling point of the solvent. The low-boiling solvent was continuously evaporated and vented through the split line. Based on this technique, Poy developed the programmed temperature vaporising injector; PTV. By introducing the sample at a low initial liner temperature many of the disadvantages of the classic hot injection techniques could be circumvented.
- Gas source inlet or gas switching valve; gaseous samples in collection bottles are connected to what is most commonly a six-port switching valve. The

carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated sample loop. Upon switching, the contents of the sample loop are inserted into the carrier gas stream.

- P/T (Purge-and-Trap) system; An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix. The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature. The trap is then heated and the volatiles are directed into the carrier gas stream. Samples requiring preconcentration or purification can be introduced via such a system, usually hooked up to the S/SL port.

The choice of carrier gas (mobile phase) is important. Hydrogen has a range of flow rates that are comparable to helium in efficiency. However, helium may be more efficient and provide the best separation if flow rates are optimized. Helium is non-flammable and works with a greater number of detectors and older instruments. Therefore, helium is the most common carrier gas used. However, the price of helium has gone up considerably over recent years, causing an increasing number of chromatographers to switch to hydrogen gas. Historical use, rather than rational consideration, may contribute to the continued preferential use of helium.

Detectors

Commonly used detectors are the flame ionization detector (FID) and the thermal conductivity detector (TCD). While TCDs are beneficial in that they are non-destructive, its low

detection limit for most analytes inhibits widespread use. FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. FIDs cannot detect water or carbon dioxide which make them ideal for environmental organic analyte analysis. FID is two to three times more sensitive to analyte detection than TCD.

The TCD relies on the thermal conductivity of matter passing around a thin wire of tungsten-rhenium with a current traveling through it. In this set up helium or nitrogen serve as the carrier gas because of their relatively high thermal conductivity which keep the filament cool and maintain uniform resistivity and electrical efficiency of the filament. When analyte molecules elute from the column, mixed with carrier gas, the thermal conductivity decreases while there is an increase in filament temperature and resistivity resulting in fluctuations in voltage ultimately causing a detector response. Detector sensitivity is proportional to filament current while it is inversely proportional to the immediate environmental temperature of that detector as well as flow rate of the carrier gas.

In a flame ionization detector (FID), electrodes are placed adjacent to a flame fueled by hydrogen / air near the exit of the column, and when carbon containing compounds exit the column they are pyrolyzed by the flame. This detector works only for organic / hydrocarbon containing compounds due to the ability of the carbons to form cations and electrons upon pyrolysis which generates a current between the electrodes. The increase in current is translated and appears as a peak in a chromatogram. FIDs have low detection limits (a few picograms per second) but they are unable to generate ions

from carbonyl containing carbons. FID compatible carrier gasses include helium, hydrogen, nitrogen, and argon.

Alkali flame detector (AFD) or alkali flame ionization detector (AFID) has high sensitivity to nitrogen and phosphorus, similar to NPD. However, the alkaline metal ions are supplied with the hydrogen gas, rather than a bead above the flame. For this reason AFD does not suffer the "fatigue" of the NPD, but provides a constant sensitivity over long period of time. In addition, when alkali ions are not added to the flame, AFD operates like a standard FID. A catalytic combustion detector (CCD) measures combustible hydrocarbons and hydrogen. Discharge ionization detector (DID) uses a high-voltage electric discharge to produce ions.

The polyarc reactor is an add-on to new or existing GC-FID instruments that converts all organic compounds to methane molecules prior to their detection by the FID. This technique can be used to improve the response of the FID and allow for the detection of many more carbon-containing compounds. The complete conversion of compounds to methane and the now equivalent response in the detector also eliminates the need for calibrations and standards because response factors are all equivalent to those of methane. This allows for the rapid analysis of complex mixtures that contain molecules where standards are not available.

Flame photometric detector (FPD) uses a photomultiplier tube to detect spectral lines of the compounds as they are burned in a flame. Compounds eluting off the column are carried into a hydrogen fueled flame which excites specific elements in the molecules, and the excited elements (P,S, Halogens, Some

Metals) emit light of specific characteristic wavelengths. The emitted light is filtered and detected by a photomultiplier tube. In particular, phosphorus emission is around 510–536 nm and sulfur emission is at 394 nm. With an atomic emission detector (AED), a sample eluting from a column enters a chamber which is energized by microwaves that induce a plasma. The plasma causes the analyte sample to decompose and certain elements generate an atomic emission spectra. The atomic emission spectra is diffracted by a diffraction grating and detected by a series of photomultiplier tubes or photo diodes.

Electron capture detector (ECD) uses a radioactive beta particle (electron) source to measure the degree of electron capture. ECD are used for the detection of molecules containing electronegative / withdrawing elements and functional groups like halogens, carbonyl, nitriles, nitro groups, and organometalics. In this type of detector either nitrogen or 5% methane in argon is used as the mobile phase carrier gas. The carrier gas passes between two electrodes placed at the end of the column, and adjacent to the cathode (negative electrode) resides a radioactive foil such as 63Ni. The radioactive foil emits a beta particle (electron) which collides with and ionizes the carrier gas to generate more ions resulting in a current. When analyte molecules with electronegative / withdrawing elements or functional groups electrons are captured which results in a decrease in current generating a detector response.

Nitrogen–phosphorus detector (NPD), a form of thermionic detector where nitrogen and phosphorus alter the work function on a specially coated bead and a resulting current is measured.

Dry electrolytic conductivity detector (DELCD) uses an air phase and high temperature (v. Coulsen) to measure chlorinated compounds.

Mass spectrometer (MS), also called GC-MS; highly effective and sensitive, even in a small quantity of sample. This detector can be used to identify the analytes in chromatograms by their mass spectrum. Some GC-MS are connected to an NMR spectrometer which acts as a backup detector. This combination is known as GC-MS-NMR. Some GC-MS-NMR are connected to an infrared spectrophotometer which acts as a backup detector. This combination is known as GC-MS-NMR-IR. It must, however, be stressed this is very rare as most analyses needed can be concluded via purely GC-MS.

Vacuum ultraviolet (VUV) represents the most recent development in gas chromatography detectors. Most chemical species absorb and have unique gas phase absorption cross sections in the approximately 120–240 nm VUV wavelength range monitored. Where absorption cross sections are known for analytes, the VUV detector is capable of absolute determination (without calibration) of the number of molecules present in the flow cell in the absence of chemical interferences.

Olfactometric detector, also called GC-O, uses a human assessor to analyse the odour activity of compounds. With an odour port or a sniffing port, the quality of the odour, the intensity of the odour and the duration of the odour activity of a compound can be assessed.

Other detectors include the Hall electrolytic conductivity detector (ElCD), helium ionization detector (HID), infrared

detector (IRD), photo-ionization detector (PID), pulsed discharge ionization detector (PDD), and thermionic ionization detector (TID).

Methods

The method is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate and/or ideal for the analysis required.

Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, column temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique. Depending on the detector(s) (see below) installed on the GC, there may be a number of detector conditions that can also be varied. Some GCs also include valves which can change the route of sample and carrier flow. The timing of the opening and closing of these valves can be important to method development.

Carrier gas selection and flow rates

Typical carrier gases include helium, nitrogen, argon, and hydrogen. Which gas to use is usually determined by the detector being used, for example, a DID requires helium as the carrier gas. When analyzing gas samples the carrier is also selected based on the sample's matrix, for example, when analyzing a mixture in argon, an argon carrier is preferred because the argon in the sample does not show up on the

chromatogram. Safety and availability can also influence carrier selection. The purity of the carrier gas is also frequently determined by the detector, though the level of sensitivity needed can also play a significant role. Typically, purities of 99.995% or higher are used. The most common purity grades required by modern instruments for the majority of sensitivities are 5.0 grades, or 99.999% pure meaning that there is a total of 10 ppm of impurities in the carrier gas that could affect the results. The highest purity grades in common use are 6.0 grades, but the need for detection at very low levels in some forensic and environmental applications has driven the need for carrier gases at 7.0 grade purity and these are now commercially available. Trade names for typical purities include "Zero Grade," "Ultra-High Purity (UHP) Grade," "4.5 Grade" and "5.0 Grade."

The carrier gas linear velocity affects the analysis in the same way that temperature does (see above). The higher the linear velocity the faster the analysis, but the lower the separation between analytes. Selecting the linear velocity is therefore the same compromise between the level of separation and length of analysis as selecting the column temperature. The linear velocity will be implemented by means of the carrier gas flow rate, with regards to the inner diameter of the column.

With GCs made before the 1990s, carrier flow rate was controlled indirectly by controlling the carrier inlet pressure, or "column head pressure." The actual flow rate was measured at the outlet of the column or the detector with an electronic flow meter, or a bubble flow meter, and could be an involved, time consuming, and frustrating process. It was not possible to vary the pressure setting during the run, and thus the flow

was essentially constant during the analysis. The relation between flow rate and inlet pressure is calculated with Poiseuille's equation for compressible fluids.

Many modern GCs, however, electronically measure the flow rate, and electronically control the carrier gas pressure to set the flow rate. Consequently, carrier pressures and flow rates can be adjusted during the run, creating pressure/flow programs similar to temperature programs.

Stationary compound selection

The polarity of the solute is crucial for the choice of stationary compound, which in an optimal case would have a similar polarity as the solute. Common stationary phases in open tubular columns are cyanopropylphenyl dimethyl polysiloxane, carbowax polyethyleneglycol, biscyanopropyl cyanopropylphenyl polysiloxane and diphenyl dimethyl polysiloxane. For packed columns more options are available.

Inlet types and flow rates

The choice of inlet type and injection technique depends on if the sample is in liquid, gas, adsorbed, or solid form, and on whether a solvent matrix is present that has to be vaporized. Dissolved samples can be introduced directly onto the column via a COC injector, if the conditions are well known; if a solvent matrix has to be vaporized and partially removed, a S/SL injector is used (most common injection technique); gaseous samples (e.g., air cylinders) are usually injected using a gas switching valve system; adsorbed samples (e.g., on adsorbent tubes) are introduced using either an external (on-

line or off-line) desorption apparatus such as a purge-and-trap system, or are desorbed in the injector (SPME applications).

Sample size and injection technique

Sample injection

The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns. In the injection system in the capillary gas chromatograph the amount injected should not overload the column and the width of the injected plug should be small compared to the spreading due to the chromatographic process. Failure to comply with this latter requirement will reduce the separation capability of the column. As a general rule, the volume injected, V_{inj}, and the volume of the detector cell, V_{det}, should be about 1/10 of the volume occupied by the portion of sample containing the molecules of interest (analytes) when they exit the column.

Some general requirements which a good injection technique should fulfill are that it should be possible to obtain the column's optimum separation efficiency, it should allow accurate and reproducible injections of small amounts of representative samples, it should induce no change in sample composition, it should not exhibit discrimination based on differences in boiling point, polarity, concentration or thermal/catalytic stability, and it should be applicable for trace analysis as well as for undiluted samples.

However, there are a number of problems inherent in the use of syringes for injection. Even the best syringes claim an accuracy of only 3%, and in unskilled hands, errors are much larger. The needle may cut small pieces of rubber from the septum as it injects sample through it. These can block the needle and prevent the syringe filling the next time it is used. It may not be obvious that this has happened. A fraction of the sample may get trapped in the rubber, to be released during subsequent injections. This can give rise to ghost peaks in the chromatogram. There may be selective loss of the more volatile components of the sample by evaporation from the tip of the needle.

Column selection

The choice of column depends on the sample and the active measured. The main chemical attribute regarded when choosing a column is the polarity of the mixture, but functional groups can play a large part in column selection. The polarity of the sample must closely match the polarity of the column stationary phase to increase resolution and separation while reducing run time. The separation and run time also depends on the film thickness (of the stationary phase), the column diameter and the column length.

Column temperature and temperature program

The column(s) in a GC are contained in an oven, the temperature of which is precisely controlled electronically. (When discussing the "temperature of the column," an analyst is technically referring to the temperature of the column oven.

The distinction, however, is not important and will not subsequently be made in this article.)

The rate at which a sample passes through the column is directly proportional to the temperature of the column. The higher the column temperature, the faster the sample moves through the column. However, the faster a sample moves through the column, the less it interacts with the stationary phase, and the less the analytes are separated.

In general, the column temperature is selected to compromise between the length of the analysis and the level of separation.

A method which holds the column at the same temperature for the entire analysis is called "isothermal." Most methods, however, increase the column temperature during the analysis, the initial temperature, rate of temperature increase (the temperature "ramp"), and final temperature are called the temperature program.

A temperature program allows analytes that elute early in the analysis to separate adequately, while shortening the time it takes for late-eluting analytes to pass through the column.

Data reduction and analysis

Qualitative analysis

Generally, chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis), which is called a chromatogram. This provides a spectrum of peaks for a sample representing the analytes present in a

sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant. Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes. However, in most modern applications, the GC is connected to a mass spectrometer or similar detector that is capable of identifying the analytes represented by the peaks.

Quantitative analysis

The area under a peak is proportional to the amount of analyte present in the chromatogram. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined.

Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of an analyte.

The relative response factor is the expected ratio of an analyte to an internal standard (or external standard) and is calculated by finding the response of a known amount of analyte and a constant amount of internal standard (a chemical added to the sample at a constant concentration, with a distinct retention time to the analyte).

In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

Applications

In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. The samples are also required to be salt-free; they should not contain ions. Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

Various temperature programs can be used to make the readings more meaningful; for example to differentiate between substances that behave similarly during the GC process.

Professionals working with GC analyze the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water. GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.

In practical courses at colleges, students sometimes get acquainted to the GC by studying the contents of Lavender oil or measuring the ethylene that is secreted by *Nicotiana benthamiana* plants after artificially injuring their leaves. These GC analyse hydrocarbons (C2-C40+). In a typical experiment, a packed column is used to separate the light gases, which are then detected with a TCD. The hydrocarbons are separated using a capillary column and detected with a FID. A complication with light gas analyses that include H_2 is

that He, which is the most common and most sensitive inert carrier (sensitivity is proportional to molecular mass) has an almost identical thermal conductivity to hydrogen (it is the difference in thermal conductivity between two separate filaments in a Wheatstone Bridge type arrangement that shows when a component has been eluted). For this reason, dual TCD instruments used with a separate channel for hydrogen that uses nitrogen as a carrier are common. Argon is often used when analysing gas phase chemistry reactions such as F-T synthesis so that a single carrier gas can be used rather than two separate ones. The sensitivity is reduced, but this is a trade off for simplicity in the gas supply.

Gas chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

Affinity chromatography

Affinity chromatography is a method of separating a biomolecule from a mixture, based on a highly specific macromolecular binding interaction between the biomolecule and another substance. The specific type of binding interaction depends on the biomolecule of interest; antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid binding interactions are frequently exploited for isolation of various biomolecules. Affinity chromatography is useful for its high selectivity and resolution of separation, compared to other chromatographic methods.

Principle

Affinity chromatography takes advantage of specific binding interactions between the analyte of interest (normally dissolved in the mobile phase), and a binding partner or ligand (immobilized on the stationary phase). In a typical affinity chromatography experiment, the ligand is attached to a solid, insoluble matrix--usually a polymer such as agarose or polyacrylamide--chemically modified to introduce reactive functional groups with which the ligand can react, forming stable covalent bonds. The stationary phase is first loaded into a column to which the mobile phase is introduced. Molecules that bind to the ligand will remain associated with the stationary phase.

A wash buffer is then applied to remove non-target biomolecules by disrupting their weaker interactions with the stationary phase, while the biomolecules of interest will remain bound. Target biomolecules may then be removed by applying a so-called elution buffer, which disrupts interactions between the bound target biomolecules and the ligand. The target molecule is thus recovered in the eluting solution.

Affinity chromatography does not require the molecular weight, charge, hydrophobicity, or other physical properties of the analyte of interest to be known, although knowledge of its binding properties is useful in the design of a separation protocol.

Batch and column setups

Binding to the solid phase may be achieved by column chromatography whereby the solid medium is packed onto a column, the initial mixture run through the column to allow settling, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using a batch treatment, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the elute.

Sometimes a hybrid method is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

The ligands used in affinity chromatography are obtained from both organic and inorganic sources. Examples of biological sources are serum proteins, lectins and antibodies. Inorganic sources as moronic acts, metal chelates and triazine dyes.

A third method, expanded bed absorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensure that the solid phase does not exit the column with the liquid phase.

Affinity columns can be eluted by changing salt concentrations, pH, pI, charge and ionic strength directly or through a gradient to resolve the particles of interest.

More recently, setups employing more than one column in series have been developed. The advantage compared to single column setups is that the resin material can be fully loaded since non-binding product is directly passed on to a consecutive column with fresh column material. These chromatographic processes are known as periodic counter-current chromatography (PCC). The resin costs per amount of produced product can thus be drastically reduced. Since one column can always be eluted and regenerated while the other column is loaded, already two columns are sufficient to make full use of the advantages. Additional columns can give additional flexibility for elution and regeneration times, at the cost of additional equipment and resin costs.

Specific uses

Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts, and purification from blood.

By using affinity chromatography, one can separate proteins that bind to a certain fragment from proteins that do not bind that specific fragment. Because this technique of purification relies on the biological properties of the protein needed, it is a useful technique and proteins can be purified many folds in one step.

Various affinity media

Many different affinity media exist for a variety of possible uses. Briefly, they are (generalized) activated/functionalized that work as a functional spacer, support matrix, and eliminates handling of toxic reagents.

Amino acid media is used with a variety of serum proteins, proteins, peptides, and enzymes, as well as rRNA and dsDNA. Avidin biotin media is used in the purification process of biotin/avidin and their derivatives.

Carbohydrate bonding is most often used with glycoproteins or any other carbohydrate-containing substance; carbohydrate is used with lectins, glycoproteins, or any other carbohydrate metabolite protein. Dye ligand media is nonspecific but mimics biological substrates and proteins. Glutathione is useful for separation of GST tagged recombinant proteins. Heparin is a generalized affinity ligand, and it is most useful for separation of plasma coagulation proteins, along with nucleic acid enzymes and lipases

Hydrophobic interaction media are most commonly used to target free carboxyl groups and proteins.

Immunoaffinity media (detailed below) utilizes antigens' and antibodies' high specificity to separate; immobilized metal affinity chromatography is detailed further below and uses interactions between metal ions and proteins (usually specially tagged) to separate; nucleotide/coenzyme that works to separate dehydrogenases, kinases, and transaminases.

Nucleic acids function to trap mRNA, DNA, rRNA, and other nucleic acids/oligonucleotides. Protein A/G method is used to purify immunoglobulins.

Speciality media are designed for a specific class or type of protein/co enzyme; this type of media will only work to separate a specific protein or coenzyme.

Immunoaffinity

Another use for the procedure is the affinity purification of antibodies from blood serum. If the serum is known to contain antibodies against a specific antigen (for example if the serum comes from an organism immunized against the antigen concerned) then it can be used for the affinity purification of that antigen. This is also known as Immunoaffinity Chromatography. For example,,if an organism is immunised against a GST-fusion protein it will produce antibodies against the fusion-protein, and possibly antibodies against the GST tag as well. The protein can then be covalently coupled to a solid support such as agarose and used as an affinity ligand in purifications of antibody from immune serum.

For thoroughness, the GST protein and the GST-fusion protein can each be coupled separately. The serum is initially allowed to bind to the GST affinity matrix. This will remove antibodies against the GST part of the fusion protein. The serum is then separated from the solid support and allowed to bind to the GST-fusion protein matrix. This allows any antibodies that recognize the antigen to be captured on the solid support. Elution of the antibodies of interest is most often achieved using a low pH buffer such as glycine pH 2.8. The eluate is

collected into a neutral tris or phosphate buffer, to neutralize the low pH elution buffer and halt any degradation of the antibody's activity. This is a nice example as affinity purification is used to purify the initial GST-fusion protein, to remove the undesirable anti-GST antibodies from the serum and to purify the target antibody.

Monoclonal antibodies can also be selected to bind proteins with great specificity, where protein is released under fairly gentle conditions. This can become of use for further research in the future.

A simplified strategy is often employed to purify antibodies generated against peptide antigens. When the peptide antigens are produced synthetically, a terminal cysteine residue is added at either the N- or C-terminus of the peptide. This cysteine residue contains a sulfhydryl functional group which allows the peptide to be easily conjugated to a carrier protein (e.g. Keyhole limpet hemocyanin (KLH)). The same cysteine-containing peptide is also immobilized onto an agarose resin through the cysteine residue and is then used to purify the antibody.

Most monoclonal antibodies have been purified using affinity chromatography based on immunoglobulin-specific Protein A or Protein G, derived from bacteria.

Immunoaffinity chromatography with monoclonal antibodies immobilized on monolithic column has been successfully used to capture extracellular vesicles (e.g., exosomes and exomeres) from human blood plasma by targeting tetraspanins and integrins found on the surface of the EVs.

Immunoaffinity chromatography is also the basis for immunochromatographic test (ICT) strips, which provide a rapid means of diagnosis in patient care. Using ICT, a technician can make a determination at a patient's bedside, without the need for a laboratory. ICT detection is highly specific to the microbe causing an infection.

Immobilized metal ion affinity chromatography

Immobilized metal ion affinity chromatography (IMAC) is based on the specific coordinate covalent bond of amino acids, particularly histidine, to metals. This technique works by allowing proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions, such as cobalt, nickel, or copper for the purification of histidine-containing proteins or peptides, iron, zinc or gallium for the purification of phosphorylated proteins or peptides. Many naturally occurring proteins do not have an affinity for metal ions, therefore recombinant DNA technology can be used to introduce such a protein tag into the relevant gene. Methods used to elute the protein of interest include changing the pH, or adding a competitive molecule, such as imidazole.

Recombinant proteins

Possibly the most common use of affinity chromatography is for the purification of recombinant proteins. Proteins with a known affinity are protein tagged in order to aid their purification. The protein may have been genetically modified so as to allow it to be selected for affinity binding; this is known as a fusion protein. Tags include hexahistidine (His), glutathione-S-transferase (GST) and maltose binding protein

(MBP). Histidine tags have an affinity for nickel, cobalt, zinc, copper and iron ions which have been immobilized by forming coordinate covalent bonds with a chelator incorporated in the stationary phase. For elution, an excess amount of a compound able to act as a metal ion ligand, such as imidazole, is used. GST has an affinity for glutathione which is commercially available immobilized as glutathione agarose. During elution, excess glutathione is used to displace the tagged protein.

Lectins

Lectin affinity chromatography is a form of affinity chromatography where lectins are used to separate components within the sample. Lectins, such as concanavalin A are proteins which can bind specific alpha-D-mannose and alpha-D-glucose carbohydrate molecules. Some common carbohydrate molecules that is used in lectin affinity chromatography are Con A-Sepharose and WGA-agarose. Another example of a lectin is wheat germ agglutinin which binds D-N-acetyl-glucosamine. The most common application is to separate glycoproteins from non-glycosylated proteins, or one glycoform from another glycoform. Although there are various ways to perform lectin affinity chromatography, the goal is extract a sugar ligand of the desired protein.

Specialty

Another use for affinity chromatography is the purification of specific proteins using a gel matrix that is unique to a specific protein. For example, the purification of E. coli β-galactosidase is accomplished by affinity chromatography using p-aminobenyl-1-thio-β-D-galactopyranosyl agarose as the affinity

matrix. p-aminobenyl-1-thio-β-D-galactopyranosyl agarose is used as the affinity matrix because it contains a galactopyranosyl group, which serves as a good substrate analog for E.Coli-B-Galactosidase. This property allows the enzyme to bind to the stationary phase of the affinity matrix and is eluted by adding increasing concentrations of salt to the column.

Alkaline phosphatase

Alkaline phosphatase from E. coli can be purified using a DEAE-Cellulose matrix. A. phosphatase has a slight negative charge, allowing it to weakly bind to the positively charged amine groups in the matrix. The enzyme can then be eluted out by adding buffer with higher salt concentrations.

Boronate affinity chromatography

Boronate affinity chromatography consists of using boronic acid or boronates to elute and quantify amounts of glycoproteins. Clinical adaptations have applied this type of chromatography for use in determining long term assessment of diabetic patients through analysis of their glycated hemoglobin.

Serum albumin purification

Of many uses of affinity chromatography, one use of it is seen in affinity purification of albumin and macroglobulin contamination. This type of purification is helpful in removing excess albumin and α_2-macroglobulin contamination, when performing mass spectrometry. In affinity purification of serum

albumin, the stationary used for collecting or attracting serum proteins can be Cibacron Blue-Sepharose. Then the serum proteins can be eluted from the adsorbent with a buffer containing thiocyanate (SCN).

Weak affinity chromatography

Weak affinity chromatography (WAC) is an affinity chromatography technique for affinity screening in drug development. WAC is an affinity-based liquid chromatographic technique that separates chemical compounds based on their different weak affinities to an immobilized target. The higher affinity a compound has towards the target, the longer it remains in the separation unit, and this will be expressed as a longer retention time. The affinity measure and ranking of affinity can be achieved by processing the obtained retention times of analyzed compounds.

The WAC technology is demonstrated against a number of different protein targets – proteases, kinases, chaperones and protein–protein interaction (PPI) targets. WAC has been shown to be more effective than established methods for fragment based screening.

History

Affinity chromatography was conceived and first developed by Pedro Cuatrecasas and Meir Wilchek.

Chapter 4

Ion and Size-exclusion Chromatography

Ion chromatography

Ion chromatography (or **ion-exchange chromatography**) separates ions and polar molecules based on their affinity to the ion exchanger. It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids. However, ion chromatography must be done in conditions that are one unit away from the isoelectric point of a protein.

The two types of ion chromatography are anion-exchange and cation-exchange. Cation-exchange chromatography is used when the molecule of interest is positively charged. The molecule is positively charged because the pH for chromatography is less than the pI (a/k/a pH(I)). In this type of chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it. Anion-exchange chromatography is when the stationary phase is positively charged and negatively charged molecules (meaning that pH for chromatography is greater than the pI) are loaded to be attracted to it. It is often used in protein purification, water analysis, and quality control. The water-soluble and charged molecules such as proteins, amino acids, and peptides bind to moieties which are oppositely charged by forming ionic bonds to the insoluble stationary phase. The

equilibrated stationary phase consists of an ionizable functional group where the targeted molecules of a mixture to be separated and quantified can bind while passing through the column—a cationic stationary phase is used to separate anions and an anionic stationary phase is used to separate cations. Cation exchange chromatography is used when the desired molecules to separate are cations and anion exchange chromatography is used to separate anions. The bound molecules then can be eluted and collected using an eluant which contains anions and cations by running higher concentration of ions through the column or changing pH of the column.

One of the primary advantages for the use of ion chromatography is only one interaction involved during the separation as opposed to other separation techniques; therefore, ion chromatography may have higher matrix tolerance. Another advantage of ion exchange is the predictability of elution patterns (based on the presence of the ionizable group). For example, when cation exchange chromatography is used, cations will elute out last. Meanwhile, the negative charged molecules will elute out first. However, there are also disadvantages involved when performing ion-exchange chromatography, such as constant evolution with the technique which leads to the inconsistency from column to column. A major limitation to this purification technique is that it is limited to ionizable group.

History

Ion chromatography has advanced through the accumulation of knowledge over a course of many years. Starting from 1947,

Spedding and Powell used displacement ion-exchange chromatography for the separation of the rare earths. Additionally, they showed the ion-exchange separation of 14N and 15N isotopes in ammonia. At the start of the 1950s, Kraus and Nelson demonstrated the use of many analytical methods for metal ions dependent on their separation of their chloride, fluoride, nitrate or sulfate complexes by anion chromatography. Automatic in-line detection was progressively introduced from 1960 to 1980 as well as novel chromatographic methods for metal ion separations. A groundbreaking method by Small, Stevens and Bauman at Dow Chemical Co. unfolded the creation of the modern ion chromatography. Anions and cations could now be separated efficiently by a system of suppressed conductivity detection. In 1979, a method for anion chromatography with non-suppressed conductivity detection was introduced by Gjerde et al. Following it in 1980, was a similar method for cation chromatography.

As a result, a period of extreme competition began within the IC market, with supporters for both suppressed and non-suppressed conductivity detection. This competition led to fast growth of new forms and the fast evolution of IC. A challenge that needs to be overcome in the future development of IC is the preparation of highly efficient monolithic ion-exchange columns and overcoming this challenge would be of great importance to the development of IC.

The boom of Ion exchange chromatography primarily began between 1935–1950 during World War II and it was through the "Manhattan project" that applications and IC were significantly extended. Ion chromatography was originally

introduced by two English researchers, agricultural Sir Thompson and chemist J T Way. The works of Thompson and Way involved the action of water-soluble fertilizer salts, ammonium sulfate and potassium chloride. These salts could not easily be extracted from the ground due to the rain. They performed ion methods to treat clays with the salts, resulting in the extraction of ammonia in addition to the release of calcium. It was in the fifties and sixties that theoretical models were developed for IC for further understanding and it was not until the seventies that continuous detectors were utilized, paving the path for the development from low-pressure to high-performance chromatography. Not until 1975 was "ion chromatography" established as a name in reference to the techniques, and was thereafter used as a name for marketing purposes. Today IC is important for investigating aqueous systems, such as drinking water. It is a popular method for analyzing anionic elements or complexes that help solve environmentally relevant problems. Likewise, it also has great uses in the semiconductor industry.

Because of the abundant separating columns, elution systems, and detectors available, chromatography has developed into the main method for ion analysis.

When this technique was initially developed, it was primarily used for water treatment. Since 1935, ion exchange chromatography rapidly manifested into one of the most heavily leveraged techniques, with its principles often being applied to majority of fields of chemistry, including distillation, adsorption, and filtration.

Principle

- Ion-exchange chromatography separates molecules based on their respective charged groups. Ion-exchange chromatography retains analyte molecules on the column based on coulombic (ionic) interactions. The ion exchange chromatography matrix consists of positively and negatively charged ions. Essentially, molecules undergo electrostatic interactions with opposite charges on the stationary phase matrix. The stationary phase consists of an immobile matrix that contains charged ionizable functional groups or ligands. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. To achieve electroneutrality, these inert charges couple with exchangeable counterions in the solution. Ionizable molecules that are to be purified compete with these exchangeable counterions for binding to the immobilized charges on the stationary phase. These ionizable molecules are retained or eluted based on their charge. Initially, molecules that do not bind or bind weakly to the stationary phase are first to wash away. Altered conditions are needed for the elution of the molecules that bind to the stationary phase. The concentration of the exchangeable counterions, which competes with the molecules for binding, can be increased or the pH can be changed. A change in pH affects the charge on the particular molecules and, therefore, alters binding. The molecules then start eluting out based

on the changes in their charges from the adjustments. Further such adjustments can be used to release the protein of interest. Additionally, concentration of counterions can be gradually varied to separate ionized molecules. This type of elution is called gradient elution. On the other hand, step elution can be used in which the concentration of counterions are varied in one step. This type of chromatography is further subdivided into cation exchange chromatography and anion-exchange chromatography. Positively charged molecules bind to cation exchange resins while negatively charged molecules bind to anion exchange resins. The ionic compound consisting of the cationic species M+ and the anionic species B- can be retained by the stationary phase.

Procedure

Before ion-exchange chromatography can be initiated, it must be equilibrated. The stationary phase must be equilibrated to certain requirements that depend on the experiment that you are working with. Once equilibrated, the charged ions in the stationary phase will be attached to its opposite charged exchangeable ions. Exchangeable ions such as Cl- or Na+. Next, a buffer should be chosen in which the desired protein can bind to. After equilibration, the column needs to be washed. The washing phase will help elute out all impurities that does not bind to the matrix while the protein of interest remains bounded. This sample buffer needs to have the same pH as the buffer used for equilibration to help bind the desired

proteins. Uncharged proteins will be eluted out of the column at a similar speed of the buffer flowing through the column. Once the sample has been loaded onto to the column and the column has been washed with the buffer to elute out all non-desired proteins, elution is carried out to elute the desired proteins that are bound to the matrix. Bound proteins are eluted out by utilizing a gradient of linearly increasing salt concentration. With increasing ionic strength of the buffer, the salt ions will compete with the desired proteins in order to bind to charged groups on the surface of the medium. This will cause desired proteins to be eluted out of the column. Proteins that have a low net charge will be eluted out first as the salt concentration increases causing the ionic strength to increase. Proteins with high net charge will need a higher ionic strength for them to be eluted out of the column. It is possible to perform ion exchange chromatography in bulk, on thin layers of medium such as glass or plastic plates coated with a layer of the desired stationary phase, or in chromatography columns. Thin layer chromatography or column chromatography share similarities in that they both act within the same governing principles; there is constant and frequent exchange of molecules as the mobile phase travels along the stationary phase. It is not imperative to add the sample in minute volumes as the predetermined conditions for the exchange column have been chosen so that there will be strong interaction between the mobile and stationary phases. Furthermore, the mechanism of the elution process will cause a compartmentalization of the differing molecules based on their respective chemical characteristics. This phenomenon is due to an increase in salt concentrations at or near the top of the column, thereby displacing the molecules at that position, while molecules bound lower are released at a later point when

the higher salt concentration reaches that area. These principles are the reasons that ion exchange chromatography is an excellent candidate for initial chromatography steps in a complex purification procedure as it can quickly yield small volumes of target molecules regardless of a greater starting volume.

Comparatively simple devices are often used to apply counterions of increasing gradient to a chromatography column. Counterions such as copper (II) are chosen most often for effectively separating peptides and amino acids through complex formation.

A simple device can be used to create a salt gradient. Elution buffer is consistently being drawn from the chamber into the mixing chamber, thereby altering its buffer concentration. Generally, the buffer placed into the chamber is usually of high initial concentration, whereas the buffer placed into the stirred chamber is usually of low concentration. As the high concentration buffer from the left chamber is mixed and drawn into the column, the buffer concentration of the stirred column gradually increase. Altering the shapes of the stirred chamber, as well as of the limit buffer, allows for the production of concave, linear, or convex gradients of counterion.

A multitude of different mediums are used for the stationary phase. Among the most common immobilized charged groups used are trimethylaminoethyl (TAM), triethylaminoethyl (TEAE), diethyl-2-hydroxypropylaminoethyl (QAE), aminoethyl (AE), diethylaminoethyl (DEAE), sulpho (S), sulphomethyl (SM), sulphopropyl (SP), carboxy (C), and carboxymethyl (CM).

Successful packing of the column is an important aspect of ion chromatography. Stability and efficiency of a final column depends on packing methods, solvent used, and factors that affect mechanical properties of the column. In contrast to early inefficient dry- packing methods, wet slurry packing, in which particles that are suspended in an appropriate solvent are delivered into a column under pressure, shows significant improvement. Three different approaches can be employed in performing wet slurry packing: the balanced density method (solvent's density is about that of porous silica particles), the high viscosity method (a solvent of high viscosity is used), and the low viscosity slurry method (performed with low viscosity solvents).

Polystyrene is used as a medium for ion- exchange. It is made from the polymerization of styrene with the use of divinylbenzene and benzoyl peroxide. Such exchangers form hydrophobic interactions with proteins which can be irreversible. Due to this property, polystyrene ion exchangers are not suitable for protein separation. They are used on the other hand for the separation of small molecules in amino acid separation and removal of salt from water. Polystyrene ion exchangers with large pores can be used for the separation of protein but must be coated with a hydrophilic substance.

Cellulose based medium can be used for the separation of large molecules as they contain large pores. Protein binding in this medium is high and has low hydrophobic character. DEAE is an anion exchange matrix that is produced from a positive side group of diethylaminoethyl bound to cellulose or Sephadex.

Agarose gel based medium contain large pores as well but their substitution ability is lower in comparison to dextrans. The ability of the medium to swell in liquid is based on the cross-linking of these substances, the pH and the ion concentrations of the buffers used.

Incorporation of high temperature and pressure allows a significant increase in the efficiency of ion chromatography, along with a decrease in time. Temperature has an influence of selectivity due to its effects on retention properties. The retention factor ($k = (t_R - t_M)/(t_M - t_{ext})$) increases with temperature for small ions, and the opposite trend is observed for larger ions.

Despite ion selectivity in different mediums, further research is being done to perform ion exchange chromatography through the range of 40–175 °C.

An appropriate solvent can be chosen based on observations of how column particles behave in a solvent. Using an optical microscope, one can easily distinguish a desirable dispersed state of slurry from aggregated particles.

Weak and strong ion exchangers

A "strong" ion exchanger will not lose the charge on its matrix once the column is equilibrated and so a wide range of pH buffers can be used. "Weak" ion exchangers have a range of pH values in which they will maintain their charge. If the pH of the buffer used for a weak ion exchange column goes out of the capacity range of the matrix, the column will lose its charge distribution and the molecule of interest may be lost. Despite

the smaller pH range of weak ion exchangers, they are often used over strong ion exchangers due to their having greater specificity. In some experiments, the retention times of weak ion exchangers are just long enough to obtain desired data at a high specificity.

Resins (often termed 'beads') of ion exchange columns may include functional groups such as weak/strong acids and weak/strong bases. There are also special columns that have resins with amphoteric functional groups that can exchange both cations and anions. Some examples of functional groups of strong ion exchange resins are quaternary ammonium cation (Q), which is an anion exchanger, and sulfonic acid (S, $-SO_2OH$), which is a cation exchanger. These types of exchangers can maintain their charge density over a pH range of 0–14. Examples of functional groups of Weak ion exchange resins include diethylaminoethyl (DEAE, $-C_2H_4N(CH_2H_5)_2$), which is an anion exchanger, and carboxymethyl (CM, $-CH_2-COOH$), which is a cation exchanger. These two types of exchangers can maintain the charge density of their columns over a pH range of 5–9.

In ion chromatography, the interaction of the solute ions and the stationary phase based on their charges determines which ions will bind and to what degree. When the stationary phase features positive groups which attracts anions, it is called an anion exchanger; when there are negative groups on the stationary phase, cations are attracted and it is a cation exchanger. The attraction between ions and stationary phase also depends on the resin, organic particles used as ion exchangers.

Each resin features relative selectivity which varies based on the solute ions present who will compete to bind to the resin group on the stationary phase. The selectivity coefficient, the equivalent to the equilibrium constant, is determined via a ratio of the concentrations between the resin and each ion, however, the general trend is that ion exchangers prefer binding to the ion with a higher charge, smaller hydrated radius, and higher polarizability, or the ability for the electron cloud of an ion to be disrupted by other charges. Despite this selectivity, excess amounts of an ion with a lower selectivity introduced to the column would cause the lesser ion to bind more to the stationary phase as the selectivity coefficient allows fluctuations in the binding reaction that takes place during ion exchange chromatography.

Typical technique

A sample is introduced, either manually or with an autosampler, into a sample loop of known volume. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. This is typically a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. Equilibration of the stationary phase is needed in order to obtain the desired charge of the column. If the column is not properly equilibrated the desired molecule may not bind strongly to the column. The target analytes (anions or cations) are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that displaces the analyte ions from the stationary phase. For example, in cation

exchange chromatography, the positively charged analyte can be displaced by adding positively charged sodium ions. The analytes of interest must then be detected by some means, typically by conductivity or UV/visible light absorbance.

Control an IC system usually requires a chromatography data system (CDS). In addition to IC systems, some of these CDSs can also control gas chromatography (GC) and HPLC.

Membrane exchange chromatography

A type of ion exchange chromatography, membrane exchange is a relatively new method of purification designed to overcome limitations of using columns packed with beads. Membrane Chromatographic devices are cheap to mass-produce and disposable unlike other chromatography devices that require maintenance and time to revalidate. There are three types of membrane absorbers that are typically used when separating substances. The three types are flat sheet, hollow fibre, and radial flow. The most common absorber and best suited for membrane chromatography is multiple flat sheets because it has more absorbent volume. It can be used to overcome mass transfer limitations and pressure drop, making it especially advantageous for isolating and purifying viruses, plasmid DNA, and other large macromolecules. The column is packed with microporous membranes with internal pores which contain adsorptive moieties that can bind the target protein. Adsorptive membranes are available in a variety of geometries and chemistry which allows them to be used for purification and also fractionation, concentration, and clarification in an

efficiency that is 10 fold that of using beads. Membranes can be prepared through isolation of the membrane itself, where membranes are cut into squares and immobilized. A more recent method involved the use of live cells that are attached to a support membrane and are used for identification and clarification of signaling molecules.

Separating proteins

Ion exchange chromatography can be used to separate proteins because they contain charged functional groups. The ions of interest (in this case charged proteins) are exchanged for another ions (usually H) on a charged solid support. The solutes are most commonly in a liquid phase, which tends to be water. Take for example proteins in water, which would be a liquid phase that is passed through a column. The column is commonly known as the solid phase since it is filled with porous synthetic particles that are of a particular charge. These porous particles are also referred to as beads, may be aminated (containing amino groups) or have metal ions in order to have a charge. The column can be prepared using porous polymers, for macromolecules over 100,000 the optimum size of the porous particle is about 1 μm. This is because slow diffusion of the solutes within the pores does not restrict the separation quality. The beads containing positively charged groups, which attract the negatively charged proteins, are commonly referred to as anion exchange resins. The amino acids that have negatively charged side chains at pH 7 (pH of water) are glutamate and aspartate. The beads that are negatively charged are called cation exchange resins, as positively charged proteins will be attracted. The amino acids

that have positively charged side chains at pH 7 are lysine, histidine and arginine. The isoelectric point is the pH at which a compound - in this case a protein - has no net charge. A protein's isoelectric point or PI can be determined using the pKa of the side chains, if the amino (positive chain) is able to cancel out the carboxyl (negative) chain, the protein would be at its PI. Using buffers instead of water for proteins that do not have a charge at pH 7, is a good idea as it enables the manipulation of pH to alter ionic interactions between the proteins and the beads. Weakly acidic or basic side chains are able to have a charge if the pH is high or low enough respectively. Separation can be achieved based on the natural isoelectric point of the protein. Alternatively a peptide tag can be genetically added to the protein to give the protein an isoelectric point away from most natural proteins (e.g., 6 arginines for binding to a cation-exchange resin or 6 glutamates for binding to an anion-exchange resin such as DEAE-Sepharose).

Elution by increasing ionic strength of the mobile phase is more subtle. It works because ions from the mobile phase interact with the immobilized ions on the stationary phase, thus "shielding" the stationary phase from the protein, and letting the protein elute.

Elution from ion-exchange columns can be sensitive to changes of a single charge- chromatofocusing. Ion-exchange chromatography is also useful in the isolation of specific multimeric protein assemblies, allowing purification of specific complexes according to both the number and the position of charged peptide tags.

Gibbs–Donnan effect

In ion exchange chromatography, the Gibbs–Donnan effect is observed when the pH of the applied buffer and the ion exchanger differ, even up to one pH unit. For example, in anion-exchange columns, the ion exchangers repeal protons so the pH of the buffer near the column differs is higher than the rest of the solvent. As a result, an experimenter has to be careful that the protein(s) of interest is stable and properly charged in the "actual" pH.

This effect comes as a result of two similarly charged particles, one from the resin and one from the solution, failing to distribute properly between the two sides; there is a selective uptake of one ion over another. For example, in a sulphonated polystyrene resin, a cation exchange resin, the chlorine ion of a hydrochloric acid buffer should equilibrate into the resin. However, since the concentration of the sulphonic acid in the resin is high, the hydrogen of HCl has no tendency to enter the column. This, combined with the need of electroneutrality, leads to a minimum amount of hydrogen and chlorine entering the resin.

Uses

Clinical utility

A use of ion chromatography can be seen in argentation chromatography. Usually, silver and compounds containing acetylenic and ethylenic bonds have very weak interactions. This phenomenon has been widely tested on olefin compounds.

The ion complexes the olefins make with silver ions are weak and made based on the overlapping of pi, sigma, and d orbitals and available electrons therefore cause no real changes in the double bond. This behavior was manipulated to separate lipids, mainly fatty acids from mixtures in to fractions with differing number of double bonds using silver ions. The ion resins were impregnated with silver ions, which were then exposed to various acids (silicic acid) to elute fatty acids of different characteristics.

Detection limits as low as 1 μM can be obtained for alkali metal ions. It may be used for measurement of HbA1c, porphyrin and with water purification. Ion Exchange Resins(IER) have been widely used especially in medicines due to its high capacity and the uncomplicated system of the separation process. One of the synthetic uses is to use Ion Exchange Resins for kidney dialysis. This method is used to separate the blood elements by using the cellulose membraned artificial kidney.

Another clinical application of ion chromatography is in the rapid anion exchange chromatography technique used to separate creatine kinase (CK) isoenzymes from human serum and tissue sourced in autopsy material (mostly CK rich tissues were used such as cardiac muscle and brain). These isoenzymes include MM, MB, and BB, which all carry out the same function given different amino acid sequences. The functions of these isoenzymes are to convert creatine, using ATP, into phosphocreatine expelling ADP. Mini columns were filled with DEAE-Sephadex A-50 and further eluted with tris-buffer sodium chloride at various concentrations (each concentration was chosen advantageously to manipulate

elution). Human tissue extract was inserted in columns for separation. All fractions were analyzed to see total CK activity and it was found that each source of CK isoenzymes had characteristic isoenzymes found within. Firstly, CK- MM was eluted, then CK-MB, followed by CK-BB. Therefore, the isoenzymes found in each sample could be used to identify the source, as they were tissue specific.

Using the information from results, correlation could be made about the diagnosis of patients and the kind of CK isoenzymes found in most abundant activity. From the finding, about 35 out of 71 patients studied suffered from heart attack (myocardial infarction) also contained an abundant amount of the CK-MM and CK-MB isoenzymes. Findings further show that many other diagnosis including renal failure, cerebrovascular disease, and pulmonary disease were only found to have the CK-MM isoenzyme and no other isoenzyme. The results from this study indicate correlations between various diseases and the CK isoenzymes found which confirms previous test results using various techniques. Studies about CK-MB found in heart attack victims have expanded since this study and application of ion chromatography.

Industrial applications

Since 1975 ion chromatography has been widely used in many branches of industry. The main beneficial advantages are reliability, very good accuracy and precision, high selectivity, high speed, high separation efficiency, and low cost of consumables. The most significant development related to ion chromatography are new sample preparation methods; improving the speed and selectivity of analytes separation;

lowering of limits of detection and limits of quantification; extending the scope of applications; development of new standard methods; miniaturization and extending the scope of the analysis of a new group of substances. Allows for quantitative testing of electrolyte and proprietary additives of electroplating baths. It is an advancement of qualitative hull cell testing or less accurate UV testing. Ions, catalysts, brighteners and accelerators can be measured. Ion exchange chromatography has gradually become a widely known, universal technique for the detection of both anionic and cationic species. Applications for such purposes have been developed, or are under development, for a variety of fields of interest, and in particular, the pharmaceutical industry. The usage of ion exchange chromatography in pharmaceuticals has increased in recent years, and in 2006, a chapter on ion exchange chromatography was officially added to the United States Pharmacopia-National Formulary (USP-NF). Furthermore, in 2009 release of the USP-NF, the United States Pharmacopia made several analyses of ion chromatography available using two techniques: conductivity detection, as well as pulse amperometric detection.

Majority of these applications are primarily used for measuring and analyzing residual limits in pharmaceuticals, including detecting the limits of oxalate, iodide, sulfate, sulfamate, phosphate, as well as various electrolytes including potassium, and sodium. In total, the 2009 edition of the USP-NF officially released twenty eight methods of detection for the analysis of active compounds, or components of active compounds, using either conductivity detection or pulse amperometric detection.

Drug development

There has been a growing interest in the application of IC in the analysis of pharmaceutical drugs. IC is used in different aspects of product development and quality control testing. For example, IC is used to improve stabilities and solubility properties of pharmaceutical active drugs molecules as well as used to detect systems that have higher tolerance for organic solvents. IC has been used for the determination of analytes as a part of a dissolution test. For instance, calcium dissolution tests have shown that other ions present in the medium can be well resolved among themselves and also from the calcium ion. Therefore, IC has been employed in drugs in the form of tablets and capsules in order to determine the amount of drug dissolve with time. IC is also widely used for detection and quantification of excipients or inactive ingredients used in pharmaceutical formulations. Detection of sugar and sugar alcohol in such formulations through IC has been done due to these polar groups getting resolved in ion column. IC methodology also established in analysis of impurities in drug substances and products. Impurities or any components that are not part of the drug chemical entity are evaluated and they give insights about the maximum and minimum amounts of drug that should be administered in a patient per day.

Size-exclusion chromatography

Size-exclusion chromatography (**SEC**), also known as **molecular sieve chromatography**, is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied

to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as **gel-filtration chromatography**, versus the name gel permeation chromatography, which is used when an organic solvent is used as a mobile phase. The chromatography column is packed with fine, porous beads which are composed of dextran polymers (Sephadex), agarose (Sepharose), or polyacrylamide (Sephacryl or BioGel P). The pore sizes of these beads are used to estimate the dimensions of macromolecules. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (Mw) results for polymers.

Applications

The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic-soluble polymers. Either technique should not be confused with gel electrophoresis, where an electric field is used to "pull" or "push" molecules through the gel depending on their electrical charges. The amount of time a solute remains within a pore is dependent on the size of the pore. Larger solutes will have access to a smaller volume and vice versa. Therefore, a smaller solute will remain within the pore for a longer period of time compared to a larger solute.

Another use of size exclusion chromatography is to examine the stability and characteristics of natural organic matter in water. In this method, Margit B. Muller, Daniel Schmitt, and

Fritz H. Frimmel tested water sources from different places in the world to determine how stable the natural organic matter is over a period of time. Even though, size exclusion chromatography is widely utilized to study natural organic material, there are limitations. One of these limitations include that there is no standard molecular weight marker; thus, there is nothing to compare the results back to. If precise molecular weight is required, other methods should be used.

Advantages

- The advantages of this method include good separation of large molecules from the small molecules with a minimal volume of eluate, and that various solutions can be applied without interfering with the filtration process, all while preserving the biological activity of the particles to separate. The technique is generally combined with others that further separate molecules by other characteristics, such as acidity, basicity, charge, and affinity for certain compounds. With size exclusion chromatography, there are short and well-defined separation times and narrow bands, which lead to good sensitivity. There is also no sample loss because solutes do not interact with the stationary phase.

Discovery

The technique was invented in 1955 by Grant Henry Lathe and Colin R Ruthven, working at Queen Charlotte's Hospital,

London. They later received the John Scott Award for this invention. While Lathe and Ruthven used starch gels as the matrix, Jerker Porath and Per Flodin later introduced dextran gels; other gels with size fractionation properties include agarose and polyacrylamide. A short review of these developments has appeared.

There were also attempts to fractionate synthetic high polymers; however, it was not until 1964, when J. C. Moore of the Dow Chemical Company published his work on the preparation of gel permeation chromatography (GPC) columns based on cross-linked polystyrene with controlled pore size, that a rapid increase of research activity in this field began. It was recognized almost immediately that with proper calibration, GPC was capable to provide molar mass and molar mass distribution information for synthetic polymers. Because the latter information was difficult to obtain by other methods, GPC came rapidly into extensive use.

Theory and method

SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping smaller molecules in the pores of the adsorbent ("stationary phase"). This process is usually performed within a column, which typically consists of a hollow tube tightly packed with micron-scale polymer beads containing pores of different sizes. These pores may be depressions on the surface or channels through the bead. As the solution travels down the column some particles enter into the pores. Larger particles cannot enter into as many pores. The larger the particles, the faster the elution. The larger molecules simply pass by the pores because

those molecules are too large to enter the pores. Larger molecules therefore flow through the column more quickly than smaller molecules, that is, the smaller the molecule, the longer the retention time.

One requirement for SEC is that the analyte does not interact with the surface of the stationary phases, with differences in elution time between analytes ideally being based solely on the solute volume the analytes can enter, rather than chemical or electrostatic interactions with the stationary phases. Thus, a small molecule that can penetrate every region of the stationary phase pore system can enter a total volume equal to the sum of the entire pore volume and the interparticle volume. This small molecule elutes late (after the molecule has penetrated all of the pore- and interparticle volume—approximately 80% of the column volume). At the other extreme, a very large molecule that cannot penetrate any the smaller pores can enter only the interparticle volume (~35% of the column volume) and elutes earlier when this volume of mobile phase has passed through the column. The underlying principle of SEC is that particles of different sizes elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near-simultaneously, particles of the same size should elute together.

However, as there are various measures of the size of a macromolecule (for instance, the radius of gyration and the hydrodynamic radius), a fundamental problem in the theory of SEC has been the choice of a proper molecular size parameter by which molecules of different kinds are separated.

Experimentally, Benoit and co-workers found an excellent correlation between elution volume and a dynamically based molecular size, the hydrodynamic volume, for several different chain architecture and chemical compositions. The observed correlation based on the hydrodynamic volume became accepted as the basis of universal SEC calibration.

Still, the use of the hydrodynamic volume, a size based on dynamical properties, in the interpretation of SEC data is not fully understood. This is because SEC is typically run under low flow rate conditions where hydrodynamic factor should have little effect on the separation. In fact, both theory and computer simulations assume a thermodynamic separation principle: the separation process is determined by the equilibrium distribution (partitioning) of solute macromolecules between two phases: a dilute bulk solution phase located at the interstitial space and confined solution phases within the pores of column packing material. Based on this theory, it has been shown that the relevant size parameter to the partitioning of polymers in pores is the mean span dimension (mean maximal projection onto a line). Although this issue has not been fully resolved, it is likely that the mean span dimension and the hydrodynamic volume are strongly correlated.

Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of the column 'working' range and is where molecules are too large to get trapped in the stationary phase. The lower end of the range is defined by the permeation limit, which defines the molecular weight of a molecule that is small enough to penetrate all pores of the

stationary phase. All molecules below this molecular mass are so small that they elute as a single band.

The filtered solution that is collected at the end is known as the **eluate**. The **void volume** includes any particles too large to enter the medium, and the solvent volume is known as the **column volume**.

Factors affecting filtration

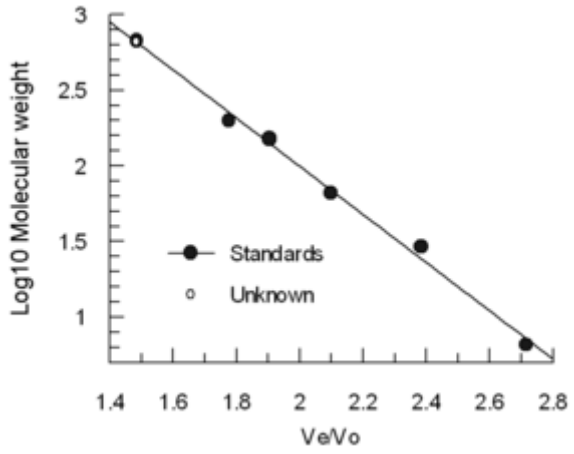
In real-life situations, particles in solution do not have a fixed size, resulting in the probability that a particle that would otherwise be hampered by a pore passing right by it. Also, the stationary-phase particles are not ideally defined; both particles and pores may vary in size. Elution curves, therefore, resemble Gaussian distributions. The stationary phase may also interact in undesirable ways with a particle and influence retention times, though great care is taken by column manufacturers to use stationary phases that are inert and minimize this issue.

Like other forms of chromatography, increasing the column length enhances resolution, and increasing the column diameter increases column capacity. Proper column packing is important for maximum resolution: An over-packed column can collapse the pores in the beads, resulting in a loss of resolution. An under-packed column can reduce the relative surface area of the stationary phase accessible to smaller species, resulting in those species spending less time trapped in pores. Unlike affinity chromatography techniques, a solvent head at the top of the column can drastically diminish

resolution as the sample diffuses prior to loading, broadening the downstream elution.

Analysis

- In simple manual columns, the eluent is collected in constant volumes, known as fractions. The more similar the particles are in size the more likely they are in the same fraction and not detected separately. More advanced columns overcome this problem by constantly monitoring the eluent.



- Standardization of a size exclusion column

The collected fractions are often examined by spectroscopic techniques to determine the concentration of the particles eluted. Common spectroscopy detection techniques are refractive index (RI) and ultraviolet (UV). When eluting spectroscopically similar species (such as during biological purification), other techniques may be necessary to identify the contents of each fraction.

The elution volume (Ve) decreases roughly linear with the logarithm of the molecular hydrodynamic volume. Columns are often calibrated using 4-5 standard samples (e.g., folded proteins of known molecular weight), and a sample containing a very large molecule such as thyroglobulin to determine the void volume. (Blue dextran is not recommended for Vo determination because it is heterogeneous and may give variable results) The elution volumes of the standards are divided by the elution volume of the thyroglobulin (Ve/Vo) and plotted against the log of the standards' molecular weights.

Applications

Biochemical applications

In general, SEC is considered a low-resolution chromatography as it does not discern similar species very well, and is therefore often reserved for the final step of a purification. The technique can determine the quaternary structure of purified proteins that have slow exchange times, since it can be carried out under native solution conditions, preserving macromolecular interactions. SEC can also assay protein tertiary structure, as it measures the hydrodynamic volume (not molecular weight), allowing folded and unfolded versions of the same protein to be distinguished. For example, the apparent hydrodynamic radius of a typical protein domain might be 14 Å and 36 Å for the folded and unfolded forms, respectively. SEC allows the separation of these two forms, as the folded form elutes much later due to its smaller size.

Polymer synthesis

SEC can be used as a measure of both the size and the polydispersity of a synthesized polymer, that is, the ability to find the distribution of the sizes of polymer molecules. If standards of a known size are run previously, then a calibration curve can be created to determine the sizes of polymer molecules of interest in the solvent chosen for analysis (often THF). In alternative fashion, techniques such as light scattering and/or viscometry can be used online with SEC to yield absolute molecular weights that do not rely on calibration with standards of known molecular weight. Due to the difference in size of two polymers with identical molecular weights, the absolute determination methods are, in general, more desirable. A typical SEC system can quickly (in about half an hour) give polymer chemists information on the size and polydispersity of the sample. The preparative SEC can be used for polymer fractionation on an analytical scale.

Drawbacks

In SEC, mass is not measured so much as the hydrodynamic volume of the polymer molecules, that is, how much space a particular polymer molecule takes up when it is in solution. However, the approximate molecular weight can be calculated from SEC data because the exact relationship between molecular weight and hydrodynamic volume for polystyrene can be found. For this, polystyrene is used as a standard. But the relationship between hydrodynamic volume and molecular weight is not the same for all polymers, so only an approximate measurement can be obtained. Another drawback is the

possibility of interaction between the stationary phase and the analyte. Any interaction leads to a later elution time and thus mimics a smaller analyte size.

When performing this method, the bands of the eluting molecules may be broadened. This can occur by turbulence caused by the flow of the mobile phase molecules passing through the molecules of the stationary phase. In addition, molecular thermal diffusion and friction between the molecules of the glass walls and the molecules of the eluent contribute to the broadening of the bands. Besides broadening, the bands also overlap with each other. As a result, the eluent usually gets considerably diluted. A few precautions can be taken to prevent the likelihood of the bands broadening. For instance, one can apply the sample in a narrow, highly concentrated band on the top of the column. The more concentrated the eluent is, the more efficient the procedure would be. However, it is not always possible to concentrate the eluent, which can be considered as one more disadvantage.

Absolute size-exclusion chromatography

Absolute size-exclusion chromatography (ASEC) is a technique that couples a light scattering instrument, most commonly multi-angle light scattering (MALS) or another form of static light scattering (SLS), but possibly a dynamic light scattering (DLS) instrument, to a size-exclusion chromatography system for absolute molar mass and/or size measurements of proteins and macromolecules as they elute from the chromatography system.

The definition of "absolute" in this case is that calibration of retention time on the column with a set of reference standards is not required to obtain molar mass or the hydrodynamic size, often referred to as hydrodynamic diameter (D_H in units of nm). Non-ideal column interactions, such as electrostatic or hydrophobic surface interactions that modulate retention time relative to standards, do not impact the final result. Likewise, differences between conformation of the analyte and the standard have no effect on an absolute measurement; for example, with MALS analysis, the molar mass of inherently disordered proteins are characterized accurately even though they elute at much earlier times than globular proteins with the same molar mass, and the same is true of branched polymers which elute late compared to linear reference standards with the same molar mass. Another benefit of ASEC is that the molar mass and/or size is determined at each point in an eluting peak, and therefore indicates homogeneity or polydispersity within the peak. For example, SEC-MALS analysis of a monodisperse protein will show that the entire peak consists of molecules with the same molar mass, something that is not possible with standard SEC analysis.

Determination of molar mass with SLS requires combining the light scattering measurements with concentration measurements. Therefore SEC-MALS typically includes the light scattering detector and either a differential refractometer or UV/Vis absorbance detector.

In addition, MALS determines the rms radius R_g of molecules above a certain size limit, typically 10 nm. SEC-MALS can therefore analyze the conformation of polymers via the relationship of molar mass to R_g. For smaller molecules, either

DLS or, more commonly, a differential viscometer is added to determine hydrodynamic radius and evaluate molecular conformation in the same manner.

In SEC-DLS, the sizes of the macromolecules are measured as they elute into the flow cell of the DLS instrument from the size exclusion column set. The hydrodynamic size of the molecules or particles are measured and not their molecular weights. For proteins a Mark-Houwink type of calculation can be used to estimate the molecular weight from the hydrodynamic size.

A major advantage of DLS coupled with SEC is the ability to obtain enhanced DLS resolution. Batch DLS is quick and simple and provides a direct measure of the average size, but the baseline resolution of DLS is a ratio of 3:1 in diameter. Using SEC, the proteins and protein oligomers are separated, allowing oligomeric resolution.

Aggregation studies can also be done using ASEC. Though the aggregate concentration may not be calculated with light scattering (an online concentration detector such as that used in SEC-MALS for molar mass measurement also determines aggregate concentration), the size of the aggregate can be measured, only limited by the maximum size eluting from the SEC columns.

Limitations of ASEC with DLS detection include flow-rate, concentration, and precision. Because a correlation function requires anywhere from 3–7 seconds to properly build, a limited number of data points can be collected across the peak.

ASEC with SLS detection is not limited by flow rate and measurement time is essentially instantaneous, and the range of concentration is several orders of magnitude larger than for DLS. However, molar mass analysis with SEC-MALS does require accurate concentration measurements. MALS and DLS detectors are often combined in a single instrument for more comprehensive absolute analysis following separation by SEC.

Chapter 5

Techniques

Thin-layer chromatography

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of an inert substrate such as glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such as heptane are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are often treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will

char most organic compounds, leaving a dark spot on the sheet. To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase, this ratio is called the retardation factor (R_f), or sometimes colloquially as *retention factor*. For the result to be quantitative the absorption of solvent must be stopped before the mobile phase reaches the end of the stationary phase. In general, a substance whose structure resembles the stationary phase will have low R_f, while one that has a similar structure to the mobile phase will have high retardation factor. Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, chemists usually apply a sample of a known compound to the sheet alongside the unknown samples.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents

A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as HPTLC, or "high-performance TLC". HPTLC typically uses thinner layers of stationary phase and smaller sample volumes, thus reducing the loss of resolution due to diffusion.

Plate preparation

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1–0.25 mm for analytical purposes and around 0.5–2.0 mm for preparative TLC.

Technique

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed, TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

To run a thin layer chromatography plate, the following procedure is carried out:

- Using a capillary tube, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from

interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber. This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.

- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results.)
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.

- Without delay, the *solvent front*, the furthest extent of solvent up the plate, is marked.
- The plate is visualized. As some plates are pre-coated with a phosphor such as zinc sulfide, allowing many compounds to be visualized by using ultraviolet light; dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

Separation process and principle

- Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the R_f value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a flash chromatography column. (A compound elutes from a column when the amount of solvent collected is equal to $1/R_f$.) Chemists often use TLC to develop a protocol for separation by chromatography and use TLC to determine which fractions contain the desired compounds.

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase. For instance, if normal-phase silica gel is used as the

stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, better able to displace the mobile phase from the available binding sites. As a consequence, the less polar compound moves higher up the plate (resulting in a higher R_f value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it becomes better at binding to the polar plate and therefore displacing solutes from it, so all compounds on the TLC plate will move higher up the plate. It is commonly said that "strong" solvents (eluents) push the analyzed compounds up the plate, whereas "weak" eluents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel-coated TLC plates, the eluent strength increases in the following order: perfluoroalkane (weakest), hexane, pentane, carbon tetrachloride, benzene/toluene, dichloromethane, diethyl ether, ethyl acetate, acetonitrile, acetone, 2-propanol/*n*-butanol, water, methanol, triethylamine, acetic acid, formic acid (strongest). For C18-coated plates the order is reverse. In other words, when the stationary phase is polar and the mobile phase is nonpolar, the method is *normal-phase* as opposed to *reverse-phase*.

This means that if a mixture of ethyl acetate and hexane as the mobile phase is used, adding more ethyl acetate results in higher R_f values for all compounds on the TLC plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate. An eluotropic series can be used as a guide in selecting a mobile phase. If a reversed order of running of the compounds

is desired, an apolar stationary phase should be used, such as C18-functionalized silica.

Analysis

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

- Fluorescent analytes, like quinine, may be detected under blacklight (366 nm)
- Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
- Iodine vapors are a general unspecific color reagent
- Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.
- Potassium permanganate – oxidation
- Bromine
- Acidic vanillin
- Phosphomolybdic acid
- In the case of lipids, the chromatogram may be transferred to a polyvinylidene fluoride membrane and then subjected to further analysis, for example mass spectrometry, a technique known as far-eastern blot.

Once visible, the R_f value, or retardation factor, of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the

initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

Applications

Characterization

In organic chemistry, reactions are qualitatively monitored with TLC. Spots sampled with a capillary tube are placed on the plate: a spot of starting material, a spot from the reaction mixture, and a cross-spot with both. A small (3 by 7 cm) TLC plate takes a couple of minutes to run. The analysis is qualitative, and it will show if the starting material has disappeared, i.e. the reaction is complete, if any product has appeared, and how many products are generated (although this might be underestimated due to co-elution). Unfortunately, TLCs from low-temperature reactions may give misleading results, because the sample is warmed to room temperature in the capillary, which can alter the reaction—the warmed sample analyzed by TLC is not the same as what is in the low-temperature flask. One such reaction is the DIBALH reduction of ester to aldehyde.

In one study TLC has been applied in the screening of organic reactions, for example in the fine-tuning of BINAP synthesis from 2-naphthol. In this method, the alcohol and catalyst solution (for instance iron(III) chloride) are placed separately on the baseline, then reacted, and then instantly analyzed.

A special application of TLC is in the characterization of radiolabeled compounds, where it is used to determine

radiochemical purity. The TLC sheet is visualized using a sheet of photographic film or an instrument capable of measuring radioactivity. It may be visualized using other means as well. This method is much more sensitive than the others and can be used to detect an extremely small amount of a compound, provided that it carries a radioactive atom.

Isolation

- Since different compounds will travel a different distance in the stationary phase, chromatography can be used to isolate components of a mixture for further analysis. The separated compounds each occupying a specific area on the plate, they can be scraped off (along with the stationary phase particles) and dissolved into an appropriate solvent. As an example, in the chromatography of an extract of green plant material (for example spinach) shown in 7 stages of development, Carotene elutes quickly and is only visible until step 2. Chlorophyll A and B are halfway in the final step and lutein the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, extracted into a solvent and placed into a spectrophotometer to determine its spectrum. The quantities extracted are small and a technique such as column chromatography is preferred to separate larger amounts. However, big preparative TLC plates with thick silica gel coatings can be used to separate more than 100 mg of material.

Examining reactions and compound stability

TLC is also used for the identification of the completion of any chemical reaction. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.

Furthermore, two-dimensional TLC is frequently used as a method to check if a compound is stable in the stationary phase (such as silica gel, which is usually slightly acidic). For this purpose, the tested compound mixture is eluted twice in a square-shaped TLC plate, first in one direction and then rotated 90°. If the target compound appears on the diagonal of the square, it is stable in silica gel and safe to purify. If it appears below the diagonal, it is decomposing on silica gel. If this is the case, purification can be attempted using neutralized silica gel (with triethylamine, for example), or an alternative stationary phase such as neutral alumina.

Supercritical fluid chromatography

Supercritical fluid chromatography (**SFC**) is a form of normal phase chromatography that uses a supercritical fluid such as carbon dioxide as the mobile phase. It is used for the analysis and purification of low to moderate molecular weight, thermally labile molecules and can also be used for the

separation of chiral compounds. Principles are similar to those of high performance liquid chromatography (HPLC), however SFC typically utilizes carbon dioxide as the mobile phase; therefore the entire chromatographic flow path must be pressurized. Because the supercritical phase represents a state in which liquid and gas properties converge, supercritical fluid chromatography is sometimes called convergence chromatography.

Applications

SFC is used in industry primarily for separation of chiral molecules, and uses the same columns as standard HPLC systems. SFC is now commonly used for achiral separations and purifications in the pharmaceutical industry.

Apparatus

SFC with CO_2 utilizes carbon dioxide pumps that require that the incoming CO_2 and pump heads be kept cold in order to maintain the carbon dioxide at a temperature and pressure that keeps it in a liquid state where it can be effectively metered at some specified flow rate. The CO_2 subsequently becomes supercritical post the injector and in the column oven when the temperature and pressure it is subjected to are raised above the critical point of the liquid and the supercritical state is achieved. SFC as a chromatographic process has been likened to a process having the combined properties of the power of a liquid to dissolve a matrix, with the chromatographic interactions and kinetics of a gas. The result is that you can get a lot of mass on column per

injection, and still maintain a high chromatographic efficiency. Typically, gradient elution is employed in analytical SFC using a polar co-solvent such as methanol, possibly with a weak acid or base at low concentrations ~1%. The effective plate counts per analysis can be observed to exceed 500K plates per metre routinely with 5 um material. The operator uses software to set mobile phase flow rate, co-solvent composition, system back pressure and column oven temperature which must exceed 40 °C for supercritical conditions to be achieved with CO_2. In addition, SFC provides an additional control parameter - pressure - by using an automated back pressure regulator. From an operational standpoint, SFC is as simple and robust as HPLC but fraction collection is more convenient because the primary mobile phase evaporates leaving only the analyte and a small volume of polar co-solvent. If the outlet CO_2 is captured, it can be recompressed and recycled, allowing for >90% reuse of CO_2.

Similar to HPLC, SFC uses a variety of detection methods including UV/VIS, mass spectrometry, FID (unlike HPLC) and evaporative light scattering.

Sample preparation

A rule-of-thumb is that any molecule that will dissolve in methanol or a less polar solvent is compatible with SFC, including polar solutes. CO_2 has polarity similar to n-heptane at its critical point, but the solvent strength can be increased by increasing density or using a polar cosolvent. In practice, when the fraction of cosolvent is high, the mobile phase is not truly supercritical, but this terminology is used regardless. This process can be sped up by using supercritical fluid.

Mobile phase

The mobile phase is composed primarily of supercritical carbon dioxide, but since CO_2 on its own is too non-polar to effectively elute many analytes, cosolvents are added to modify the mobile phase polarity. Cosolvents are typically simple alcohols like methanol, ethanol, or isopropyl alcohol. Other solvents such as acetonitrile, chloroform, or ethyl acetate can be used as modifiers. For food-grade materials, the selected cosolvent is often ethanol or ethyl acetate, both of which are generally recognized as safe (GRAS). The solvent limitations are system and column based.

Drawbacks

There have been a few technical issues that have limited adoption of SFC technology, first of which is the high pressure operating conditions. High-pressure vessels are expensive and bulky, and special materials are often needed to avoid dissolving gaskets and O-rings in the supercritical fluid. A second drawback is difficulty in maintaining pressure (backpressure regulation). Whereas liquids are nearly incompressible, so their densities are constant regardless of pressure, supercritical fluids are highly compressible and their physical properties change with pressure - such as the pressure drop across a packed-bed column. Currently, automated backpressure regulators can maintain a constant pressure in the column even if flow rate varies, mitigating this problem. A third drawback is difficulty in gas/liquid separation during collection of product. Upon depressurization, the CO_2 rapidly turns into gas and aerosolizes any dissolved analyte in

the process. Cyclone separators have lessened difficulties in gas/liquid separations.

Expanded bed adsorption

Expanded bed adsorption (EBA) is a preparative chromatographic technique which makes processing of viscous and particulate liquids possible.

Principle

The protein binding principles in EBA are the same as in classical column chromatography and the common ion-exchange, hydrophobic interaction and affinity chromatography ligands can be used. After the adsorption step is complete, the fluidized bed is washed to flush out any remaining particulates. Elution of the adsorbed proteins was commonly performed with the eluent flow in the reverse direction; that is, as a conventional packed bed, in order to recover the adsorbed solutes in a smaller volume of eluent. However, a new generation of EBA columns has been developed, which maintain the bed in the expanded state during this phase, producing high-purity, high yields of e.g. MAbs [monoclonal antibodies] in even smaller volumes of eluent. Process duration at manufacturing scale has also been cut considerably (under 7 hours in some cases).

EBA may be considered to combine both the "Removal of Insolubles" and the "Isolation" steps of the 4-step downstream processing heuristic. The major limitations associated with

EBA technology is biomass interactions and aggregations onto adsorbent during processing.

Where classical column chromatography uses a solid phase made by a packed bed, EBA uses particles in a fluidized state, ideally expanded by a factor of 2. Expanded bed adsorption is, however, different from fluidised bed chromatography in essentially two ways: one, the EBA resin contains particles of varying size and density which results in a gradient of particle size when expanded; and two, when the bed is in its expanded state, local loops are formed. Particles such as whole cells or cell debris, which would clog a packed bed column, readily pass through a fluidized bed. EBA can therefore be used on crude culture broths or slurries of broken cells, thereby bypassing initial clearing steps such as centrifugation and filtration, which is mandatory when packed beds are used. In older EBA column designs, the feed flow rate is kept low enough that the solid packing remains stratified and does not fluidize completely. Hence EBA can be modelled as frontal adsorption in a packed bed, rather than as a well-mixed, continuous-flow adsorber.

Paper chromatography

Paper chromatography is an analytical method used to separate coloured chemicals or substances. Erwin Chargaff credits in Weintraub's history of the man the 1944 article by Consden, Gordon and Martin with sparking his discovery of Chargaff's rules, an important precursor to Watson and Crick's discovery of the double-helix structure of DNA, for which they were awarded the Nobel Prize in Physiology or Medicine in 1962. It is now primarily used as a teaching tool, having been

replaced in the laboratory by other chromatography methods such as thin-layer chromatography (TLC).

A paper chromatography variant, two-dimensional chromatography involves using two solvents and rotating the paper 90° in between. This is useful for separating complex mixtures of compounds having similar polarity, for example, amino acids. The setup has three components. The mobile phase is a solution that travels up the stationary phase, due to capillary action. The mobile phase is generally a mixture of non-polar organic solvent, while the stationary phase is polar inorganic solvent water. Here paper is used to support the stationary phase, water. Polar water molecules are held inside the void space of the cellulose network of the host paper. The difference between TLC and paper chromatography is that the stationary phase in TLC is a layer of adsorbent (usually silica gel, or aluminium oxide), and the stationary phase in paper chromatography is less absorbent paper.

R_f value, solutes, and solvents

The retention factor (R_f) may be defined as the ratio of the distance travelled by the solute to the distance travelled by the solvent. It is used in chromatography to quantify the amount of retardation of a sample in a stationary phase relative to a mobile phase. R_f values are usually expressed as a fraction of two decimal places.

- If R_f value of a solution is zero, the solute remains in the stationary phase and thus it is immobile.
- If R_f value = 1 then the solute has no affinity for the stationary phase and travels with the solvent front.

For example, if a compound travels 9.9 cm and the solvent front travels 12.7 cm, the R_f value = (9.9/12.7) = 0.779 or 0.78. R_f value depends on temperature and the solvent used in experiment, so several solvents offer several R_f values for the same mixture of compound. A solvent in chromatography is the liquid the paper is placed in, and the solute is the ink which is being separated.

Pigments and polarity

Paper chromatography is one method for testing the purity of compounds and identifying substances. Paper chromatography is a useful technique because it is relatively quick and requires only small quantities of material. Separations in paper chromatography involve the principle of partition. In paper chromatography, substances are distributed between a stationary phase and a mobile phase. The stationary phase is the water trapped between the cellulose fibers of the paper. The mobile phase is a developing solution that travels up the stationary phase, carrying the samples with it. Components of the sample will separate readily according to how strongly they adsorb onto the stationary phase versus how readily they dissolve in the mobile phase.

When a colored chemical sample is placed on a filter paper, the colors separate from the sample by placing one end of the paper in a solvent. The solvent diffuses up the paper, dissolving the various molecules in the sample according to the polarities of the molecules and the solvent. If the sample contains more than one color, that means it must have more than one kind of molecule. Because of the different chemical structures of each kind of molecule, the chances are very high

that each molecule will have at least a slightly different polarity, giving each molecule a different solubility in the solvent. The unequal solubility causes the various color molecules to leave solution at different places as the solvent continues to move up the paper. The more soluble a molecule is, the higher it will migrate up the paper. If a chemical is very non-polar it will not dissolve at all in a very polar solvent. This is the same for a very polar chemical and a very non-polar solvent.

It is very important to note that when using water (a very polar substance) as a solvent, the more polar the color, the higher it will rise on the papers.

Types

Descending

Development of the chromatogram is done by allowing the solvent to travel down the paper. Here, mobile phase is placed in solvent holder at the top. The spot is kept at the top and solvent flows down the paper from above.

Ascending

Here the solvent travels up the chromatographic paper. Both descending and ascending paper chromatography are used for the separation of organic and inorganic substances. The sample and solvent move upward.

Ascending-descending

This is the hybrid of both of the above techniques. The upper part of ascending chromatography can be folded over a rod in order to allow the paper to become descending after crossing the rod.

Circular chromatography

A circular filter paper is taken and the sample is deposited at the center of the paper. After drying the spot, the filter paper is tied horizontally on a Petri dish containing solvent, so that the wick of the paper is dipped in the solvent. The solvent rises through the wick and the components are separated into concentric rings.

Two-dimensional

In this technique a square or rectangular paper is used. Here the sample is applied to one of the corners and development is performed at a right angle to the direction of the first run.

History of paper chromatography

The discovery of paper chromatography in 1943 by Martin and Synge provided, for the first time, the means of surveying constituents of plants and for their separation and identification. Erwin Chargaff credits in Weintraub's history of the man the 1944 article by Consden, Gordon and Martin. There was an explosion of activity in this field after 1945.

Argentation chromatography

Argentation chromatography is chromatography using a stationary phase that contains silver salts. Silver-containing stationary phases are well suited for separating organic compounds on the basis of the number and type of alkene (olefin) groups. The technique is employed for gas chromatography and various types of liquid chromatography, including thin layer chromatography. Analytes containing alkene groups elute more slowly that the analogous compounds lacking alkenes. Separations are also sensitive to the type of alkene. The technique is especially useful in the analysis of fats and fatty acids, which are well known to exist in both saturated and unsaturated (alkene-containing) forms.

Theory

- Silver ions form alkene complexes. The binding is reversible, but sufficient to impede the elution of the alkene-containing analytes.

Displacement chromatography

Displacement chromatography is a chromatography technique in which a sample is placed onto the head of the column and is then displaced by a solute that is more strongly sorbed than the components of the original mixture. The result is that the components are resolved into consecutive “rectangular” zones of highly concentrated pure substances rather than solvent-separated “peaks”. It is primarily a preparative technique;

higher product concentration, higher purity, and increased throughput may be obtained compared to other modes of chromatography.

Discovery

The advent of displacement chromatography can be attributed to Arne Tiselius, who in 1943 first classified the modes of chromatography as frontal, elution, and displacement. Displacement chromatography found a variety of applications including isolation of transuranic elements and biochemical entities. The technique was redeveloped by Csaba Horváth, who employed modern high-pressure columns and equipment. It has since found many applications, particularly in the realm of biological macromolecule purification.

Principle

The basic principle of displacement chromatography is: there are only a finite number of binding sites for solutes on the matrix (the stationary phase), and if a site is occupied by one molecule, it is unavailable to others. As in any chromatography, equilibrium is established between molecules of a given kind bound to the matrix and those of the same kind free in solution. Because the number of binding sites is finite, when the concentration of molecules free in solution is large relative to the dissociation constant for the sites, those sites will mostly be filled. This results in a downward-curvature in the plot of bound *vs* free solute, in the simplest case giving a Langmuir isotherm. A molecule with a high affinity for the matrix (the displacer) will compete more effectively for binding

sites, leaving the mobile phase enriched in the lower-affinity solute. Flow of mobile phase through the column preferentially carries off the lower-affinity solute and thus at high concentration the higher-affinity solute will eventually displace all molecules with lesser affinities.

Mode of operation

Loading

At the beginning of the run, a mixture of solutes to be separated is applied to the column, under conditions selected to promote high retention.

The higher-affinity solutes are preferentially retained near the head of the column, with the lower-affinity solutes moving farther downstream. The fastest moving component begins to form a pure zone downstream. The other components also begin to form zones, but the continued supply of the mixed feed at head of the column prevents full resolution.

Displacement

After the entire sample is loaded, the feed is switched to the displacer, chosen to have higher affinity than any sample component. The displacer forms a sharp-edged zone at the head of the column, pushing the other components downstream.

Each sample component now acts as a displacer for the lower-affinity solutes, and the solutes sort themselves out into a series of contiguous bands (a "displacement train"), all moving

downstream at the rate set by the displacer. The size and loading of the column are chosen to let this sorting process reach completion before the components reach the bottom of the column. The solutes appear at the bottom of the column as a series of contiguous zones, each consisting of one purified component, with the concentration within each individual zone effectively uniform.

Regeneration

After the last solute has been eluted, it is necessary to strip the displacer from the column. Since the displacer was chosen for high affinity, this can pose a challenge. On reverse-phase materials, a wash with a high percentage of organic solvent may suffice.

Large pH shifts are also often employed. One effective strategy is to remove the displacer by chemical reaction; for instance if hydrogen ion was used as displacer it can be removed by reaction with hydroxide, or a polyvalent metal ion can be removed by reaction with a chelating agent.

For some matrices, reactive groups on the stationary phase can be titrated to temporarily eliminate the binding sites, for instance weak-acid ion exchangers or chelating resins can be converted to the protonated form.

For gel-type ion exchangers, selectivity reversal at very high ionic strength can also provide a solution. Sometimes the displacer is specifically designed with a titratable functional group to shift its affinity. After the displacer is washed out, the column is washed as needed to restore it to its initial state for the next run.

Comparison with elution chromatography

Common fundamentals

In any form of chromatography, the rate at which the solute moves down the column is a direct reflection of the percentage of time the solute spends in the mobile phase. To achieve separation in either elution or displacement chromatography, there must be appreciable differences in the affinity of the respective solutes for the stationary phase. Both methods rely on movement down the column to amplify the effect of small differences in distribution between the two phases. Distribution between the mobile and stationary phases is described by the binding isotherm, a plot of solute bound to (or partitioned into) the stationary phase as a function of concentration in the mobile phase. The isotherm is often linear, or approximately so, at low concentrations, but commonly curves (concave-downward) at higher concentrations as the stationary phase becomes saturated.

Characteristics of elution mode

In elution mode, solutes are applied to the column as narrow bands and, at low concentration, move down the column as approximately Gaussian peaks. These peaks continue to broaden as they travel, in proportion to the square root of the distance traveled. For two substances to be resolved, they must migrate down the column at sufficiently different rates to overcome the effects of band spreading. Operating at high

concentration, where the isotherm is curved, is disadvantageous in elution chromatography because the rate of travel then depends on concentration, causing the peaks to spread and distort.

Retention in elution chromatography is usually controlled by adjusting the composition of the mobile phase (in terms of solvent composition, pH, ionic strength, and so forth) according to the type of stationary phase employed and the particular solutes to be separated. The mobile phase components generally have lower affinity for the stationary phase than do the solutes being separated, but are present at higher concentration and achieve their effects due to mass action. Resolution in elution chromatography is generally better when peaks are strongly retained, but conditions that give good resolution of early peaks lead to long run-times and excessive broadening of later peaks unless gradient elution is employed. Gradient equipment adds complexity and expense, particularly at large scale.

Advantages and disadvantages of displacement mode

In contrast to elution chromatography, solutes separated in displacement mode form sharp-edged zones rather than spreading peaks. Zone boundaries in displacement chromatography are self-sharpening: if a molecule for some reason gets ahead of its band, it enters a zone in which it is more strongly retained, and will then run more slowly until its zone catches up. Furthermore, because displacement chromatography takes advantage of the non-linearity of the isotherms, loadings are deliberately high; more material can be separated on a given column, in a given time, with the purified

components recovered at significantly higher concentrations. Retention conditions can still be adjusted, but the displacer controls the migration rate of the solutes. The displacer is selected to have higher affinity for the stationary phase than does any of the solutes being separated, and its concentration is set to approach saturation of the stationary phase and to give the desired migration rate of the concentration wave. High-retention conditions can be employed without gradient operation, because the displacer ensures removal of all solutes of interest in the designed run time.

Because of the concentrating effect of loading the column under high-retention conditions, displacement chromatography is well suited to purify components from dilute feed streams. However, it is also possible to concentrate material from a dilute stream at the head of a chromatographic column and then switch conditions to elute the adsorbed material in conventional isocratic or gradient modes. Therefore, this approach is not unique to displacement chromatography, although the higher loading capacity and less dilution allow greater concentration in displacement mode.

A disadvantage of displacement chromatography is that non-idealities always give rise to an overlap zone between each pair of components; this mixed zone must be collected separately for recycle or discard to preserve the purity of the separated materials. The strategy of adding spacer molecules to form zones between the components (sometimes termed "carrier displacement chromatography") has been investigated and can be useful when suitable, readily removable spacers are found. Another disadvantage is that the raw chromatogram, for instance a plot of absorbance or refractive index*vs* elution

volume, can be difficult to interpret for contiguous zones, especially if the displacement train is not fully developed. Documentation and troubleshooting may require additional chemical analysis to establish the distribution of a given component. Another disadvantage is that the time required for regeneration limits throughput.

According to John C. Ford's article in the *Encyclopedia of Chromatography*, theoretical studies indicate that at least for some systems, optimized overloaded elution chromatography offers higher throughput than displacement chromatography, though limited experimental tests suggest that displacement chromatography is superior (at least before consideration of regeneration time).

Applications

Historically, displacement chromatography was applied to preparative separations of amino acids and rare earth elements and has also been investigated for isotope separation.

Proteins

The chromatographic purification of proteins from complex mixtures can be quite challenging, particularly when the mixtures contain similarly retained proteins or when it is desired to enrich trace components in the feed. Further, column loading is often limited when high resolutions are required using traditional modes of chromatography (e.g. linear gradient, isocratic chromatography). In these cases, displacement chromatography is an efficient technique for the

purification of proteins from complex mixtures at high column loadings in a variety of applications.

An important advance in the state of the art of displacement chromatography was the development of low molecular mass displacers for protein purification in ion exchange systems. This research was significant in that it represented a major departure from the conventional wisdom that large polyelectrolyte polymers are required to displace proteins in ion exchange systems.

Low molecular mass displacers have significant operational advantages as compared to large polyelectrolyte displacers. For example, if there is any overlap between the displacer and the protein of interest, these low molecular mass materials can be readily separated from the purified protein during post-displacement processing using standard size-based purification methods (e.g. size exclusion chromatography, ultrafiltration). In addition, the salt-dependent adsorption behavior of these low MW displacers greatly facilitates column regeneration. These displacers have been employed for a wide variety of high resolution separations in ion exchange systems. In addition, the utility of displacement chromatography for the purification of recombinantgrowth factors, antigenicvaccine proteins and antisense oligonucleotides has also been demonstrated. There are several examples in which displacement chromatography has been applied to the purification of proteins using ion exchange, hydrophobic interaction, as well as reversed-phase chromatography.

Displacement chromatography is well suited for obtaining mg quantities of purified proteins from complex mixtures using

standard analytical chromatography columns at the bench scale. It is also particularly well suited for enriching trace components in the feed. Displacement chromatography can be readily carried out using a variety of resin systems including, ion exchange, HIC and RPLC

Two-dimensional chromatography

Two-dimensional chromatography represents the most thorough and rigorous approach to evaluation of the proteome. While previously accepted approaches have utilized elution mode chromatographic approaches such as cation exchange to reversed phase HPLC, yields are typically very low requiring analytical sensitivities in the picomolar to femtomolar range. As displacement chromatography offers the advantage of concentration of trace components, two dimensional chromatography utilizing displacement rather than elution mode in the upstream chromatography step represents a potentially powerful tool for analysis of trace components, modifications, and identification of minor expressed components of the proteome.

Electrochromatography

Electrochromatography is a chemical separation technique in analytical chemistry, biochemistry and molecular biology used to resolve and separate mostly large biomolecules such as proteins. It is a combination of size exclusion chromatography (gel filtration chromatography) and gel electrophoresis. These separation mechanisms operate essentially in superposition along the length of a gel filtration column to which an axial

electric field gradient has been added. The molecules are separated by size due to the gel filtration mechanism and by electrophoretic mobility due to the gel electrophoresis mechanism. Additionally there are secondary chromatographic solute retention mechanisms.

Capillary electrochromatography

Capillary electrochromatography (CEC) is an electrochromatography technique in which the liquid mobile phase is driven through a capillary containing the chromatographic stationary phase by electroosmosis. It is a combination of high-performance liquid chromatography and capillary electrophoresis. The capillaries is packed with HPLC stationary phase and a high voltage is applied to achieve separation is achieved by electrophoretic migration of the analyte and differential partitioning in the stationary phase.

Capillary electrochromatography

Capillary electrochromatography (**CEC**) is a chromatographic technique in which the mobile phase is driven through the chromatographic bed by electroosmosis. Capillary electrochromatography is a combination of two analytical techniques, high-performance liquid chromatography and capillary electrophoresis. Capillary electrophoresis aims to separate analytes on the basis of their mass-to-charge ratio by passing a high voltage across ends of a capillary tube, which is filled with the analyte. High-performance liquid chromatography separates analytes by passing them, under high pressure, through a column filled with stationary phase.

The interactions between the analytes and the stationary phase and mobile phase lead to the separation of the analytes. In capillary electrochromatography capillaries, packed with HPLC stationary phase, are subjected to a high voltage. Separation is achieved by electrophoretic migration of solutes and differential partitioning.

Instrumentation

The components of a capillary electrochromatograph are a sample vial, source and destination vials, a packed capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. The capillary is packed with stationary phase. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial (sample is introduced into the capillary via capillary action, pressure, or siphoning). The migration of the analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different migration times in an electropherogram.

Advantages

Avoiding the use of pressure to introduce the mobile phase into the column, results in a number of important advantages. Firstly, the pressure driven flow rate across a column depends directly on the square of the particle diameter and inversely on the length of the column. This restricts the length of the column and size of the particle, particle size is seldom less than 3 micrometer and the length of the column is restricted to 25 cm. Electrically driven flow rate is independent of length of column and size. A second advantage of using electroosmosis to pass the mobile phase into the column is the plug-like flow velocity profile of EOF, which reduces the solute dispersion in the column, increasing column efficiency.

Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (**MEKC**) is a chromatography technique used in analytical chemistry. It is a modification of capillary electrophoresis (CE), extending its functionality to neutral analytes, where the samples are separated by differential partitioning between micelles (pseudo-stationary phase) and a surrounding aqueous buffer solution (mobile phase).

The basic set-up and detection methods used for MEKC are the same as those used in CE. The difference is that the solution contains a surfactant at a concentration that is greater than the critical micelle concentration (CMC). Above this

concentration, surfactant monomers are in equilibrium with micelles. In most applications, MEKC is performed in open capillaries under alkaline conditions to generate a strong electroosmotic flow. Sodium dodecyl sulfate (SDS) is the most commonly used surfactant in MEKC applications. The anionic character of the sulfate groups of SDS causes the surfactant and micelles to have electrophoretic mobility that is counter to the direction of the strong electroosmotic flow. As a result, the surfactant monomers and micelles migrate quite slowly, though their net movement is still toward the cathode. During a MEKC separation, analytes distribute themselves between the hydrophobic interior of the micelle and hydrophilic buffer solution as shown in *figure 1*.

Applications

The simplicity and efficiency of MEKC have made it an attractive technique for a variety of applications. Further improvements can be made to the selectivity of MEKC by adding chiral selectors or chiral surfactants to the system. Unfortunately, this technique is not suitable for protein analysis because proteins are generally too large to partition into a surfactant micelle and tend to bind to surfactant monomers to form SDS-protein complexes.

Recent applications of MEKC include the analysis of uncharged pesticides, essential and branched-chain amino acids in nutraceutical products, hydrocarbon and alcohol contents of the marjoram herb.

MEKC has also been targeted for its potential to be used in combinatorial chemical analysis. The advent of combinatorial

chemistry has enabled medicinal chemists to synthesize and identify large numbers of potential drugs in relatively short periods of time. Small sample and solvent requirements and the high resolving power of MEKC have enabled this technique to be used to quickly analyze a large number of compounds with good resolution.

Traditional methods of analysis, like high-performance liquid chromatography (HPLC), can be used to identify the purity of a combinatorial library, but assays need to be rapid with good resolution for all components to provide useful information for the chemist. The introduction of surfactant to traditional capillary electrophoresis instrumentation has dramatically expanded the scope of analytes that can be separated by capillary electrophoresis.

MEKC can also be used in routine quality control of antibiotics in pharmaceuticals or feedstuffs.

Reversed-phase chromatography

Reversed-phase chromatography (also called **RPC**, **reverse-phase chromatography**, or **hydrophobic chromatography**) includes any chromatographic method that uses a hydrophobic stationary phase. RPC refers to liquid (rather than gas) chromatography.

Stationary phases

In the 1970s, most liquid chromatography was performed using a solid support stationary phase (also called a column)

containing unmodified silica or alumina resins. This type of technique is now referred to as normal-phase chromatography. Since the stationary phase is hydrophilic in this technique, molecules with hydrophilic properties contained within the mobile phase will have a high affinity for the stationary phase, and therefore will adsorb to the column packing. Hydrophobic molecules experience less of an affinity for the column packing, and will pass through to be eluted and detected first. Elution of the hydrophilic molecules adsorbed to the column packing requires the use of more hydrophilic or more polar solvents in the mobile phase to shift the distribution of the particles in the stationary phase towards that of the mobile phase.

Reversed-phase chromatography is a technique using alkyl chains covalently bonded to the stationary phase particles in order to create a hydrophobic stationary phase, which has a stronger affinity for hydrophobic or less polar compounds. The use of a hydrophobic stationary phase is essentially the reverse of normal phase chromatography, since the polarity of the mobile and stationary phases have been inverted – hence the term reversed-phase chromatography. Reversed-phase chromatography employs a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydro*philic* molecules in the mobile phase will pass through the column and are eluted first. Hydrophobic molecules can be eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent, which reduces hydrophobic interactions. The more hydrophobic the molecule, the more strongly it will bind to the stationary phase, and the

higher the concentration of organic solvent that will be required to elute the molecule.

Many of the mathematical and experimental considerations used in other chromatographic methods also apply to RPC (for example, the separation resolution is dependent on the length of the column). It can be used for the separation of a wide variety of molecules. It is not typically used for separation of proteins, because the organic solvents used in RPC can denature many proteins. For this reason, normal phase chromatography is more commonly used for separation of proteins. However, the denaturation of proteins may actually be beneficial in the later analysis of the samples obtained from the chromatography. If an enzymatic digestion with trypsin is performed on the proteins analysed, linearised protein is more suitable for this. Hence, the denaturation of proteins using appropriate solvents which cause the unfolding of the proteins can actually be intentional before taking the fractionated sample through mass spectrometry.

Today, RPC is a frequently used analytical technique. There are a variety of stationary phases available for use in RPC, allowing great flexibility in the development of separation methods.

Silica-based stationary phases

Any inert polar substance that achieves sufficient packing can be used for reversed-phase chromatography. The most popular column is an octadecyl carbon chain (C18)-bonded silica (USP classification L1). This is followed by C8-bonded silica (L7), pure silica (L3), cyano-bonded silica (L10) and phenyl-bonded

silica (L11). Note that C18, C8 and phenyl are dedicated reversed-phase resins, while cyano columns can be used in a reversed-phase mode depending on analyte and mobile phase conditions. Not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping). While the overall retention mechanism remains the same, subtle differences in the surface chemistries of different stationary phases will lead to changes in selectivity.

Modern columns have different polarity. PFP is pentafluorphenyl. CN is cyano. NH2 is amino. ODS is octadecyl or C18. ODCN is a mixed mode column consisting of C18 and nitrile. SCX is strong cationic exchange (used for separation of organic amines). SAX is strong anionic exchange (used for separation of carboxylic acid compounds).

Mobile phases

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed-phase column. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, and tetrahydrofuran (THF). Other solvents can be used such as ethanol or 2-propanol (isopropyl alcohol). Elution can be performed isocratically (the water-solvent composition does not change during the separation process) or by using a solution gradient (the water-solvent composition changes during the separation process, usually by decreasing the polarity). The pH of the mobile phase can have an important role on the

retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed-phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed-phase ion-pairing chromatography.

Two-dimensional chromatography

Two-dimensional chromatography is a type of chromatographic technique in which the injected sample is separated by passing through two different separation stages. Two different chromatographic columns are connected in sequence, and the effluent from the first system is transferred onto the second column. Typically the second column has a different separation mechanism, so that bands that are poorly resolved from the first column may be completely separated in the second column. (For instance, a C18 reversed-phase chromatography column may be followed by a phenyl column.) Alternately, the two columns might run at different temperatures. During the second stage of separation the rate at which the separation occurs must be faster than the first stage, since there is still only a single detector. The plane surface is amenable to sequential development in two directions using two different solvents.

History

Modern two-dimensional chromatographic techniques are based on the results of the early developments of Paper chromatography and Thin-layer chromatography which involved liquid mobile phases and solid stationary phases.

These techniques would later generate modern Gas chromatography and Liquid chromatography analysis. Different combinations of one dimensional GC and LC produced the analytical chromatographic technique that is known as two-dimensional chromatography.

The earliest form of 2D-chromatography came in the form of a multi-step TLC separation in which a thin sheet of cellulose is used first with one solvent in one direction, then, after the paper has been dried, another solvent is run in a direction at right angles to the first. This methodology first appeared in the literature with a 1944 publication by A. J. P. Martin and coworkers detailing an efficient method for separating amino acids- "...but the two-dimensional chromatogram is especially convenient, in that it shows at a glance information that can be gained otherwise only as the result of numerous experiments" (Biochem J., 1944, 38, 224).

Examples

Two-dimensional separations can be carried out in gas chromatography or liquid chromatography. Various different coupling strategies have been developed to "resample" from the first column into the second. Some important hardware for two-dimensional separations are Deans' switch and Modulator, which selectively transfer the first dimension eluent to second dimension column

The chief advantage of two-dimensional techniques is that they offer a large increase in peak capacity, without requiring extremely efficient separations in either column. (For instance, if the first column offers a peak capacity (k_1)of 100 for a 10-

minute separation, and the second column offers a peak capacity of 5 (k_2) in a 5-second separation, then the combined peak capacity may approach $k_1 \times k_2$=500, with the total separation time still ~ 10 minutes). 2D separations have been applied to the analysis of gasoline and other petroleum mixtures, and more recently to protein mixtures.

Tandem mass spectrometry

- Tandem mass spectrometry (Tandem MS or MS/MS) uses two mass analyzers in sequence to separate more complex mixtures of analytes. The advantage of tandem MS is that it can be much faster than other two-dimensional methods, with times ranging from milliseconds to seconds. Because there is no dilution with solvents in MS, there is less probability of interference, so tandem MS can be more sensitive and have a higher signal-to-noise ratio compared to other two-dimensional methods. The main disadvantage associated with tandem MS is the high cost of the instrumentation needed. Prices can range from $500,000 to over $1 million. Many form of tandem MS involve a mass selection step and a fragmentation step. The first mass analyzer can be programmed to only pass molecules of a specific mass-to-charge ratio. Then the second mass analyzer can fragment the molecule to determine its identity. This can be especially useful for separating molecules of the same mass (i.e. proteins of the same mass or molecular isomers). Different types of mass analyzers can be coupled to achieve varying effects. One example would be a TOF-Quadrople system. Ions

can be sequentially fragmented and/or analyzed in a quadrupole as they leave the TOF in order of increasing m/z. Another prevalent tandem mass spectrometer is the quadruple-quadrupole-quadrupole (Q-Q-Q) analyzer. The first quadrupole separates by mass, collisions take place in the second quadruple, and the fragments are separated by mass in the third quadrupole.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a two-dimensional chromatography technique that combines the separation technique of gas chromatography with the identification technique of mass spectrometry. GC-MS is the single most important analytical tool for the analysis of volatile and semi-volatile organic compounds in complex mixtures. It works by first injecting the sample into the GC inlet where it is vaporized and pushed through a column by a carrier gas, typically helium. The analytes in the sample are separated based upon their interaction with the coating of the column, or the stationary phase, and the carrier gas, or the mobile phase. The compounds eluted from the column are converted into ions via electron impact (EI) or chemical ionization (CI) before traveling through the mass analyzer. The mass analyzer serves to separate the ions on a mass-to-charge basis. Popular choices perform the same function but differ in the way that they accomplish the separation. The analyzers typically used with GC-MS are the time-of-flight mass analyzer and the quadrupole mass analyzer. After leaving the mass analyzer, the analytes reach the detector and produce a signal that is read by a computer and used to create a gas chromatogram and

mass spectrum. Sometimes GC-MS utilizes two gas chromatographers in particularly complex samples to obtain considerable separation power and be able to unambiguously assign the specific species to the appropriate peaks in a technique known as GCxGC-(MS). Ultimately, GC-MS is a technique utilized in many analytical laboratories and is a very effective and adaptable analytical tool.

Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC/MS) couples high resolution chromatographic separation with MS detection. As the system adopts the high separation of HPLC, analytes which are in the liquid mobile phase are often ionized by various soft ionization methods including atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), which attains the gas phase ionization required for the coupling with MS. These ionization methods allow the analysis of a wider range of biological molecules, including those with larger masses, thermally unstable or nonvolatile compounds where GC-MS is typically incapable of analyzing.

LC-MS provides high selectivity as unresolved peaks can be isolated by selecting a specific mass. Furthermore, better identification is also attained by mass spectra and the user does not have to rely solely on the retention time of analytes. As a result, molecular mass and structural information as well as quantitative data can all be obtained via LC-MS. LC-MS can therefore be applied to various fields, such as impurity identification and profiling in drug development and pharmaceutical manufacturing, since LC provides efficient

separation of impurities and MS provides structural characterization for impurity profiling.

Common solvents used in normal or reversed phase LC such as water, acetonitrile, and methanol are all compatible with ESI, yet a LC grade solvent may not be suitable for MS. Furthermore, buffers containing inorganic ions should be avoided as they may contaminate the ion source. Nonetheless, the problem can be resolved by 2D LC-MS, as well as other various issues including analyte coelusion and UV detection responses.

Liquid chromatography-liquid chromatography

Two dimensional liquid chromatography (2D-LC) combines two separate analyses of liquid chromatography into one data analysis. Modern 2-D liquid chromatography has its origins in the late 1970s to early 1980s. During this time, the hypothesized principles of 2D-LC were being proven via experiments conducted along with supplementary conceptual and theoretical work. It was shown that 2D-LC could offer quite a bit more resolving power compared to the conventional techniques of one-dimensional liquid chromatography. In the 1990s, the technique of 2D-LC played an important role in the separation of extremely complex substances and materials found in the proteomics and polymer fields of study. Unfortunately, the technique had been shown to have a significant disadvantage when it came to analysis time. Early work with 2D-LC was limited to small portion of liquid phase separations due to the long analysis time of the machinery. Modern 2D-LC techniques tackled that disadvantage head on, and have significantly reduced what was once a damaging

feature. Modern 2D-LC has an instrumental capacity for high resolution separations to be completed in an hour or less. Due to the growing need for instrumentation to perform analysis on substances of growing complexity with better detection limits, the development of 2D-LC pushes forward. Instrumental parts have become a mainstream industry focus and are much easier to attain then before. Prior to this, 2D-LC was performed using components from 1D-LC instruments, and would lead to results of varying degrees in both accuracy and precision. The reduced stress on instrumental engineering has allowed for pioneering work in the field and technique of 2D-LC.

The purpose of employing this technique is to separate mixtures that one dimensional liquid chromatography otherwise cannot separate effectively. Two dimensional liquid chromatography is better suited to analyzing complex mixtures samples such as urine, environmental substances and forensic evidence such as blood.

Difficulties in separating mixtures can be attributed to the complexity of the mixture in the sense that separation cannot occur due to the number of different effluents in the compound. Another problem associated with one dimensional liquid chromatography involves the difficulty associated to resolving closely related compounds. Closely related compounds have similar chemical properties that may prove difficult to separate based on polarity, charge, etc. Two dimensional liquid chromatography provides separation based on more than one chemical or physical property. Using an example from Nagy and Vekey, a mixture of peptides can be separated based on their basicity, but similar peptides may not elute well. Using a subsequent LC technique, the similar

basicity between the peptides can be further separated by employing differences in apolar character.

As a result, to be able to separate mixtures more efficiently, a subsequent LC analysis must employ very different separation selectivity relative to the first column. Another requirement to effectively use 2D liquid chromatography, according to Bushey and Jorgenson, is to employ highly orthogonal techniques which means that the two separation techniques must be as different as possible.

There are two major classifications of 2D liquid chromatography. These include: Comprehensive 2D liquid chromatography (LCxLC) and Heart-cutting 2D liquid chromatrography (LC-LC). In comprehensive 2D-LC, all the peaks from a column elution are fully sampled, but it has been deemed unnecessary to transfer the entire sample from the first to the second column. A portion of the sample is sent to waste while the rest is sent to the sampling valve. In heart-cutting 2D-LC specific peaks are targeted with only a small portion of the peak being injected onto a second column. Heart-cutting 2D-LC has proven to be quite useful for sample analysis of substances that are not very complex provided they have similar retention behavior. Compared to comprehensive 2D-LC, heart-cutting 2D-LC provides an effective technique with much less system setup and a much lower operating cost. Multiple heart-cutting (mLC-LC) may be utilized to sample multiple peaks from first dimensional analysis without risking temporary overlap of second dimensional analysis. Multiple heart-cutting (mLC-LC) utilizes a setup of multiple sampling loops.

For 2D-LC, peak capacity is a very important issue. This can be generated using Gradient elution separation with much greater efficiency than an isocratic separation given a reasonable amount of time. While isocratic elution is much easier on a fast time scale, it is preferable to perform a gradient elution separation in the second dimension. The mobile phase strength is varied from a weak eluent composition to a stronger one. Based on linear solvent strength theory (LSST) of gradient elution for reversed phase chromatography, the relationship between retention time, instrumental variables and solute parameters is shown below.

$t_R=t_0 +t_D + t_0/b*\ln(b*(k_0-t_d/t_0) + 1)$

While a lot of pioneering work has been completed in the years since 2D-LC became a major analytical chromatographic technique, there are still many modern problems to be considered. Large amounts of experimental variables have yet to be decided on, and the technique is constantly in a state of development.

Gas chromatography - gas chromatography

Comprehensive two-dimensional gas chromatography is an analytical technique that separates and analyzes complex mixtures. It has been utilized in fields such as: flavor, fragrance, environmental studies, pharmaceuticals, petroleum products and forensic science. GCxGC provides a high range of sensitivity and produces a greater separation power due to the increased peak capacity.

Chapter 6

Hyphenated Methods

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (**GC-MS**) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples, including that of material samples obtained from planet Mars during probe missions as early as the 1970s. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification. Like liquid chromatography–mass spectrometry, it allows analysis and detection even of tiny amounts of a substance.

GC-MS has been regarded as a "gold standard" for forensic substance identification because it is used to perform a 100% specific test, which positively identifies the presence of a particular substance. A nonspecific test merely indicates that any of several in a category of substances is present. Although a nonspecific test could statistically suggest the identity of the substance, this could lead to false positive identification. However, the high temperatures (300°C) used in the GC-MS

injection port (and oven) can result in thermal degradation of injected molecules, thus resulting in the measurement of degradation products instead of the actual molecule(s) of interest.

History

The first on-line coupling of gas chromatography to a mass spectrometer was reported in 1959. The development of affordable and miniaturizedcomputers has helped in the simplification of the use of this instrument, as well as allowed great improvements in the amount of time it takes to analyze a sample. In 1964, Electronic Associates, Inc. (EAI), a leading U.S. supplier of analog computers, began development of a computer controlled quadrupole mass spectrometer under the direction of Robert E. Finnigan. By 1966 Finnigan and collaborator Mike Uthe's EAI division had sold over 500 quadrupole residual gas-analyzer instruments. In 1967, Finnigan left EAI to form the Finnigan Instrument Corporation along with Roger Sant, T. Z. Chou, Michael Story, Lloyd Friedman, and William Fies. In early 1968, they delivered the first prototype quadrupole GC/MS instruments to Stanford and Purdue University. When Finnigan Instrument Corporation was acquired by Thermo Instrument Systems (later Thermo Fisher Scientific) in 1990, it was considered "the world's leading manufacturer of mass spectrometers".

Instrumentation

- The GC-MS is composed of two major building blocks: the gas chromatograph and the mass

spectrometer. The gas chromatograph utilizes a capillary column whose properties regarding molecule separation depend on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio.

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (*i.e.* have the same retention time), which results in two or more molecules that co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass

spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically increases certainty that the analyte of interest is in the sample.

Purge and trap GC-MS

For the analysis of volatile compounds, a purge and trap (P&T) concentrator system may be used to introduce samples. The target analytes are extracted by mixing the sample with water and purge with inert gas (e.g. Nitrogen gas) into an airtight chamber, this is known as purging or sparging. The volatile compounds move into the headspace above the water and are drawn along a pressure gradient (caused by the introduction of the purge gas) out of the chamber. The volatile compounds are drawn along a heated line onto a 'trap'. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. P&T GC-MS is particularly suited to volatile organic compounds (VOCs) and BTEX compounds (aromatic compounds associated with petroleum).

A faster alternative is the "purge-closed loop" system. In this system the inert gas is bubbled through the water until the concentrations of organic compounds in the vapor phase are at equilibrium with concentrations in the aqueous phase. The gas phase is then analysed directly.

Types of mass spectrometer detectors

The most common type of mass spectrometer (MS) associated with a gas chromatograph (GC) is the quadrupole mass spectrometer, sometimes referred to by the Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD). Another relatively common detector is the ion trap mass spectrometer. Additionally one may find a magnetic sector mass spectrometer, however these particular instruments are expensive and bulky and not typically found in high-throughput service laboratories. Other detectors may be encountered such as time of flight (TOF), tandem quadrupoles (MS-MS) (see below), or in the case of an ion trap MS where n indicates the number mass spectrometry stages.

GC-tandem MS

When a second phase of mass fragmentation is added, for example using a second quadrupole in a quadrupole instrument, it is called tandem MS (MS/MS). MS/MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background.

The first quadrupole (Q1) is connected with a collision cell (Q2) and another quadrupole (Q3). Both quadrupoles can be used in scanning or static mode, depending on the type of MS/MS analysis being performed. Types of analysis include product ion scan, precursor ion scan, selected reaction monitoring (SRM) (sometimes referred to as multiple reaction monitoring (MRM)) and neutral loss scan. For example: When Q1 is in static mode (looking at one mass only as in SIM), and Q3 is in

scanning mode, one obtains a so-called product ion spectrum (also called "daughter spectrum"). From this spectrum, one can select a prominent product ion which can be the product ion for the chosen precursor ion. The pair is called a "transition" and forms the basis for SRM. SRM is highly specific and virtually eliminates matrix background.

Ionization

After the molecules travel the length of the column, pass through the transfer line and enter into the mass spectrometer they are ionized by various methods with typically only one method being used at any given time. Once the sample is fragmented it will then be detected, usually by an electron multiplier, which essentially turns the ionized mass fragment into an electrical signal that is then detected.

The ionization technique chosen is independent of using full scan or SIM.

Electron ionization

By far the most common and perhaps standard form of ionization is electron ionization (EI). The molecules enter into the MS (the source is a quadrupole or the ion trap itself in an ion trap MS) where they are bombarded with free electrons emitted from a filament, not unlike the filament one would find in a standard light bulb. The electrons bombard the molecules, causing the molecule to fragment in a characteristic and reproducible way. This "hard ionization" technique results in the creation of more fragments of low mass-to-charge ratio (m/z) and few, if any, molecules approaching the molecular

mass unit. Hard ionization is considered by mass spectrometrists as the employ of molecular electron bombardment, whereas "soft ionization" is charge by molecular collision with an introduced gas. The molecular fragmentation pattern is dependent upon the electron energy applied to the system, typically 70 eV (electronvolts). The use of 70 eV facilitates comparison of generated spectra with library spectra using manufacturer-supplied software or software developed by the National Institute of Standards (NIST-USA). Spectral library searches employ matching algorithms such as Probability Based Matching and dot-product matching that are used with methods of analysis written by many method standardization agencies. Sources of libraries include NIST, Wiley, the AAFS, and instrument manufacturers.

Cold electron ionization

The "hard ionization" process of electron ionization can be softened by the cooling of the molecules before their ionization, resulting in mass spectra that are richer in information. In this method named cold electron ionization (cold-EI) the molecules exit the GC column, mixed with added helium make up gas and expand into vacuum through a specially designed supersonic nozzle, forming a supersonic molecular beam (SMB). Collisions with the make up gas at the expanding supersonic jet reduce the internal vibrational (and rotational) energy of the analyte molecules, hence reducing the degree of fragmentation caused by the electrons during the ionization process. Cold-EI mass spectra are characterized by an abundant molecular ion while the usual fragmentation pattern is retained, thus making cold-EI mass spectra compatible with library search identification techniques. The enhanced molecular ions increase the

identification probabilities of both known and unknown compounds, amplify isomer mass spectral effects and enable the use of isotope abundance analysis for the elucidation of elemental formulas.

Chemical ionization

In chemical ionization (CI) a reagent gas, typically methane or ammonia is introduced into the mass spectrometer. Depending on the technique (positive CI or negative CI) chosen, this reagent gas will interact with the electrons and analyte and cause a 'soft' ionization of the molecule of interest. A softer ionization fragments the molecule to a lower degree than the hard ionization of EI. One of the main benefits of using chemical ionization is that a mass fragment closely corresponding to the molecular weight of the analyte of interest is produced.

In positive chemical ionization (PCI) the reagent gas interacts with the target molecule, most often with a proton exchange. This produces the species in relatively high amounts.

In negative chemical ionization (NCI) the reagent gas decreases the impact of the free electrons on the target analyte. This decreased energy typically leaves the fragment in great supply.

Analysis

A mass spectrometer is typically utilized in one of two ways: full scan or selective ion monitoring (SIM). The typical GC-MS instrument is capable of performing both functions either

individually or concomitantly, depending on the setup of the particular instrument.

The primary goal of instrument analysis is to quantify an amount of substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library. This is best performed by a computer because there are a myriad of visual distortions that can take place due to variations in scale. Computers can also simultaneously correlate more data (such as the retention times identified by GC), to more accurately relate certain data. Deep learning was shown to lead to promising results in the identification of VOCs from raw GC-MS data

Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. All values above 3% are assigned. The total mass of the unknown compound is normally indicated by the parent peak. The value of this parent peak can be used to fit with a chemical formula containing the various elements which are believed to be in the compound. The isotope pattern in the spectrum, which is unique for elements that have many natural isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and must be consistent with the characteristics recorded by GC-MS. Typically, this identification is done automatically by programs which come with the instrument,

given a list of the elements which could be present in the sample. A “full spectrum” analysis considers all the “peaks” within a spectrum. Conversely, selective ion monitoring (SIM) only monitors selected ions associated with a specific substance. This is done on the assumption that at a given retention time, a set of ions is characteristic of a certain compound. This is a fast and efficient analysis, especially if the analyst has previous information about a sample or is only looking for a few specific substances. When the amount of information collected about the ions in a given gas chromatographic peak decreases, the sensitivity of the analysis increases. So, SIM analysis allows for a smaller quantity of a compound to be detected and measured, but the degree of certainty about the identity of that compound is reduced.

Full scan MS

When collecting data in the full scan mode, a target range of mass fragments is determined and put into the instrument's method. An example of a typical broad range of mass fragments to monitor would be m/z 50 to m/z 400.

The determination of what range to use is largely dictated by what one anticipates being in the sample while being cognizant of the solvent and other possible interferences. A MS should not be set to look for mass fragments too low or else one may detect air (found as m/z28 due to nitrogen), carbon dioxide (m/z 44) or other possible interference. Additionally if one is to use a large scan range then sensitivity of the instrument is decreased due to performing fewer scans per second since each scan will have to detect a wide range of mass fragments.

Full scan is useful in determining unknown compounds in a sample. It provides more information than SIM when it comes to confirming or resolving compounds in a sample. During instrument method development it may be common to first analyze test solutions in full scan mode to determine the retention time and the mass fragment fingerprint before moving to a SIM instrument method.

Selective ion monitoring

In selective ion monitoring (SIM) certain ion fragments are entered into the instrument method and only those mass fragments are detected by the mass spectrometer. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g. three fragments) during each scan. More scans can take place each second. Since only a few mass fragments of interest are being monitored, matrix interferences are typically lower. To additionally confirm the likelihood of a potentially positive result, it is relatively important to be sure that the ion ratios of the various mass fragments are comparable to a known reference standard.

Applications

Environmental monitoring and cleanup

GC-MS is becoming the tool of choice for tracking organic pollutants in the environment. The cost of GC-MS equipment has decreased significantly, and the reliability has increased at

the same time, which has contributed to its increased adoption in environmental studies.

Criminal forensics

GC-MS can analyze the particles from a human body in order to help link a criminal to a crime. The analysis of fire debris using GC-MS is well established, and there is even an established American Society for Testing and Materials (ASTM) standard for fire debris analysis. GCMS/MS is especially useful here as samples often contain very complex matrices and results, used in court, need to be highly accurate.

Law enforcement

GC-MS is increasingly used for detection of illegal narcotics, and may eventually supplant drug-sniffing dogs. A simple and selective GC-MS method for detecting marijuana usage was recently developed by the Robert Koch-Institute in Germany. It involves identifying an acid metabolite of tetrahyhydrocannabinol (THC), the active ingredient in marijuana, in urine samples by employing derivatization in the sample preparation. GC-MS is also commonly used in forensic toxicology to find drugs and/or poisons in biological specimens of suspects, victims, or the deceased. In drug screening, GC-MS methods frequently utilize liquid-liquid extraction as a part of sample preparation, in which target compounds are extracted from blood plasma.

Sports anti-doping analysis

GC-MS is the main tool used in sports anti-doping laboratories to test athletes' urine samples for prohibited performance-enhancing drugs, for example anabolic steroids.

Security

A post–September 11 development, explosive detection systems have become a part of all USairports. These systems run on a host of technologies, many of them based on GC-MS. There are only three manufacturers certified by the FAA to provide these systems, one of which is Thermo Detection (formerly Thermedics), which produces the EGIS, a GC-MS-based line of explosives detectors. The other two manufacturers are Barringer Technologies, now owned by Smith 's Detection Systems, and Ion Track Instruments, part of General Electric Infrastructure Security Systems.

Chemical warfare agent detection

As part of the post-September 11 drive towards increased capability in homeland security and public health preparedness, traditional GC-MS units with transmission quadrupole mass spectrometers, as well as those with cylindrical ion trap (CIT-MS) and toroidal ion trap (T-ITMS) mass spectrometers have been modified for field portability and near real-time detection of chemical warfare agents (CWA) such as sarin, soman, and VX. These complex and large GC-MS systems have been modified and configured with resistively heated low thermal mass (LTM) gas chromatographs that reduce analysis time to less than ten percent of the time

required in traditional laboratory systems. Additionally, the systems are smaller, and more mobile, including units that are mounted in mobile analytical laboratories (MAL), such as those used by the United States Marine Corps Chemical and Biological Incident Response Force MAL and other similar laboratories, and systems that are hand-carried by two-person teams or individuals, much ado to the smaller mass detectors. Depending on the system, the analytes can be introduced via liquid injection, desorbed from sorbent tubes through a thermal desorption process, or with solid-phase micro extraction (SPME).

Chemical engineering

GC-MS is used for the analysis of unknown organic compound mixtures. One critical use of this technology is the use of GC-MS to determine the composition of bio-oils processed from raw biomass. GC-MS is also utilized in the identification of continuous phase component in a smart material, Magnetorheological (MR) fluid.

Food, beverage and perfume analysis

Foods and beverages contain numerous aromatic compounds, some naturally present in the raw materials and some forming during processing. GC-MS is extensively used for the analysis of these compounds which include esters, fatty acids, alcohols, aldehydes, terpenes etc. It is also used to detect and measure contaminants from spoilage or adulteration which may be harmful and which is often controlled by governmental agencies, for example pesticides.

Astrochemistry

Several GC-MS have left earth. Two were brought to Mars by the Viking program. Venera 11 and 12 and Pioneer Venus analysed the atmosphere of Venus with GC-MS. The Huygens probe of the Cassini–Huygens mission landed one GC-MS on Saturn's largest moon, Titan. The MSL Curiosity rover's Sample analysis at Mars (SAM) instrument contains both a gas chromatograph and quadrupol mass spectrometer that can be used in tandem as a GC-MS. The material in the comet67P/Churyumov–Gerasimenko was analysed by the Rosetta mission with a chiral GC-MS in 2014.

Medicine

Dozens of congenital metabolic diseases also known as inborn errors of metabolism (IEM) are now detectable by newborn screening tests, especially the testing using gas chromatography–mass spectrometry. GC-MS can determine compounds in urine even in minor concentration. These compounds are normally not present but appear in individuals suffering with metabolic disorders. This is increasingly becoming a common way to diagnose IEM for earlier diagnosis and institution of treatment eventually leading to a better outcome. It is now possible to test a newborn for over 100 genetic metabolic disorders by a urine test at birth based on GC-MS.

In combination with isotopic labeling of metabolic compounds, the GC-MS is used for determining metabolic activity. Most applications are based on the use of C as the labeling and the measurement of C-C ratios with an isotope ratio mass

spectrometer (IRMS); an MS with a detector designed to measure a few select ions and return values as ratios.

Liquid chromatography–mass spectrometry

Liquid chromatography–mass spectrometry (**LC–MS**) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity. This tandem technique can be used to analyze biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin. Therefore, LC-MS may be applied in a wide range of sectors including biotechnology, environment monitoring, food processing, and pharmaceutical, agrochemical, and cosmetic industries.

In addition to the liquid chromatography and mass spectrometry devices, an LC-MS system contains an interface that efficiently transfers the separated components from the LC column into the MS ion source. The interface is necessary because the LC and MS devices are fundamentally incompatible. While the mobile phase in a LC system is a pressurized liquid, the MS analyzers commonly operate under

high vacuum (around 10 Torr / 10 "Hg). Thus, it is not possible to directly pump the eluate from the LC column into the MS source. Overall, the interface is a mechanically simple part of the LC-MS system that transfers the maximum amount of analyte, removes a significant portion of the mobile phase used in LC and preserves the chemical identity of the chromatography products (chemically inert). As a requirement, the interface should not interfere with the ionizing efficiency and vacuum conditions of the MS system. Nowadays, most extensively applied LC-MS interfaces are based on atmospheric pressure ionization (API) strategies like electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). These interfaces became available in the 1990s after a two decade long research and development process.

History of LC-MS

The coupling of chromatography with MS is a well developed chemical analysis strategy dating back from the 1950s. Gas chromatography (GC)–MS was originally introduced in 1952, when A. T. James and A. J. P. Martin were trying to develop tandem separation - mass analysis techniques. In GC, the analytes are eluted from the separation column as a gas and the connection with electron ionization (EI) or chemical ionization (CI) ion sources in the MS system was a technically simpler challenge. Because of this, the development of GC-MS systems was faster than LC-MS and such systems were first commercialized in the 1970s. The development of LC-MS systems took longer than GC-MS and was directly related to the development of proper interfaces. V. L. Tal'roze and

collaborators started the development of LC-MS in the early 1970s, when they first used capillaries to connect LC columns and MS ion sources. A similar strategy was investigated by McLafferty and collaborators in 1973. This was the first and most obvious way of coupling LC with MS, and was known as the capillary inlet interface. This pioneer interface for LC-MS had the same analysis capabilities of GC-MS and was limited to rather volatile analytes and non-polar compounds with low molecular mass (below 400 Da). In the capillary inlet interface, the evaporation of the mobile phase inside the capillary was one of the main issues. Within the first years of development of LC-MS, on-line and off-line alternatives were proposed as coupling alternatives. In general, off-line coupling involved fraction collection, evaporation of solvent, and transfer of analytes to the MS using probes. Off-line analyte treatment process was time consuming and there was an inherent risk of sample contamination. Rapidly, it was realized that the analysis of complex mixtures would require the development of a fully automated on-line coupling solution in LC-MS.

Moving-belt interface

The moving-belt interface (MBI) was developed in 1977. This interface consisted of an endless moving belt receiving the LC column effluent. On the belt, the solvent was evaporated by gently heating and efficiently exhausting the solvent vapors under reduced pressure in two vacuum chambers. After removing the liquid phase, the analytes would desorb from the belt and migrate to the MS ion source to be analysed. MBI was successfully used for LC-MS applications between 1978 and 1990 because it allowed coupling of LC to MS devices using EI, CI, and fast-atom bombardment (FAB) ion sources. The most

common MS systems connected by MBI interfaces to LC columns were magnetic sector and quadropole instruments. MBI interfaces for LC-MS allowed MS to be widely applied in the analysis of drugs, pesticides, steroids, alkaloids, and polycyclic aromatic hydrocarbons. This interface is no longer used because of its mechanical complexity and the difficulties associated to belt renewal. Particle beam interfaces took over the wide applications of MBI for LC-MS in 1988.

Direct liquid introduction interface

The direct liquid introduction (DLI) interface was developed in 1980. This interface was thought as a solution to the evaporation of liquid inside the capillary inlet interface. In DLI, a nebulizer was used to disintegrate part of the effluent coming from the column. A small diaphragm was used to form a liquid jet composed of small droplets that were subsequently dried in a desolvation chamber. A microbore capillary column was used to transfer the nebulized liquid product to the MS ion source. The analytes were ionized using a solvent assisted chemical ionization source, where the LC solvents acted as reagent gases. To use this interface, it was necessary to split the flow coming out of the LC column because only a small portion of the effluent (10 to 50 µl/min out of 1 ml/min) could be analyzed on-line without breaking the MS vacuum. One of the main operational problems of the DLI interface was the frequent clogging of the diaphragm orifices. The DLI interface was used between 1982 and 1985 for the analysis of pesticides, corticosteroids, metabolites in horse urine, erythromycin, and vitamin B_{12}. However, this interface was replaced by the thermospray interface, which removed the flow rate limitations and the issues with the clogging diaphragms.

Thermospray interface

The thermospray (TSP) interface was developed in 1983 by Vestal laboratories at the University of Houston. The interface resulted from a long term research project intended to find a LC-MS interface capable of handling high flow rates (1 ml/min) and avoiding the flow split in DLI interfaces. The TSP interface was composed by a heated probe, a desolvation chamber, and an ion exchange skimmer.

The LC effluent passed through the heated probe and emerged as a jet of vapor and small droplets flowing into the desolvation chamber at low pressure. The ionization of solutes occurred by direct evaporation or ion-molecule reactions induced by the solvent. This interface was able to handle up to 2 ml/min of eluate from the LC column and would efficiently introduce it into the MS vacuum system. TSP was also more suitable for LC-MS applications involving reversed phase liquid chromatography (RT-LC). The TSP system had a dual function acting as an interface and a solvent-mediated chemical ionization source.

With time, the mechanical complexity of TSP was simplified, and this interface became popular as the first ideal LC-MS interface for pharmaceutical applications comprising the analysis of drugs, metabolites, conjugates, nucleosides, peptides, natural products, and pesticides. The introduction of TSP marked a significant improvement for LC-MS systems and was the most widely applied interface until the beginning of the 1990s, when it began to be replaced by interfaces involving atmospheric pressure ionization (API).

FAB based interfaces

The frit FAB and continuous flow-FAB (CF-FAB) interfaces were developed in 1985 and 1986 respectively. Both interfaces were similar, but they differed in that the first used a porous frit probe as connecting channel, while CF-FAB used a probe tip. From these, the CF-FAB was more successful as a LC-MS interface and was useful to analyze non-volatile and thermally labile compounds. In these interfaces, the LC effluent passed through the frit or CF-FAB channels to form a uniform liquid film at the tip. There, the liquid was bombarded with ion beams or high energy atoms (fast atom). For stable operation, the FAB based interfaces were able to handle liquid flow rates of only 1–15 µl and were also restricted to microbore and capillary columns. In order to be used in FAB MS ionization sources, the analytes of interest should be mixed with a matrix (e.g., glycerol) that could be added before or after the separation in the LC column. FAB based interfaces were extensively used to characterize peptides, but lost applicability with the advent of electrospray based interfaces in 1988.

Liquid chromatography

Liquid chromatography is a method of physical separation in which the components of a liquid mixture are distributed between two immiscible phases, i.e., stationary and mobile. The practice of LC can be divided into five categories, i.e., adsorption chromatography, partition chromatography, ion-exchange chromatography, size-exclusion chromatography, and affinity chromatography. Among these, the most widely used variant is the reverse-phase (RP) mode of the partition

chromatography technique, which makes use of a nonpolar (hydrophobic) stationary phase and a polar mobile phase. In common applications, the mobile phase is a mixture of water and other polar solvents (e.g., methanol, isopropanol, and acetonitrile), and the stationary matrix is prepared by attaching long-chain alkyl groups (e.g., n-octadecyl or C_{18}) to the surface of irregularly or spherically shaped 5 µm diameter silica particles.

In HPLC, typically 20 µl of the sample of interest are injected into the mobile phase stream delivered by a high pressure pump. The mobile phase containing the analytes permeates through the stationary phase bed in a definite direction. The components of the mixture are separated depending on their chemical affinity with the mobile and stationary phases. The separation occurs after repeated sorption and desorption steps occurring when the liquid interacts with the stationary bed. The liquid solvent (mobile phase) is delivered under high pressure (up to 400 bar or 300.000 torr) into a packed column containing the stationary phase. The high pressure is necessary to achieve a constant flow rate for reproducible chromatography experiments. Depending on the partitioning between the mobile and stationary phases, the components of the sample will flow out of the column at different times. The column is the most important component of the LC system and is designed to withstand the high pressure of the liquid. Conventional LC columns are 100–300 mm long with outer diameter of 6.4 mm (1/4 inch) and internal diameter of 3.0–4.6 mm. For applications involving LC-MS, the length of chromatography columns can be shorter (30–50 mm) with 3–5 µm diameter packing particles. In addition to the conventional model, other LC columns are the narrow bore, microbore,

microcapillary, and nano-LC models. These columns have smaller internal diameters, allow for a more efficient separation, and handle liquid flows under 1 ml/min (the conventional flow-rate). In order to improve separation efficiency and peak resolution, ultra performance liquid chromatography (UPLC) can be used instead of HPLC. This LC variant uses columns packed with smaller silica particles (~1.7 µm diameter) and requires higher operating pressures in the range of 310.000 to 775.000 torr (6000 to 15000 psi).

Mass spectrometry

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio (*m/z)* of charged particles (ions). Although there are many different kinds of mass spectrometers, all of them make use of electric or magnetic fields to manipulate the motion of ions produced from an analyte of interest and determine their *m/z.* The basic components of a mass spectrometer are the ion source, the mass analyzer, the detector, and the data and vacuum systems. The ion source is where the components of a sample introduced in a MS system are ionized by means of electron beams, photon beams (UV lights), laser beams or corona discharge. In the case of electrospray ionization, the ion source moves ions that exist in liquid solution into the gas phase. The ion source converts and fragments the neutral sample molecules into gas-phase ions that are sent to the mass analyzer. While the mass analyzer applies the electric and magnetic fields to sort the ions by their masses, the detector measures and amplifies the ion current to calculate the abundances of each mass-resolved ion. In order to generate a

mass spectrum that a human eye can easily recognize, the data system records, processes, stores, and displays data in a computer.

The mass spectrum can be used to determine the mass of the analytes, their elemental and isotopic composition, or to elucidate the chemical structure of the sample. MS is an experiment that must take place in gas phase and under vacuum (1.33 * 10 to 1.33 * 10 pascal). Therefore, the development of devices facilitating the transition from samples at higher pressure and in condensed phase (solid or liquid) into a vacuum system has been essential to develop MS as a potent tool for identification and quantification of organic compounds like peptides. MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds. Among the many different kinds of mass analyzers, the ones that find application in LC-MS systems are the quadrupole, time-of-flight (TOF), ion traps, and hybrid quadrupole-TOF (QTOF) analyzers.

Interfaces

The interface between a liquid phase technique (HPLC) with a continuously flowing eluate, and a gas phase technique carried out in a vacuum was difficult for a long time. The advent of electrospray ionization changed this. Currently, the most common LC-MS interfaces are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo-ionization (APPI). These are newer MS ion sources that facilitate the transition from a high pressure environment (HPLC) to high vacuum conditions

needed at the MS analyzer. Although these interfaces are described individually, they can also be commercially available as dual ESI/APCI, ESI/APPI, or APCI/APPI ion sources. Various deposition and drying techniques were used in the past (e.g., moving belts) but the most common of these was the off-line MALDI deposition. A new approach still under development called direct-EI LC-MS interface, couples a nano HPLC system and an electron ionization equipped mass spectrometer.

Electrospray ionization (ESI)

ESI interface for LC-MS systems was developed by Fenn and collaborators in 1988. This ion source/ interface can be used for the analysis of moderately polar molecules (e.g., metabolites, xenobiotics, and peptides). The liquid eluate coming out of the LC column is pumped through a metal capillary kept at 3 to 5 kV. The liquid is nebulized at the tip of the capillary and a fine spray of charged droplets is formed. To avoid contamination, this capillary is usually perpendicularly located at the inlet of the MS system.

The heat created by the electric potential is used to rapidly evaporate the droplets in an atmosphere of dry nitrogen. Later, the ionized analytes are transferred into the high vacuum chamber of the MS as the charged ions flow through a series of small apertures with the aid of focusing voltages. Positively and negatively charged ions can be detected and it is possible to switch between the negative and positive modes of operation. Most ions produced in the ESI interface are multiply charged. The use of 1–3 mm ID microbore columns is recommended for LC-MS systems using electrospray ionization

(ESI) interfaces because optimal operation is achieved with flow rates in the 50-200 µl/min range.

Atmospheric pressure chemical ionization (APCI)

The development of the APCI interface for LC-MS started with Horning and collaborators in the early 1973. However, its commercial application was introduced at the beginning of the 1990s after Henion and collaborators improved the LC-APCI-MS interface in 1986.

The APCI ion source/ interface can be used to analyze small, neutral, relatively non-polar, and thermally stable molecules (e.g., steroids, lipids, and fat soluble vitamins). These compounds are not well ionized using ESI. In addition, APCI can also handle mobile phase streams containing buffering agents. The liquid from the LC system is pumped through a capillary and there is also nebulization at the tip, where a corona discharge takes place. First, the ionizing gas surrounding the interface and the mobile phase solvent are subject to chemical ionization at the ion source. Later, these ions react with the analyte and transfer their charge.

The sample ions then pass through small orifice skimmers by means of or ion-focusing lenses. Once inside the high vacuum region, the ions are subject to mass analysis. This interface can be operated in positive and negative charge modes and singly-charged ions are mainly produced. APCI ion source can also handle flow rates between 500 and 2000 µl/min and it can be directly connected to conventional 4.6 mm ID columns.

Atmospheric pressure photoionization (APPI)

The APPI interface for LC-MS was developed simultaneously by Bruins and Syage in 2000. APPI is another LC-MS ion source/ interface for the analysis of neutral compounds that cannot be ionized using ESI. This interface is similar to the APCI ion source, but instead of a corona discharge, the ionization occurs by using photons coming from a discharge lamp. In the direct-APPI mode, singly charged analyte molecular ions are formed by absorption of a photon and ejection of an electron. In the dopant-APPI mode, an easily ionizable compound (Dopant) is added to the mobile phase or the nebulizing gas to promote a reaction of charge-exchange between the dopant molecular ion and the analyte. The ionized sample is later transferred to the mass analyzer at high vacuum as it passes through small orifice skimmers.

Applications

The coupling of MS with LC systems is attractive because liquid chromatography can separate delicate and complex natural mixtures, which chemical composition needs to be well established (e.g., biological fluids, environmental samples, and drugs). Further, LC-MS has applications in volatile explosive residue analysis. Nowadays, LC-MS has become one of the most widely used chemical analysis techniques because more than 85% of natural chemical compounds are polar and thermally labile and GC-MS cannot process these samples. As an example, HPLC-MS is regarded as the leading analytical technique for proteomics and pharmaceutical laboratories.

Other important applications of LC-MS include the analysis of food, pesticides, and plant phenols.

Pharmacokinetics

LC-MS is widely used in the field of bioanalysis and is specially involved in pharmacokinetic studies of pharmaceuticals. Pharmacokinetic studies are needed to determine how quickly a drug will be cleared from the body organs and the hepatic blood flow. MS analyzers are useful in these studies because of their shorter analysis time, and higher sensitivity and specificity compared to UV detectors commonly attached to HPLC systems. One major advantage is the use of tandem MS-MS, where the detector may be programmed to select certain ions to fragment. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression in LC-MS, the LC separation can be quite quick.

Proteomics/metabolomics

LC-MS is used in proteomics as a method to detect and identify the components of a complex mixture. The bottom-up proteomics LC-MS approach generally involves protease digestion and denaturation using trypsin as a protease, urea to denature the tertiary structure, and iodoacetamide to modify the cysteine residues. After digestion, LC-MS is used for peptide mass fingerprinting, or LC-MS/MS (tandem MS) is used to derive the sequences of individual peptides. LC-MS/MS is most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometry. Samples of complex biological

(e.g., human serum) may be analyzed in modern LC-MS/MS systems, which can identify over 1000 proteins. However, this high level of protein identification is possible only after separating the sample by means of SDS-PAGE gel or HPLC-SCX. Recently, LC-MS/MS has been applied to search peptide biomarkers. An example is the recent discovery and validation of peptide biomarkers for four major bacterial respiratory tract pathogens (*Staphylococcus aureus,Moraxella catarrhalis*; *Haemophilus influenzae* and *Streptococcus pneumoniae*).

LC-MS has emerged as one of the most commonly used techniques in global metabolite profiling of biological tissue (e.g., blood plasma, serum, urine). LC-MS is also used for the analysis of natural products and the profiling of secondary metabolites in plants. In this regard, MS-based systems are useful to acquire more detailed information about the wide spectrum of compounds from a complex biological samples. LC-Nuclear magnetic resonance (NMR) is also used in plant metabolomics, but this technique can only detect and quantify the most abundant metabolites. LC-MS has been useful to advance the field of plant metabolomics, which aims to study the plant system at molecular level providing a non-biased characterization of the plant metabolome in response to its environment. The first application of LC-MS in plant metabolomics was the detection of a wide range of highly polar metabolites, oligosaccharides, amino acids, amino sugars, and sugar nucleotides from *Cucurbita maxima*phloem tissues. Another example of LC-MS in plant metabolomics is the efficient separation and identification of glucose, sucrose, raffinose, stachyose, and verbascose from leaf extracts of *Arabidopsis thaliana*.

Drug development

LC-MS is frequently used in drug development because it allows quick molecular weight confirmation and structure identification. These features speed up the process of generating, testing, and validating a discovery starting from a vast array of products with potential application. LC-MS applications for drug development are highly automated methods used for peptide mapping, glycoprotein mapping, lipodomics, natural products dereplication, bioaffinity screening, *in vivo* drug screening, metabolic stability screening, metabolite identification, impurity identification, quantitative bioanalysis, and quality control.

Pyrolysis–gas chromatography–mass spectrometry

Pyrolysis–gas chromatography–mass spectrometry is a method of chemical analysis in which the sample is heated to decomposition to produce smaller molecules that are separated by gas chromatography and detected using mass spectrometry.

How it works

Pyrolysis is the thermal decomposition of materials in an inert atmosphere or a vacuum. The sample is put into direct contact with a platinum wire, or placed in a quartz sample tube, and rapidly heated to 600–1000 °C. Depending on the application even higher temperatures are used. Three different heating techniques are used in actual pyrolyzers: Isothermal furnace,

inductive heating (Curie Point filament), and resistive heating using platinum filaments. Large molecules cleave at their weakest bonds, producing smaller, more volatile fragments. These fragments can be separated by gas chromatography. Pyrolysis GC chromatograms are typically complex because a wide range of different decomposition products is formed. The data can either be used as fingerprint to prove material identity or the GC/MS data is used to identify individual fragments to obtain structural information.

To increase the volatility of polar fragments, various methylating reagents can be added to a sample before pyrolysis.

Besides the usage of dedicated pyrolyzers, pyrolysis GC of solid and liquid samples can be performed directly inside programmable temperature vaporizer (PTV) injectors that provide quick heating (up to 60 °C/s) and high maximum temperatures of 600-650 °C.

This is sufficient for many pyrolysis applications. The main advantage is that no dedicated instrument has to be purchased and pyrolysis can be performed as part of routine GC analysis. In this case quartz GC inlet liners can be used. Quantitative data can be acquired, and good results of derivatization inside the PTV injector are published as well.

Applications

Pyrolysis gas chromatography is useful for the identification of involatile compounds. These materials include polymeric materials, such as acrylics or alkyds. The way in which the

polymer fragments, before it is separated in the GC, can help in identification. Pyrolysis gas chromatography is also used for environmental samples, including fossils. Pyrolysis GC is used in forensic laboratories to analyze evidence found of crime scenes such as paints, adhesives, plastics, synthetic fibres and soil extracts.

Chapter 7

Stubs

Äkta Explorer

The **ÄKTA explorer** was the first high end FPLC (Fast Protein Liquid Chromatography) system that was developed for Life Science research by the Swedish company Pharmacia in 1994. Its purpose was to simplify and automatize protein purification. It was followed by a line of similar devices (the "Äkta" line). The product line name was transferred together with the sale of Pharmacia first to Amersham and then to GE Healthcare. Although protein purification is possible with a large range of chromatographic devices, the Äkta line represents together with BioRad's NGC line the only devices that were specifically designed for this purpose both from the hardware and software perspective. Main users of these devices are the pharmaceutical industry and academic researchers.

Anion-exchange chromatography

Anion-exchange chromatography is a process that separates substances based on their charges using an ion-exchange resin containing positively charged groups, such as diethyl-aminoethyl groups (DEAE). In solution, the resin is coated with positively charged counter-ions (cations). Anion exchange resins will bind to negatively charged molecules, displacing the counter-ion. Anion exchange chromatography is commonly used to purify proteins, amino acids, sugars/carbohydrates

and other acidic substances with a negative charge at higher pH levels. The tightness of the binding between the substance and the resin is based on the strength of the negative charge of the substance.

General technique for protein purification

A slurry of resin, such as DEAE-Sephadex is poured into the column. The matrix that is used is insoluble with charged groups that are covalently attached. These charged groups are referred to as exchangers like cation and anion exchangers. After it settles, the column is pre-equilibrated in buffer before the protein mixture is applied. DEAE-Sephadex is a positively-charged slurry that will have electrostatic interactions with the negatively charged atoms, making them elute later than the positively-charged molecules in the interested sample. This is a separation technique used widely to discover specific proteins, or enzymes in the body. Unbound proteins are collected in the flow-through and/or in subsequent buffer washes. Proteins that bind to the positively charged resin are retained and can be eluted in one of two ways. First, the salt concentration in the elution buffer is gradually increased. The negative ions in the salt solution (e.g. Cl) compete with protein in binding to the resin. Second, the pH of the solution can be gradually decreased which results in a more positive charge on the protein, releasing it from the resin. Both of these techniques can displace the negatively charged protein which is then eluted into test tubes fractions with the buffer.

The separation of proteins will depend on the differences in total charge. Composition of ionizable side chain groups will determine the total charge of the protein at a particular pH. At the isoelectric point (pI), the total charge on the protein is 0 and it will not bind to the matrix. If the pH is above the pI, the protein will have a negative charge and bind to the matrix in an anion exchange column. The stability of the protein at values above or below the pI, will determine if an anion exchange column or cation exchange column should be used. If it is stable at pH values below the pI, the cation exchange column be used. If it is stable at pH values above the pI then the anion exchange column can be used.

Aqueous normal-phase chromatography

Aqueous normal-phase chromatography (**ANP**) is a chromatographic technique that involves the mobile phase region between reversed-phase chromatography (RP) and organic normal-phase chromatography (ONP).

Principle

In normal-phase chromatography, the stationary phase is polar and the mobile phase is nonpolar. In reversed phase we have just the opposite; the stationary phase is nonpolar and the mobile phase is polar. Typical stationary phases for normal-phase chromatography are silica or organic moieties with cyano and amino functional groups. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl

(C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. The designations for the reversed phase materials refer to the length of the hydrocarbon chain.

In normal-phase chromatography, the least polar compounds elute first and the most polar compounds elute last. The mobile phase consists of a nonpolar solvent such as hexane or heptane mixed with a slightly more polar solvent such as isopropanol, ethyl acetate or chloroform. Retention decreases as the amount of polar solvent in the mobile phase increases. In reversed phase chromatography, the most polar compounds elute first with the most nonpolar compounds eluting last. The mobile phase is generally a binary mixture of water and a miscible polar organic solvent like methanol, acetonitrile or THF. Retention increases as the amount of the polar solvent (water) in the mobile phase increases. Normal phase chromatography, an adsorptive mechanism, is used for the analysis of solutes readily soluble in organic solvents, based on their polar differences such as amines, acids, metal complexes, etc.. Reversed-phase chromatography, a partition mechanism, is typically used for separations by non-polar differences.

The "hydride surface" distinguishes the support material from other silica materials; most silica materials used for chromatography have a surface composed primarily of silanols (-Si-OH). In a "hydride surface" the terminal groups are primarily -Si-H. The hydride surface can also be functionalized with carboxylic acids and long-chain alkyl groups. Mobile phases for ANPC are based on an organic solvent (such as methanol or acetonitrile) with a small amount of water; thus,

the mobile phase is both "aqueous" (water is present) and "normal" (less polar than the stationary phase). Thus, polar solutes (such as acids and amines) are most strongly retained, with retention decreasing as the amount of water in the mobile phase increases.

Typically the amount of the nonpolar component in the mobile phase must be 60% or greater with the exact point of increased retention depending on the solute and the organic component of the mobile phase. A true ANP stationary phase will be able to function in both the reversed phase and normal phase modes with only the amount of water in the eluent varying. Thus a continuum of solvents can be used from 100% aqueous to pure organic. ANP retention has been demonstrated for a variety of polar compounds on the hydride based stationary phases. Recent investigations have demonstrated that silica hydride materials have a very thin water layer (about 0.5 monolayer) in comparison to HILIC phases that can have from 6–8 monolayers. In addition the substantial negative charge on the surface of hydride phases is the result of hydroxide ion adsorption from the solvent rather than silanols.

Features

An interesting feature of these phases is that both polar and nonpolar compounds can be retained over some range of mobile phase composition (organic/aqueous). The retention mechanism of polar compounds has recently been shown to be the result of the formation of a hydroxide layer on the surface of the silica hydride. Thus positively charged analytes are attracted to the negatively charged surface and other polar analytes are likely to be retained through displacement of

hydroxide or other charged species on the surface. This property distinguishes it from a pure HILIC (hydrophilic interaction chromatography) columns where separation by polar differences is obtained through partitioning into a water-rich layer on the surface, or a pure RP stationary phase on which separation by nonpolar differences in solutes is obtained with very limited secondary mechanisms operating.

Another important feature of the hydride-based phases is that for many analyses it is usually not necessary to use a high pH mobile phase to analyze polar compounds such as bases. The aqueous component of the mobile phase usually contains from 0.1 to 0.5% formic or acetic acid, which is compatible with detector techniques that include mass spectral analysis.

AUFS

Absorbance Units Full Scale (**AUFS**) is a ubiquitous unit of UV absorbance intensity.

Usage

AUFS is an arbitrary but ubiquitous unit of UV absorbance intensity. It can be used in chemical analysis to quantify components in a mixture, as each components' integrated peak area correspond to their relative abundance.

Application areas

- Analytical chemistry

- Chromatography

Chromatography in blood processing

Chromatography is a physical method of separation that distributes the components you want to separate between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction. Cold ethanol precipitation, developed by Cohn in 1946, manipulates pH, ionic strength, ethanol concentration and temperature to precipitate different protein fractions from plasma. Chromatographic techniques utilise ion exchange, gel filtration and affinity resins to separate proteins. Since the 1980s it has emerged as an effective method of purifying blood components for therapeutic use.

Human blood plasma

Blood plasma is the liquid component of blood, which contains dissolved proteins, nutrients, ions, and other soluble components. In whole blood, red blood cells, white blood cells, and platelets are suspended within the plasma. The goal of plasma purification and processing is to extract specific materials that are present in blood, and use them for restoration and repair. There are several components that make up blood plasma, one of which is the protein albumin. Albumin is a highly water-soluble protein with considerable structural stability. It serves as a transportation device for materials such as hormones, enzymes, fatty acids, metal ions, and medicinal products. It is also used for therapeutic purposes, being essential in restoration and maintenance of

circulating blood volume in imperative situations such as severe trauma or surgery. With little room for error, extremely pure samples that are lacking impurities needs to be at hand in good amount. Human blood plasma is important for the body so the nutrients etc. can be stored.

Development of chromatography

Traditionally, the Cohn process incorporating cold ethanol fractionation has been used for albumin purification. However, chromatographic methods for separation started being adopted in the early 1980s. Developments were ongoing in the time period between when Cohn fractionation started being used, in 1946, and when chromatography started being used, in 1983. In 1962, the Kistler & Nistchmann process was created which was a spinoff of the Cohn process. Chromatographic processes began to take shape in 1983. In the 1990s, the Zenalb and the CSL Albumex processes were created which incorporated chromatography with a few variations.

The general approach to using chromatography for plasma fractionation for albumin is: recovery of supernatant I, delipidation, anion exchange chromatography, cation exchange chromatography, and gel filtration chromatography.

The recovered purified material is formulated with combinations of sodium octanoate and sodium N-acetyl tryptophanate and then subjected to viral inactivation procedures, including pasteurisation at 60 °C.

This is a more efficient alternative than the Cohn process for four main reasons: 1) smooth automation and a relatively

inexpensive plant was needed, 2) easier to sterilize equipment and maintain a good manufacturing environment, 3) chromatographic processes are less damaging to the albumin protein, and 4) a more successful albumin end result can be achieved.

Compared with the Cohn process, the albumin purity went up from about 95% to 98% using chromatography, and the yield increased from about 65% to 85%. Small percentage increases make a difference in regard to sensitive measurements like purity. There is one big drawback in using chromatography, which has to do with the economics of the process. Although the method was efficient from the processing aspect, acquiring the necessary equipment is a big task. Large machinery is necessary, and for a long time the lack of equipment availability was not conducive to its widespread use. The components are more readily available now but it is still a work in progress and will possibly be ready in the future to help the world.

Bridging Methods

Integrating traditional and modern methods is a useful way to process albumin.

There are three main steps that combine Cohn fractionation with chromatography: 1) factors I, II, and III are removed via cold ethanol fractionation, 2) Sepharose fast flow ion exchange and sepharose fast flow chromatography procedures are run, and 3) gel filtration is run. The result is albumin with 9% lower aluminum levels with a processing time that is almost twice as fast.

Although it was hard to make chromatographic processing methods widely adopted, global expansion is a work in progress. Various blood components must be readily available at various medical treatment centers around the world. The Institute of Transfusion Medicine in Skopje, North Macedonia is a plasma fractionation center in the Balkans. Their modernized albumin purification process consists of five steps:

- Starting material is plasma that has been pretreated by centrifugation,
- A round of gel filtration is run,
- ion exchange on DEAE Sepharose is run to bind the albumin to the column,
- Albumin is eluted with a sodium acetate buffer, and
- Final polishing with gel filtration.

The end result is a highly pure and safe batch of albumin that is 100% non-pyrogenic, sterile, and free of active HIV virus. The product purity is greater than 98% and the protein content is about 50 g/L.

Non-chromatographic processing methods

Other plasma processing methods exist, but generally do not provide the resolution or purity of chromatographic methods. Two-phase liquid extraction may be performed using polyethylene glycol (PEG)-phosphate Aqueous two-phase systems, with a PEG-rich top layer and a phosphate-rich bottom layer. Although this method is somewhat useful for protein recovery, it does not work as well for the recovery of

other blood components. Membrane fractionation has the advantage of minimal protein loss yet high removal of pathological plasma components.

This method incorporates processes such as thermofiltration and applying pulsate flow. The latest two-stage membrane system utilizes a high flow recirculation circuit that is effective for removal of LDL cholesterol. It may prove useful for patients that have clogged arteries and other cardiovascular problems involving cholesterol.

Batch adsorption, e.g. onto ion exchange media, is only useful when dealing with smaller samples of plasma, typically 200 mL or less. Batch adsorption recovers the product in a larger volume of elution buffer than does column chromatography or frontal chromatography, and the resulting more dilute product requires concentration, typically on a membrane system, which can lead to loss of product by irreversible adsorption to the membrane.

Centrifugal partition chromatography

Centrifugal partition chromatography is a special chromatographic technique where both stationary and mobile phase are liquid, and the stationary phase is immobilized by a strong centrifugal force. Centrifugal partition chromatography consists of a series-connected network of extraction cells, which operates as elemental extractors, and the efficiency is guaranteed by the cascade.

History

In the 1940s Craig invented the first apparatus to conduct countercurrent partitioning; he called this Countercurrent Distribution Craig apparatus consists of a series of glass tubes that are designed and arranged such that the lighter liquid phase is transferred from one tube to the next. The next major milestone was Droplet countercurrent chromatography (DCCC). It uses only gravity to move the mobile phase through the stationary phase which is held in long vertical tubes connected in series. The modern era of CCC began with the development of the planetary centrifuge by Ito which was first introduced in 1966 as a closed helical tube which was rotated on a "planetary" axis as is turned on a "sun" axis.

Centrifugal partition chromatography was introduced in Japan in 1982; the first instrument was built at Sanki Eng. Ltd. in Kyoto. The first instrument consisted of twelve cartridges arranged around the rotor of a centrifuge; the inner volume of each cartridge was about 15 mL for 50 channels. In 1999 Kromaton was developed of the first FCPC with Radial Cells. During cell development, the Z cell was completed in 2005 and the twin cell in 2009. In 2017 RotaChrom designed its top performing CPC cells through Computed Fluid Dynamic simulation software.

After thousands of simulations, this tool revealed the drawbacks of conventional CPC cell designs and highlighted the unparallel load capacity and scalable cell design of RotaChrom.

Operation

- The extraction cells consist of hollow bodies with inlets and outlets of liquid connection. The cells are first filled with the liquid chosen to be the stationary phase. Under rotation, the pumping of the mobile phase is started, which enters the cells from the inlet. When entering the flow of mobiles phase forms small droplets according to the Stokes' law, which is called atomization. These droplets fall through the stationary phase, creating a high interface area, which is called the extraction. At the end of the cells, these droplets unite due to the surface tension, which is called settling.

Comparison with countercurrent chromatography

Countercurrent chromatography and centrifugal partition chromatography are two different instrumental realization of the same liquid–liquid chromatographic theory. Countercurrent chromatography usually uses a planetary gear motion without rotary seals, while centrifugal partition chromatography uses circular rotation with rotary seals for liquid connection. CCC has interchanging mixing and settling zones in the coil tube, so atomization, extraction and settling are time and zone separated. Inside centrifugal partition chromatography, all three steps happen continuously in one time, inside the cells.

Advantages of centrifugal partition chromatography:

- Higher flow rate for same volume size Laboratory scale example: 250 mL centrifugal partition

chromatography has optimal flow rate of 5–15 mL/min, 250 mL countercurrent chromatography has optimal flow rate of 1–3 mL/min. Process scale example: 25 L countercurrent chromatography has optimal flow rate of 100–300 ml/min, 25 L centrifugal partition chromatography has optimal flow rate of 1000–3000 ml/min.

- Higher productivity (due to higher flow rate and faster separation time)
- Scalable up to tonnes per month
- Better stationary phase retention for most phases

Disadvantages of centrifugal partition chromatography:

- Higher pressure than CCC (typical operation pressures of 40-160 bar vs 5–25 bar)
- Rotary seal wear over time

Laboratory scale

Centrifugal partition chromatography has been extensively used for isolation and purification of natural products for 40 years. Due to the ability to get very high selectivity, and the ability to tolerate samples containing particulated matter, it is possible to work with direct extracts of biomass, opposed to traditional liquid chromatography, where impurities degrade the solid stationary phase so that separation become impossible.

There are numerous laboratory scale centrifugal partition chromatography manufacturers around the world, like Gilson (Armen Instrument), Kromaton (Rousselet Robatel), and AECS-

QUIKPREP. These instruments operate at flow rates of 1–500 mL/min. with stationary phase retentions of 40–80%.

Production scale

Centrifugal partition chromatography does not uses any solid stationary phase, so it guarantees a cost-effective separation for the highest industrial levels. As opposed to countercurrent chromatography, it is possible to get very high flow rates (for example 10 liters / min) with active stationary phase ratio of >80%, which guarantees good separation and high productivity.

As in centrifugal partition chromatography, material is dissolved, and loaded the column in mass / volume units, loading capability can be much higher than standard solid-liquid chromatographic techniques, where material is loaded to the active surface area of the stationary phase, which takes up less than 10% of the column.

Industrial instrument like Gilson (Armen Instrument), Kromaton (Rousselet Robatel)and Rotachrom (RotaChrom) differ from laboratory scale instruments by the applicable flow rate with satisfactory stationary phase retention (70-90%). Industrial instruments have flow rates of multiple liter / minutes, while able to purify materials from 10 kg to tonnes per month.

Operating the production scale equipment requires industrial volume solvent preparation (mixer/settler) and solvent recovery equipment.

Chiral analysis

Chiral analysis refers to the quantification of component enantiomers of racemic drug substances or pharmaceutical compounds. Other synonyms commonly used include **enantiomer analysis**, **enantiomeric analysis**, and **enantioselective analysis**. Chiral analysis includes all analytical procedures focused on the characterization of the properties of chiral drugs. Chiral analysis is usually performed with chiral separation methods where the enantiomers are separated on an analytical scale and simultaneously assayed for each enantiomer.

Many compounds of biological and pharmacological interest are chiral. Pharmacodynamic, pharmacokinetic, and toxicological properties of the enantiomers of racemic chiral drugs has expanded significantly and become a key issue for both the pharmaceutical industry and regulatory agencies. Typically one of the enantiomers is more active pharmacologically (eutomer). In several cases, unwanted side effects or even toxic effects may occur with the inactive enantiomer (distomer). Even if the side effects are not that serious, the inactive enantiomer has to be metabolized, this puts an unnecessary burden on the already stressed out system of the patient. Large differences in activity between enantiomers reveal the need to accurate assessment of enantiomeric purity of pharmaceutical, agrochemicals, and other chemical entities like fragrances and flavors become very important. Moreover, the moment a racemic therapeutic is placed in a biological system, a chiral environment, it is no more 50:50 due enantioselective absorption, distribution, metabolism, and elimination (ADME)

process. Hence to track the individual enantiomeric profile there is a need for chiral analysis tool.

Chiral technology is an active subject matter related to asymmetric synthesis and enantioselective analysis, particularly in the area of chiral chromatography. As a consequence of the advances in chiral technology, a number of pharmaceuticals currently marketed as racemic drugs are undergoing re-assessment as chiral specific products or chiral switches. Despite the choice to foster either a single enantiomer or racemic drug, in the current regulatory environment, there will be a need for enantioselective investigations.

This poses a big challenge to pharmaceutical analysts and chromatographers involved in drug development process. In pharmaceutical research and development stereochemical analytical methodology may be required to comprehend enantioselective drug action and disposition, chiral purity assessment, study stereochemical stability during formulation and production, assess dosage forms, enantiospecific bioavailability and bioequivalence investigations of chiral drugs.

Besides pharmaceutical applications chiral analysis plays a major role in the study of biological and environmental samples and also in the forensic field. Chiral analysis methods and applications between the period 2010 and 2020 are exhaustively reviewed recently. There are number of articles, columns, and interviews in LCGC relating to emerging trends in chiral analysis and its application in drug discovery and development process.

For chiral examination there is a need to have the right chiral environment. This could be provided as a plane polarized light, an additional chiral compound or by exploiting the inborn chirality of nature. The chiral analytical strategies incorporate physical, biological, and separation science techniques. The most frequently employed technique in enantioselective analysis involve the separation science techniques, in particular chiral chromatographic methods or chiral chromatography.. Today wide range of CSPs are available commercially based on various chiral selectors including polysaccharides, cyclodextrins, glycopeptide antibiotics, proteins, Pirkle, crown ethers, etc. to achieve analysis of chiral molecules.

Chiral chromatography

- This term has become very popular and commonly used in practice. But the appropriate expression is "enantioselective chromatography". Chiral chromatography has advanced to turn into the most preferred technique for the determination of enantiomeric purity as well as separation of pure enantiomers both on analytical and preparative scale. Chiral chromatographic assay is the first step in any study pertaining to enantioselective synthesis or separation. This includes the use of techniques viz. gas chromatography (GC), high performance liquid chromatography (HPLC), chiral supercritical fluid chromatography (SFC), capillary electrophoresis (CE) and thin-layer chromatography (TLC).

Principle - separation of enantiomers

In an isotopic/achiral environment, enantiomers exhibit identical physicochemical properties, and therefore are indistinguishable under these conditions. For the separation of chiral molecules the challenge is to construct the right chiral environment. In a chromatographic system there are three variables namely, the chiral analyte (CA), mobile phase and stationary phase, that can be manipulated to provide the crucial chiral environment. The strategy is to make these variables to interact with a chiral auxiliary (chiral selector, CS) whereby it forms a diastereomeric complex which has different physicochemical properties and makes it possible to separate the enantiomers. Based on the nature of the diastereomeric complex formed between the CS-CA species, enantiomer separation mythologies are categorized as indirect and direct enantiomer separation mode

Indirect separation of enantiomer

- Indirect enantiomer separation involves the interaction between the chiral analyte (CA) of interest and the suitable reactive CS (in this case it is an enantiopure chiral derivatizing agent, CDA) leading to the formation of a covalent diastereomeric complex that can be separated with an achiral chromatographic technique. Therapeutic agents often contain reactive functional groups (amino, hydroxyl, epoxy, carbonyl and carboxylic acid, etc.) in their

structures. They are converted into covalently bonded diastereomeric derivatives using enantiomerically pure chiral derivatizing agent. The diastereomers thus formed unlike enantiomers, exhibit different physicochemical properties in an achiral environment and are eventually separated as a result of differential retention time on a stationary phase. The success of this approach depends on the availability of stable enantiopure chiral derivatizing agent (CDA) and on the presence of a suitable reactive functional group in the chiral drug molecule for covalent formation of diastereomeric derivative. The reaction of a racemic, (R,S)- Drug with a chirally and chemically pure chiral derivatizing agent, (R')-CDA, will afford diastereomeric products, (R)-Drug-(R')-CDA + (S)-Drug-(R')- CDA. The chiral derivatization reaction scheme is illustrated in the box on the right hand side.

In contrast to enantiomers, diastereomers have different physicochemical properties that make them separable on regular achiral stationary phases. The major benefit of the indirect methodology is that conventional achiral stationary phase/mobile phase system may be used for the separation of the generated diastereomers. Thus, considerable flexibility in chromatographic conditions is available to achieve the desired separation and to eliminate interferences from metabolites and endogenous substances. Moreover, the sensitivity of the method can be enhanced by sensible choice of the CDA and the chromatographic detection system. But this indirect approach to enantiomeric analysis has some potential problems. These include availability of a suitable functional group on the

enantiomer for derivatization, enantiomeric purity of the CDA, racemization of the CDA during derivatization, and racemization of the analyte during the derivatization. Currently, however, the application of indirect analytical approaches is in decline.

Direct separation of enantiomers

Direct enantiomer separation involves the formation of a transient rather than covalent diastereomeric complexation between the chiral selector/discriminator and the analyte (drug enantiomer). In this approach, the subtle energy differences between the reversibly formed noncovalent diastereomeric complexes are exploited for chiral recognition. The direct chromatographic enantiomer separation may be achieved in two different ways, the chiral mobile phase additive and chiral stationary phase mode.

Chiral mobile phase additive (CMPA)

In this approach, an enantiomerically pure compound, the chiral selector, is added to the mobile phase and separation happens on a conventional achiral column. When a mixture of enantiomers is introduced into the chromatographic system, the individual enantiomers form transient diastereomeric complexes with the chiral mobile phase additive. In the chiral mobile phase additive technique, two possible mechanisms may operate: one possibility is that CMPA and the enantiomers may form diastereomers in the mobile phase. Another is that the stationary phase may be coated with the CMPA, leading to diastereomeric interactions with the enantiomeric pairs during chromatographic separation process. It is observed that both

the mechanisms may happen depending on the characteristic of the stationary phase and mobile phase employed. Of late this method finds limited application.

Chiral stationary phase (CSP)

In the direct enantiomer separation the most popular approach is use of chiral stationary phases. In this case the site of the chiral selector is on the stationary phase. Stationary phase consist of an inert solid support (usually silica microparticles) on to the surface of which a single enantiomer of a chiral molecule (selector) is either coated/adsorbed or chemically linked and that forms the chiral stationary phase. Commonly used chiral selectors include polysaccharides, proteins, cyclodextrins, etc. An interesting review of chiral stationary phase development and application in chiral analysis appeared in LCGC magazine, 2011.

Chiral recognition

Chiral recognition implies the ability of chiral stationery phases to interact differently with mirror-image molecules, leading to their separation. The mechanism of enantiomeric resolution using CSPs is generally attributed to the “three-point" interaction model (fig.1.) between the analyte and the chiral selector in the stationary phase. Also known as the Dalgliesh model. Under this model, for chiral recognition, and hence enantiomeric resolution to happen on a CSP one of the enantiomers of the analyte must be involved in three simultaneous interactions. This means to say the one of enantiomers is able to have a good interaction with the

complimentary sites on the chiral selector attached to the CSP. While Its mirror-image partner may only interact at two or one such sites. In the figure, enantiomer (a), has the correct configuration of the ligands (X, Y and Z) for three-point interactions with the complimentary sites (X', Y' and Z') on the CSP, while its mirror image (b) can only interact at one site. The dotted lines (-----) indicate interaction with complimentary sites.

The diastereomeric complexes thus formed will have different energies of interaction. The enantiomer forming the more stable complex will have less energy and stay longer in the stationary phase compared to the less stable complex with higher energy. The success of chiral separation basically depends in manipulating the subtle energy differences between the reversibly formed non-covalent transient diastereomeric complexes. The energy difference reflects the magnitude of enantioselectivity.

Mobile phase has a major role in stabilizing the diastereomeric complex and thus in chiral separation. This simplified bimolecular interaction model is a treatment suitable for theoretical purposes. Mobile phase plays a key role in chiral recognition mechanism. Components of MP (such as bulk solvents, modifiers, buffer salts, additives) not only influence the conformational flexibility of CS and CA molecules but also their degree of ionization. The types of interaction involved in the analyte-selector interaction vary depending on the nature of the CSP used. These may include hydrogen bonding, dipole-dipole, π-π, electrostatic, hydrophobic or steric interactions, and inclusion complex formation.

Polysaccharide CSPs

Background

It is surprising to note that In 1980, there was no single chiral stationary phase available in the market for performing chiral chromatography. However, In late 1980s the subject of enantioselective chromatography attracted growing interest, particularly under the drive of the institution of Okamoto in Japan, the teams of Pirkle, and Armstrong in the US, Schurig and König in Germany, Lindner in Austria, and Francotte in Switzerland . The Polysaccharides, amylose and cellulose, form the most abundant chiral polymers on earth. These naturally occurring polysaccharides form basis for an important class of chiral selectors.

Chemistry

- Amylose and cellulose cannot be used as such due to poor resolution and difficulty in handling. But the carbamate and benzoate derivatives of these polymers, especially amylose and cellulose, demonstrate excellent properties as chiral selectors for chromatographic separation. A large number of polysaccharide-based CSPs are commercially available for chiral separation. These CSPs showed tremendous chiral recognition capability to resolve a wide range of chiral analytes.

These CSPs are compatible with NP/RP and SFC and also used for analytical, semi-preparative and preparative separations.

Many screening research studies conducted at different labs go to suggest that the four CSPs namely Chiralcel OD, Chiralcel OJ, Chiralpak AD, and Chiralpak As are capable of resolving more than 80% of the chiral separations due to their adaptability and high loading capacity. These four polysaccharide chiral stationary stationary phases are referred to as the "golden four".

Polysaccharide CSPs are prepared with high quality silica support on to which the polymeric chiral selector (amylose/cellulose dr.) is physically coated (coated CSP) or chemically immobilized (immobilized CSP). Separations can be done in normal phase, reversed-phase, and polar organic mode. While working with coated polysaccharide CSP solvent selection should be done with caution. One should not use drastic solvents such as dichloromethane, chloroform, toluene, ethyl acetate, THF; 1,4-dioxane; acetone; DMSO, etc. These so called "non-standard" solvents will dissolve the silica and irreversibly destroy the stationary phase.

These immobilized CSP are much more rugged and the "non-standard" solvents can be employed. Thus expanding the choice of co-solvent.

The major strength of immobilized CSPs are high solvent versatility in selection of mobile phase composition, enhanced sample solubility, high selectivity, robustness and extended durability, excellent column efficiency, and broad application domain in the resolution of enantiomers. Solvent is a key factor in HPLC MD. More solvents to play with means better sample solubility, Improves resolution, and enables effective chiral method development.

Mechanism

Number of chiral environments are created within the polymer. Cavities are formed between adjacent glucose units, and spaces/channels between polysaccharide chains.

These chiral cavities or channels give the chiral discrimination capability to polysaccharide CSPs. The mechanism of Chiral discrimination is not well understood but believed to involve hydrogen bonding and dipole-dipole interaction between the analyte molecule and the ester or carbamate linkage of the CSP.

Application

Some of the applications of these CSPs include the direct chiral analysis of β-adrenergic blockers such as metoprolol and celiprolol, the calcium channel blocker, felodipine and the anticonvulsant agent, ethotoin.

Macrocyclic CSPs

An interesting way of achieving chiral distinction on a CSP is the use of selectors with chiral cavity. These chiral selectors are attached to the stationary phase support material. In this category, there are basically three types of cavity chiral selectors namely cyclodextrins, crown ethers and macrocyclic glycopeptide antibiotics. Among these cyclodextrin based CSP is popular. In this type of CSPs the enantioselective guest-host interaction governs the chiral distinction.

Cyclodextrin-type CSP

- Cyclodextrins (CDs) are cyclic oligosaccharides of six, seven, or eight glucose units designated as α, β, and γ cyclodextrins respectively. Depicted in the diagram below. Daniel Armstrong is considered the pioneer of micelle and cyclodextrin-based separations. Cyclodextrins are covalently attached to silica by Armstrong process and provide stable CSPs. The primary hydroxyl groups are used to anchor the CD molecules to the modified silica surface. CDs are chiral because of innate chirality of the building blocks, glucose units. In cyclodextrin the glucose units are α-(1,4)- connected. The shape of CD looks like a shortened cone (see the sketch). The inner surface of the cone forms moderately hydrophobic pocket. The width of the CD-cavity is identified with the quantity of glucose units present. In cyclodextrins, secondary hydroxyl groups (OH-2 and - 3) line the upper rim of the cavity, and an essential 6-hydroxyl group is positioned at the lower rim. The hydroxyl group offer chiral binding points, which appear to be fundamental for enantioselectivity. Apolar glyosidic oxygen makes the pit hydrophobic and guarantees inclusion complexing of the hydrophobic moiety of analytes. Interactions between the polar area of an analyte and secondary hydroxyl groups at the mouth of the pit, joined with the hydrophobic connections inside the pit, give a unique two-point fit and lead to enantioselectivity. Selectivity of a cyclodextrin phase is dependent on two key factors namely the size and structure of the

analyte since it is based on a simple fit-unfit geometric criteria. An aromatic ring or cycloalkyl ring should be attached near the stereogenic center of the analyte. Substituents at or near the analyte chiral center must be able to interact with the hydroxyl groups at the entrance of the CD cavity through H-bonding. α-Cyclodextrin holds small aromatic molecules, whereas β-cyclodextrin incorporates both naphthyl groups and substituted phenyl groups. The aqueous compatibility of CD and its unique molecular structure make the CD- bonded phase highly suitable for use in chiral HPLC analysis of drugs. One further benefit of CD is that they are generally less expensive than the other CSPs. Some of the major shortcomings of CD CSPs is that it is limited to compounds that can enter into CD cavity, minor structural changes in analyte leads to unpredictable effect on resolution, often poor efficiency and cannot invert elution order.

Enantiomers of propranolol, metoprolol, chlorpheniramine, verapamil, hexobarbitaI, methadone and much more drugs have been separated using immobilized β-cyclodextrin.

Initially natural CDs have been used as the chiral selector. Later, modified cyclodextrin structures have been prepared by derivatizing the secondary hydroxyl groups present on the CD molecule. Incorporation of these additional functional groups may improve the chiral recognition capability by possibly modifying the chiral pocket and creating extra auxiliary interaction site. This approach enabled to expand the range of target chiral analytes that could be separated. A number of

chiral pharmaceuticals has been resolved using derivatized CDs including ibuprofen, suprofen, flurbiprofen from NSAID category and b-blockers like metoprolol and atenolol.

Glycopeptide-type CSP

Armstrong introduced macrocyclic glycopeptides (also known as glycopeptide antibiotics) as a new class of chiral selector for liquid chromatography in 1994. At present, vancomycin, teicoplanin and ristocetin are available under the brand names Chirobiotic V, Chirobiotic T and Chirobiotic R respectively. These cyclic glycopeptides have multiple chiral centers and a cup-like inclusion area to which a floating sugar lid is attached. Similar to protein chiral selectors, the amphoteric cyclic glycopeptides comprises of peptide and carbohydrate binding sites leading to possibilities for different modes of interaction beside the formation of inclusion complexation. In this chiral selector the cavities are shallower than that of CDs and hence the interactions are weaker, allows more rapid solute exchange between phases, higher column efficiency. operates in normal phase, reversed-phase and polar organic phase.

The complex structural nature of glycopeptide antibiotic class of CSP has made the understanding of the mechanism of chiral recognition at molecular level tricky. For instance, vancomycin molecule has 18 stereogenic centers in the molecule and offers a complex cyclodextrin-like chiral environment. In comparison to a single basket of cyclodextrins, vancomycin consists of three baskets, resulting in a more complex inclusion of appropriate guest molecules. The attractive forces include π-π interactions, hydrogen bonding, ionic interactions, and dipole

stacking. A carboxylic acid and a secondary amine group located on the rim of the cup and can participate in ionic interactions. Vancomycin stationary phases operate in reversed, normal and polar organic phase modes.

Wide range of chiral analysis has been done using chirobiotic CSPs. The antihypertensive drugs viz. oxprenolol, pindolol, propranolol have been separated using vancomycin and teicoplanin chirobiotic CSPS. The NSAID drugs ketoprofen and ibuprofen has been separated using ristocetin CSP.

Crown ether-type CSP

Crown ethers, like cyclodextrin-type CSPs contain a chiral cavity. Crown ethers are immobilized on the silica surface to form chiral stationary phase. Crown ethers contain oxygen atoms within the cavity. The cyclic structure that contains apolar ethylene groups between oxygen forms hydrophobic inner cavity. Cram *et al.*, introduced CSP based on chiral crown ethers and accomplished separation of amino acid. The crucial chiral recognition principle underlying crown ether-based enantiomer separation is based on the formation of numerous hydrogen bonds between the protonated primary amino group of the analyte and the ether oxygens of the crown structure. This structural requirement confines the application of crown ether-type CSPs to chiral compounds having primary amino groups adjoining the chiral centers, such as amino acids, amino acid derivatives. Progress in the field of crown ether-type CSPs have been reviewed.

Protein-type CSP

Proteins are complex, high-molecular weight biopolymers. They are inherently chiral being composed of L-amino acids and possess ordered 3D-structure. They are known to bind/interact stereoselectively with small molecules reversibly, making them extremely versatile CSPs for chiral separation of drug molecules. Hermansson made use of this property to develop number of CSPs by immobilizing proteins on to silica surface. They operate under reverse phase mode (phosphate buffer and organic modifiers).

Protein polymer remains in twisted form because of the different intramolecular bonding. These bonding create different type of chiral loops/grooves present in the protein molecule. Separation mechanism of proteins depends on unique combination of hydrophobic and polar interactions by which the analytes are oriented to chiral surfaces. H-bonding and charge transfer may also contribute to enantioselectivity. The mechanism of chiral distinction by proteins is mostly not well established due to their complex nature. Several proteins based CSP have been employed for chiral drug analysis including α-acid glycoprotein (enantiopac; chiral-AGP), ovomucoid protein (Ultron ES DVM), human serum albumin (HSA). α-AGP CSP (chiral AGP), has been employed for the quantification of atenolol enantiomers in biological matrices, for pharmacokinetic investigation of racemic metoprolol. The major weakness of protein based CSPs include low loading capacity, protein phases are expensive, extremely fragile, delicate to handle, very low column efficiency, cannot invert elution order.

Pirkle-type CSP

Pirkle and co-workers pioneered the development of a variety of CSPs based on charge-transfer complexation and simultaneous hydrogen bonding. These phases are also referred to as Brush-type CSPs. The Pirkle phases are based on aromatic π-acid (3,5-dinitrobenzoyl ring) and π- basic (naphthalene) derivative. In addition to π-π interaction sites, they have hydrogen-bonding and dipole-dipole interaction sites provided by an amide, urea or ester functionality. Strong three-point interaction, according to Dalgleish's model, enables enantioseparation. These phases are classified into π-electron-acceptor, π-electron-donor or π-electron acceptor-donor phase.

A number of Pirkle-type CSPs are commercially available. They are used most often in the normal phase mode. The ionic form of the DNPBG (3,5-dinitrobenzoyl-phenylglycine) CSP has been successfully employed to achieve separation of racemic propranolol in biological fluid. Many compounds of pharmaceutical interest including enantiomers of naproxen and metoprolol has been separated using Pirkle CSP.

See also

- Chiral resolution
- Chiral chromatography
- Chiral drugs
- Chiral switch
- Enantiomer
- Chirality