Electrophoresis

The term electrophoresis means any experimental technique that is based on movement of charged particles (ions, molecules, macromolecules) in electric field in liquid medium. Any electrically charged particle dissolved in aqueous solution, when placed to a constant electric field, will start to migrate towards the electrode bearing the opposite charge; the speed of the particle movement will be directly proportional to the applied voltage and particle charge, but inversely proportional to the particle size. Any molecules that differ in size and/or charge can be separated from each other in this way. The electrophoretic analysis can in principle be applied to any particles that are charged under given experimental condition, such as small cations or anions, organic acids, amino acids, peptides, saccharides, lipids, proteins, nucleotides, nucleic acids, even the whole subcellular particles or the whole cells. In practice, however, the by far commonest subjects of electrophoretic separation are proteins and nucleic acids.

Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a solution stabilized by a porous matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities

Discovery of electrophoresis

The movement of particles under spatially uniform electric field in a fluid is called electrophoresis. In 1807, Ferdinand Frederic Reuss observed clay particles dispersed in water to migrate on applying constant electric field for the first time. It is caused by a charged interface present between the particle surface and the surrounding fluid. The rate of migration of particle depends on the strength of the field, on the net charge size and shape of the molecules and also on the ionic strength, viscosity and temperature of medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species and as a separation technique. It provides the basis for a number of analytical techniques used for separating molecules by size, charge, or binding affinity, example- for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix. Gel matrix used mainly is polyacrylamide and agarose. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

Techniques of electrophoresis

Gel electrophoresis

Gel electrophoresis can provide information about the molecular weights and charges of proteins, the subunit structures of proteins, and the purity of a particular protein preparation. It is relatively simple to use and it is highly reproducible. The most common use of gel electrophoresis is the qualitative analysis of complex mixtures of proteins. Microanalytical methods and sensitive, linear image analysis systems make gel electrophoresis popular for quantitative and preparative purposes as well. The technique provides the highest resolution of all methods available for separating proteins. Polypeptides differing in molecular weight by as little as a few hundreds of daltons and proteins differing by less than 0.1 pH unit in their isoelectric points are routinely resolved in gels.

Agarose gel electrophoresis

In particular, agarose gel electrophoresis is generally used to separate DNA (single-stranded, double-stranded, and supercoiled) and RNA. Since DNA is negatively charged, it migrates in an electric field toward the positively charged cathode. The agarose matrix retards DNA migration roughly proportionally to DNA length when the DNA being separated is small. Longer oligonucleotides have a harder time traveling through the matrix, while shorter oligonucleotides (and small molecules such as ATP) breeze right through it.

Types of agarose

There are a few different types of agarose available. For analytical purposes, such as running digested plasmids to see whether a ligation was successful, you can usually use agarose from USB. However, if you want to recover your DNA and/or perform some in-gel reactions, you should use the low melting agarose (the Nu Sieve GTG, etc). These specific agarose protocols are usually provided with the reagent and are available online.

Agarose can be used for isoelectric focusing and separation of large proteins or protein complexes. Agarose is a highly purified polysaccharide derived from agar. For protein IEF applications, the critical qualities arelow EEO and good clarity at the working concentration. When used in a thin horizontal format for IEF, agarose gels must be supported on a plastic backing and cooled during electrophoresis. Agarose is normally purchased as a dry powder. It dissolves when the suspended powder is heated to near boiling and it remains liquid until the temperature drops to about 40 ºC, when it gels or "sets." There are specific types of agarose that have melting and gelling temperatures considerably lower than those of standard agarose. These properties improve the recovery of material from a gel after separation for subsequent enzymatic treatments of the separated material. The pore size and sieving characteristics of a gel are determined by adjusting the concentration of agarose in the gel. The higher the concentration, the smaller the pore size. Working concentrations are normally in the range of 0.4–4% w/v.

Agarose gels are relatively fragile and should be handled carefully. The

gels are hydrocolloids, held together by hydrogen and hydrophobic bonds, and tend to be somewhat brittle (Fig 1.5a). An agarose gel should always be handled with some form of support for the entire gel, such as a tray or wide spatula, because the gel will break if it bends too far.

Agarose and polyacrylamide gels are cross-linked, sponge like structures. Although they are up to 99.5% water, the size of the pores of these gels is similar to the sizes of many proteins and nucleic acids. As molecules are forced through the gel by the applied voltage, larger molecules are retarded by the gel more than are smaller molecules. For any particular gel, molecules smaller than a matrix-determined size are not retarded at all; they move almost as if in free solution. At the other extreme, molecules larger than a matrix-determined size cannot enter the gel at all. Gels can be tailored to sieve molecules of a wide range of sizes by appropriate choice of matrix concentration. The average pore size of a gel is determined by the percentage of solids in the gel and, for polyacrylamide, the amount of cross-linker and total amount of polyacrylamide used Polyacrylamide, which makes a small-pore gel, is used to separate most proteins, ranging in molecular weight from <5,000 to >200,000, and polynucleotides from <5 bases up to ~2,000 base pairs in size. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins, and protein complexes.

Various types of agarose can separate nucleic acids from 50 to 30,000 base pairs and, with pulsed-field techniques, up to chromosome- and similar-

sized pieces >5 × 106 base pairs long. Whichever matrix is selected, it is important that it be electrically neutral. Charged matrices may interact chromatographically with molecules and retard migration. The presence of fixed charged groups on the matrix will also cause the flow of water toward one or the other electrode, usually the cathode. This phenomenon, called electro endosmosis (often abbreviated EEO in supplier literature), usually decreases the resolution of separation.

Polyacrylamide gels electrophoresis

Polyacrylamide gels are physically tougher than agarose gels. The gel forms when a mixed solution of acrylamide and cross-linker monomers copolymerize into long chains that are covalently cross-linked. The gel structure is held together by the cross-linker (Fig 1.5b). The most common cross-linker is N,N'-methylene bisacrylamide (“bis” for short). Other cross-linkers that can be cleaved after polymerization are available (e.g.N,N'-bis-[acryloyl]-cystamine can be cleaved by disulphide reducing agents); they aid in recovering separated species from the gel by allowing the polymerized acrylamide to be solubilized. Because polymerization of acrylamide is a free-radical catalyzed reaction, preparation of polyacrylamide gels is somewhat more complex than that of agarose gels. Some of the technical issues are discussed in the following sections.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly practiced gel electrophoresis technique used for proteins. The method provides an easy way to estimate the number of polypeptides in a sample and thus assess the complexity of the sample or the purity of a preparation. SDS-PAGE is particularly useful for monitoring the fractions obtained during chromatographic or other purification procedures. It also allows samples from different sources to be compared for protein content. One of the more important features of SDS-PAGE is that it is a simple, reliable method with which to estimate the molecular weights of proteins.

SDS-PAGE requires that proteins be denatured to their constituent polypeptide chains, so that is limited in the information it can provide. In those situations where it «is desirable to maintain biological activity or antigenicity, non-denaturing electrophoresis systems must be employed. However, the gel patterns from non-denaturing gels are more difficult to interpret than are those from SDS-PAGE. Non-denaturing systems also give information about the charge isomers of proteins, but this information is best obtained by isoelectric focusing (IEF; see the entry on IEF in the AES website). An IEF run will often show heterogeneity due to structural modifications that is not apparent in other types of electrophoresis. Proteins thought to be a single species by SDS-PAGE analysis are sometimes found by IEF to consist of multiple species. A true determination of the purity of a protein preparation is obtained with two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) that combines IEF

with SDS-PAGE. Since 2-D PAGE is capable of resolving over 2,000 proteins in a single gel it is important as the primary tool of proteomics research where multiple proteins must be separated for parallel analysis (see the Application Focus on 2-D PAGE on this website). Proteins can be definitively identified by immunoblotting, which combines antibody specificity with the high resolution of gel electrophoresis. Finally, gel electrophoresis lends itself to protein purification for which purpose8various devices have been developed.

Polymerizing the gel

The free-radical vinyl polymerization of acrylamide gel can be initiated either by chemical peroxide or by a photochemical method. The most common method uses ammonium persulphate as the initiat or peroxide and the quaternary amine, N,N,N',N'-tetramethylethylene diamine (TEMED) as the catalyst.

For photochemical polymerization, riboflavin and long-wave UV light are the initiator, and TEMED is the catalyst. Shining long-wavelength ultraviolet light on the gel mixture, usually from a fluorescent light, starts the photochemical reaction. Photochemical polymerization is used when the ionic strength in the gel must be very low, because only a minute amount of riboflavin is required. It is also used if the protein studied is sensitive to ammonium persulphate or the by-products of peroxide-initiated polymerization.

Polymerization of acrylamide generates heat. Rapid polymerization can generate too much heat, causing convection inconsistencies in the gel structure

and occasionally breaking glass plates. It is a particular problem for high-concentration gels (>20%T). To prevent excessive heating, the concentration of initiator-catalyst reagents should be adjusted so that complete polymerization requires 20–60 min.

Gas chromatography

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 (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

Gas chromatography is in principle similar to column

chromatography (as well as other forms of chromatography such as HPLC, TLC), but has several notable differences. First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is "Gas–liquid chromatography", referring to the mobile and stationary phases, respectively.) Second, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Finally, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

2. Origins of Gas Chromatography

The development of GC as an analytical technique was pioneered by Martin and Synge 1941; they suggested the use of gas-liquid partition chromatograms for analytical purposes**.** When dealing with liquid-liquid partition chromatography, they predicted that the mobile phase need not be a liquid but may be a vapor. Very refined separations of volatile substances on a column in which a permanent gas is made to flow over a gel impregnated with a non-volatile solvent would be much faster and thus, the columns much more efficient and separation times much shorter.

3. Why Choose Gas Chromatography

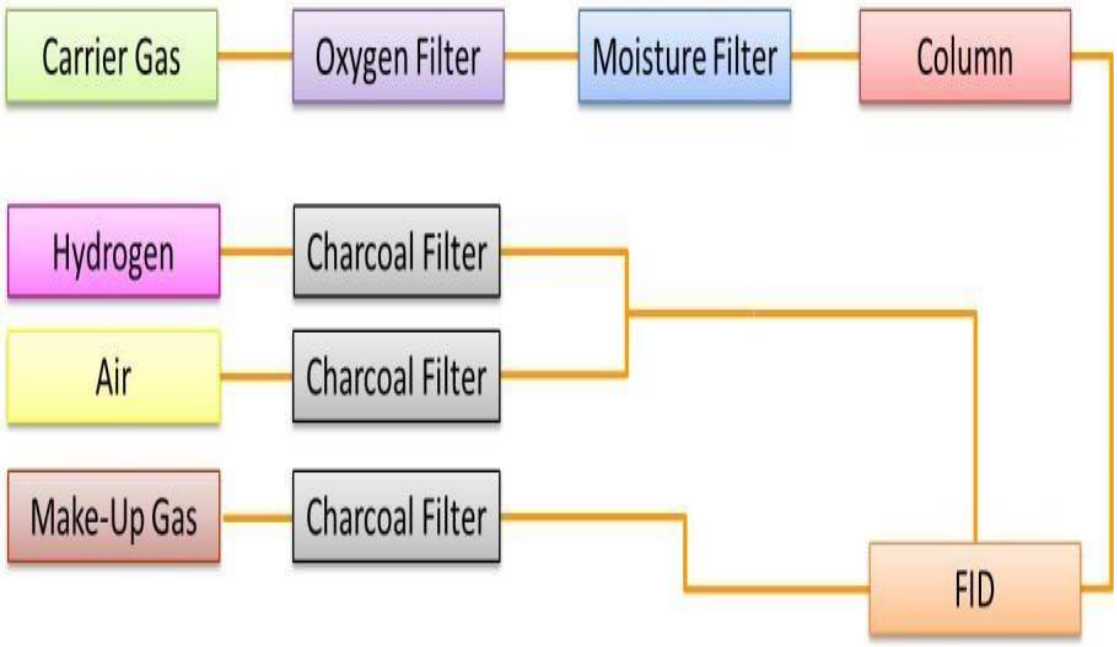
The two main chromatographic techniques used in modern analytical chemistry are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).

HPLC uses a liquid mobile phase to transport the sample components (**analyte**) through the column, which is packed with a solid stationary phase material.

In contrast, gas chromatography uses a gaseous mobile phase to transport sample components through either packed columns or hollow capillary columns containing a polymeric liquid stationary phase. In most cases, GC columns have smaller internal diameter and are longer than HPLC columns. GC has developed into a sophisticated technique since the pioneering work of Martin and James in 1951, and is capable of separating very complex mixtures of volatile analytes.

Gas Inlets:

Gas is fed from cylinders through supply piping to the instrument. It is usual to filter gases to ensure high gas purity and the gas supply may be regulated at the bench to ensure an appropriate supply pressure.



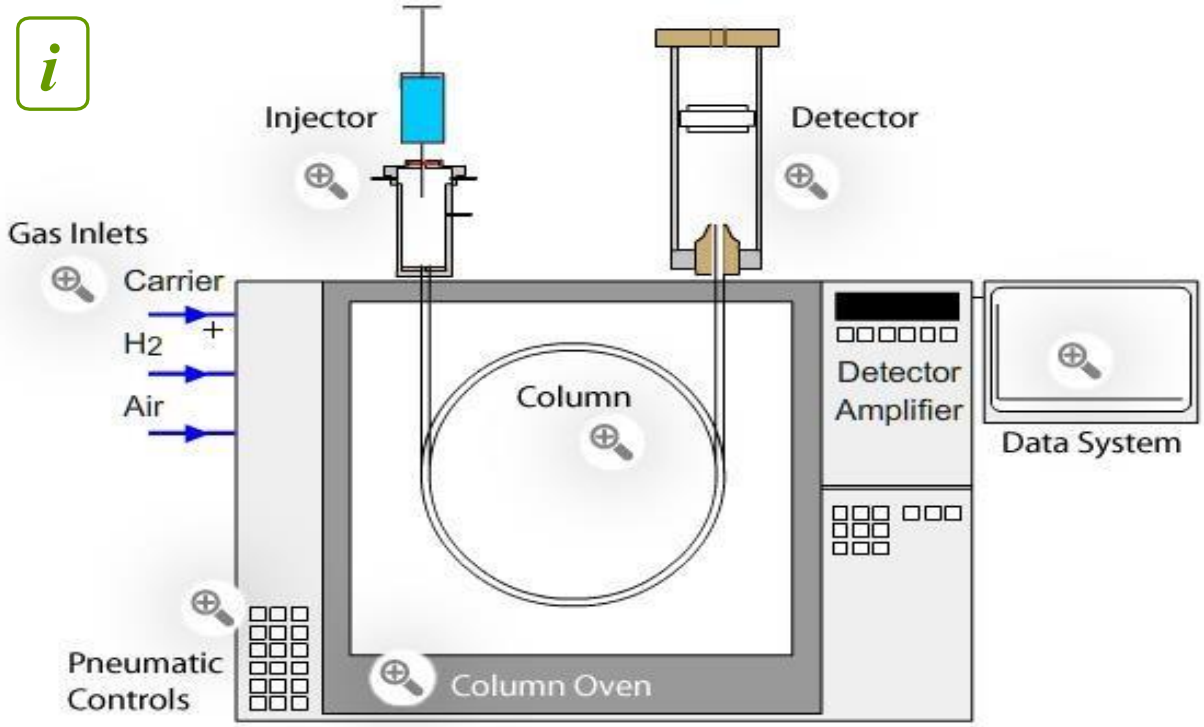
Gas filters: required for a GC instrument with Flame Ionization (FID) detector.

Required gases might Carrier: (H_2, He, N_2)

Make-up gas: (H_2, He, N_2)

Detector Fuel Gas: (H_2/Air, Ar, Ar/CH_4, N_2) depending on the detector type

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.



GC instrument

1- Pneumatic controls:

The gas supply is regulated to the correct pressure (or flow) and then fed to the required part of the instrument. Control is usually required to regulate the gas coming into the instrument and then to supply the various parts of the instrument. A GC fitted with a Split/Splitless inlet, capillary GC column and Flame Ionization detector may have the following different gas specifications:

Carrier gas supply pressure, column inlet pressure (column carrier gas flow), inlet split flow, inlet septum purge flow, detector air flow, detector hydrogen flow, detector make-up gas flow. Modern GC instruments have Electronic Pneumatic pressure controllers – older instruments may have manual pressure

control via regulators**.**

2- Column:

In GC, retention of analyte molecules occurs due to stronger interactions with the stationary phase than the mobile phase. This is unique in GC and, therefore, interactions between the stationary phase and analyte are of great importance. The interaction types can be divided into three broad categories:

- Dispersive
- Dipole
- Hydrogen bonding

The sample is separated into its constituent components in the column. Columns vary in length and internal diameter depending on the application type and can be either packed or capillary. Packed columns (typical dimension 1.5 m x 4 mm) are packed with a solid support coated with immobilized liquid stationary phase material (GLC). Capillary columns (typical dimension 30m×0.32mm×0.1mm film thickness) are long hollow silica tubes with the inside wall of the column coated with immobilized liquid stationary phase material of various film thickness.

Many different stationary phase chemistries are available to suit a host of applications. Columns may also contain solid stationary phase particles (GSC) for particular application types.

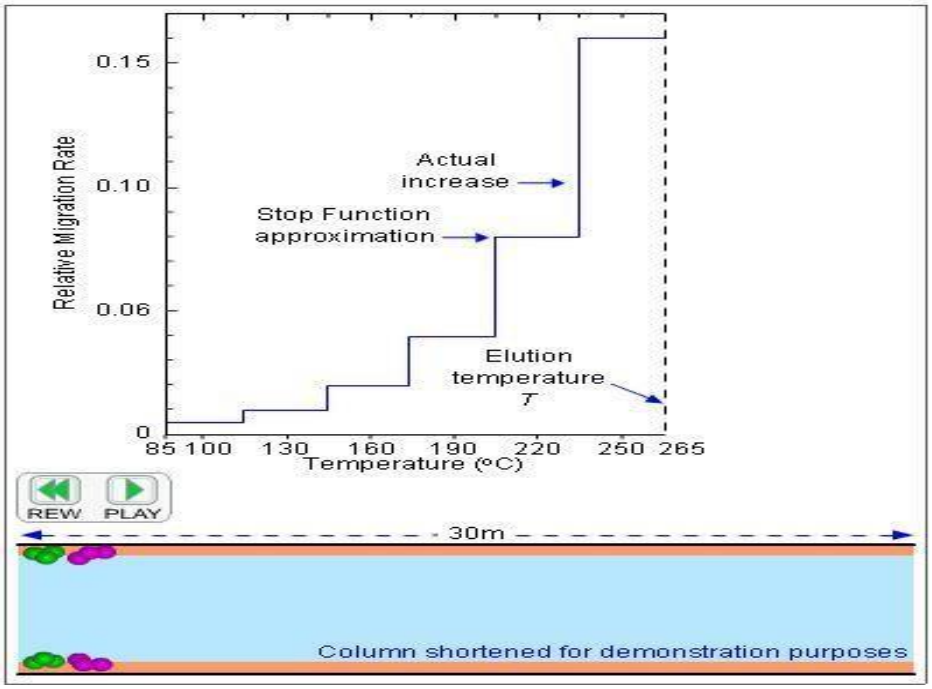
3- Column Oven:

Temperature in GC is controlled via a heated oven. The oven heats rapidly to

give excellent thermal control. The oven is cooled using a fan and vent arrangement usually at the rear of the oven.

A hanger or cage is usually included to support the GC column and to prevent it touching the oven walls as this can damage the column.

The injector and detector connections are also contained in the GC oven. For Isothermal operation, the GC is held at a steady temperature during the analysis. In temperature programmed GC (pTGC) the oven temperature is increased according to the temperature program during the analysis.



GC temperature

4- Detector:

The detector responds to a physicochemical property of the analyte, amplifies this response and generates an electronic signal for the data system to produce a chromatogram.

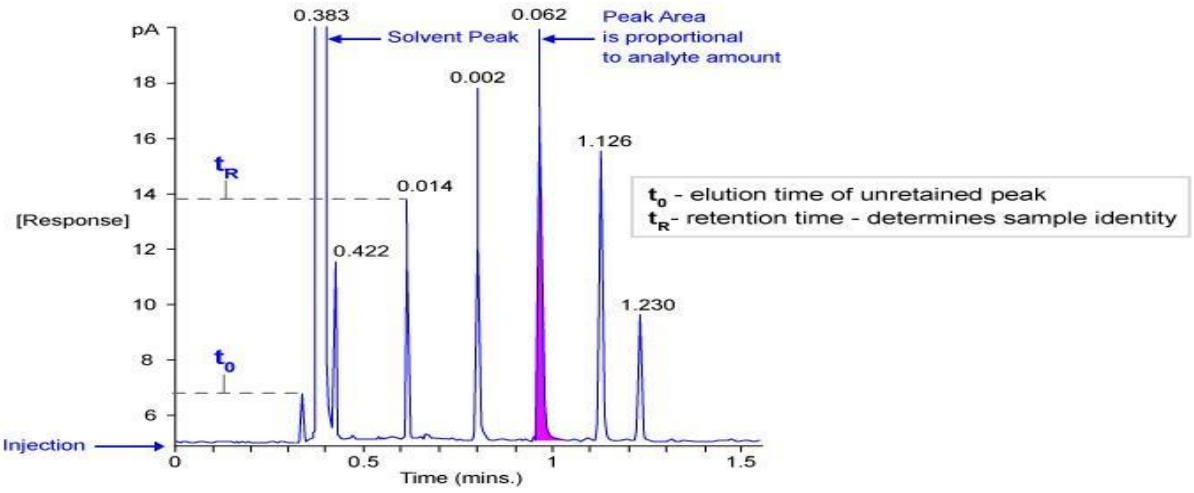
Many different detector types exist and the choice is based mainly on application, analyte chemistry and required sensitivity – also on whether quantitative or qualitative data is required.

Detector choices include:

- Flame Ionization (FID)
- Electron Capture (ECD)
- Flame Photometric (FPD)
- Nitrogen Phosphorous (NPD)
- Thermal Conductivity (TCD
- Mass Spectrometer (MS)

5- Data System:

The data system receives the analogue signal from the detector and digitizes it to form the record of the chromatographic separation known as the 'Chromatogram'



(Figure 4). The data system can also be used to perform various quantitative and qualitative operations on the chromatogram – assisting with sample identification and quantitation.

The following information gives an indication of the type of sample (analyte) analyzed by either GC and HPLC and relative strengths and limitations of each technique.

GC: Samples analyzed by GC must be volatile (have a significant vapor pressure below 250 °C)

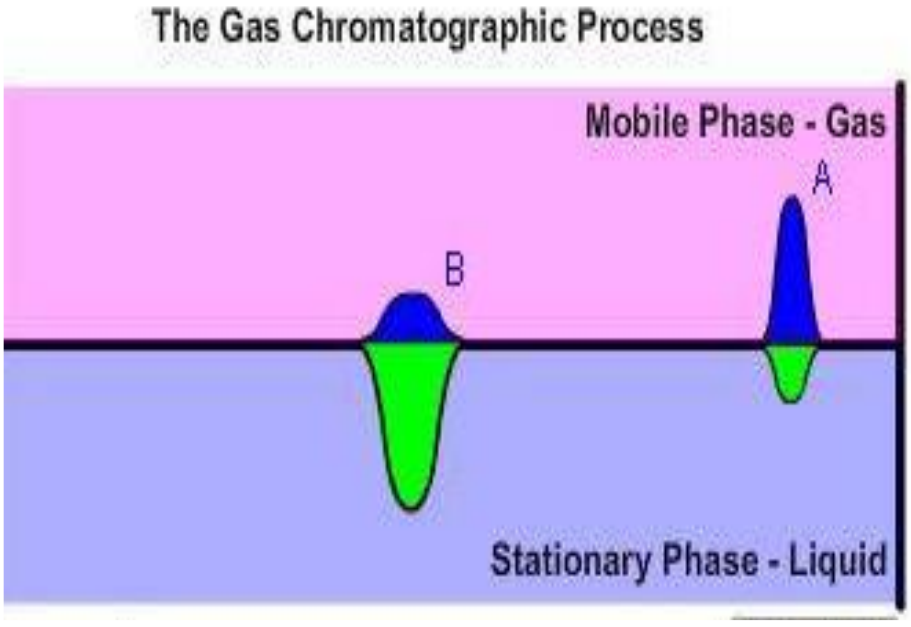
- **Derivatization** to increase volatility is possible but can be cumbersome and introduces possible **quantitative** errors
- Most GC analytes are under 500 Da Molecular Weight for volatility purposes
- Highly polar analytes may be less volatile than suspected when dissolved in a

polar solvent or in the presence of other polar species due to intermolecular forces such as hydrogen bonding.

4. Gas Chromatography Separation Mechanism

In Gas Chromatography (GC) the mobile phase is a gas and the stationary phase is either a solid - Gas solid chromatography (GSC) or an immobilized polymeric liquid - Gas Liquid Chromatography (GLC). Of the two types of GC, GLC is by far the most common as will be seen.

(Figure 5) shows a typical separation process in GC. Each sample component 'partitions' between the gaseous mobile phase and liquid stationary phase (often coated onto the inner wall of a long thin capillary tube). The rate and degree of partitioning depends upon the chemical affinity of the analyte for the stationary phase and the analyte vapor pressure – which is governed by the column temperature.



Gas chromatographic process

From it can be seen that component A has a lower affinity for the stationary phase and therefore is moved through the column more quickly than component B, which spends more of its time in the stationary phase – in this way separation is achieved.

In GC, analyte separation is achieved by optimizing the differences in stationary phase affinity and the relative vapor pressures of the analytes. In practice these parameters are manipulated by changing the chemical nature of the stationary phase and the column temperature

The Distribution Coefficient (Partition Coefficient) (K_c)

The 'distribution coefficient' measures the tendency of an analyte to be attracted to the stationary phase (Equation 1). Large K_c values lead to longer retention analyte times. The value of K_c can be controlled by the chemical nature of the stationary phase and the column temperature.

[CS]

[CM]

Where: C_S = concentration of analyte in the stationary phase and C_M = concentration of analyte in the mobile phase

5. The Gas Chromatograph

Instrumentation for Gas chromatography has continually evolved since

the inception of the technique in 1951 and the introduction of the first commercial systems in 1954.

Most modern commercial GC systems operate in the following way :

- An inert carrier gas, such as helium, is supplied from gas cylinders to the GC where the pressure is regulated using manual or electronic (pneumatic) pressure controls
- The regulated carrier gas is supplied to the inlet and subsequently flows through the column and into the detector
- The sample is injected into the (usually) heated injection port where it is volatilized and carried into the column by the carrier gas
- The sample is separated inside the column - usually a long silica based column with small internal diameter. The sample separates by differential partition of the analytes between the mobile and stationary phases, based on relative vapor pressure and solubility in the immobilized liquid stationary phase
- On elution from the column, the carrier gas and analytes pass into a detector, which responds to some physicochemical property of the analyte and generates an electronic signal measuring the amount of analyte present
- The data system then produces an integrated chromatogram
- Gas chromatography uses ovens that are temperature programmable. The temperature of the GC oven typically ranges from 5 °C to 400 °C but can go as low as -25 °C with cryogenic cooling

6. The Chromatogram

As the components elute from the column they pass into a detector – where some physicochemical property of the analyte produces a response from the detector. This response is amplified and plotted against time – giving rise to a 'chromatogram')

Components (such as the injection solvent) that are not retained within the column **elute** at the 'dead time' or 'hold up time' $\mathbf{t_0}$. There are various ways of measuring this parameter using unretained compounds such as methane or hexane.

Those compounds (analytes and sample components) that are retained elute as approximately '**Gaussian'** shaped peaks later in the chromatogram. Retention times provide the **qualitative** aspect of the chromatogram and the retention time of a compound will always be the same under identical chromatographic conditions. The chromatographic peak height or peak area is related to the quantity of analyte. For determination of the actual amount of the compound, the area or height is compared against standards of known concentration

7. GC Advantages and Disadvantages

Gas chromatography has several important advantages which are listed opposite. GC techniques produce fast analyses because of the highly efficient nature of the separations achieved – this will be studied further in the Band Broadening Section. It can be argued that modern GC produces the fastest

separations of all chromatographic techniques. A column has been produced to separate 970 components within a reasonable analysis time - proving that very complex separations may be carried out using GC.

By using a combination of oven temperature and stationary phase chemistry (polarity) very difficult separations may also be carried out – including separations of chiral and other positional isomers.

GC is excellent for quantitative analysis with a range of sensitive and linear detectors to choose from.

GC is however limited to the analysis of volatile samples. Some highly polar analytes can be derivatized to impart a degree of volatility, but this process can be difficult and may incur quantitative errors.

A practical upper temperature limit for conventional GC columns is around 350-380 ˚C. Analyte boiling points rarely exceed 400 ˚C in GC analysis and the upper Molecular Weight is usually around 500 Da.

Advantages

- Fast analysis.
- High efficiency – leading to high resolution.
- Sensitive detectors (ppb).
- Non-destructive – enabling coupling to Mass Spectrometers (MS) - an instrument that measures the masses of individual molecules that have been converted into ions, i.e. molecules that have been electrically charged.

- High quantitative accuracy (<1% RSD typical).
- Requires small samples (<1 mL).
- Rugged and reliable techniques.
- Well established with extensive literature and applications.

Disadvantages

- Limited to volatile samples.
- Not suitable for samples that degrade at elevated temperatures (thermally labile).
- Not suited to preparative chromatography.
- Requires MS detector for analyte structural elucidation (characterization).
- Most non-MS detectors are destructive.

High performance liquid chromatography

High-performance liquid chromatography (HPLC) is an analytical technique to separate, identify, and quantify components in a mixture. It is the single biggest chromatography technique essential to most laboratories worldwide.

The components of a basic high-performance liquid chromatography [HPLC] system are shown in the simple diagram.

A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute. An injector [sample manager or auto sampler] is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to *see* the separated compound bands as they elute from the HPLC column [most compounds have no colour, so we cannot see them with our eyes]. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate

containing that purified compound for further study. This is called preparative chromatography [discussed in the section on HPLC Scale].

Note that high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector [ELSD]. The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer [MS] to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

HPLC Operation:

A simple way to understand how we achieve the separation of the compounds contained in a sample.

Mobile phase enters the column from the left, passes through the particle bed, and exits at the right. Flow direction is represented by green arrows. First, consider the top image; it represents the column at time zero [the moment of injection], when the sample enters the column and begins to form a band. The sample shown here, a mixture of yellow, red, and blue dyes, appears at the inlet of the column as a single black band. [In reality, this sample could be anything that can be dissolved in a solvent; typically the compounds would be colourless and the column wall opaque, so we would need a detector to see the separated compounds as they elute.]

After a few minutes, during which mobile phase flows continuously and steadily past the packing material particles, we can see that the individual dyes have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the dyes or analytes. Notice that the yellow dye band moves the fastest and is about to exit the column. The yellow dye likes [is attracted to] the mobile phase more than the other dyes. Therefore, it moves at a *faster* speed, closer to that of the mobile phase. The blue dye band likes the packing material more than the mobile phase. Its stronger attraction to the particles causes it to move significantly *slower*. In other words, it is the most retained compound in this sample mixture. The red dye band has an intermediate attraction for the mobile

phase and therefore moves at an *intermediate* speed through the column. Since each dye band moves at different speed, we are able to separate it chromatographically.

Detector

As the separated dye bands leave the column, they pass immediately into the detector. The detector contains a flow cell that *sees* [detects] each separated compound band against a background of mobile phase. [In reality, solutions of many compounds at typical HPLC analytical concentrations are colourless.] An appropriate detector has the ability to sense the presence of a compound and send its corresponding electrical signal to a computer data station. A choice is made among many different types of detectors, depending upon the characteristics and concentrations of the compounds that need to be separated and analyzed, as discussed earlier.

Chromatogram

A chromatogram is a representation of the separation that has chemically [chromatographically] occurred in the HPLC system. A series of peaks rising from a baseline is drawn on a time axis. Each peak represents the detector

response for a different compound. The chromatogram is plotted by the computer data station.

The yellow band has completely passed through the detector flow cell; the electrical signal generated has been sent to the computer data station. The resulting chromatogram has begun to appear on screen. Note that the chromatogram begins when the sample was first injected and starts as a straight line set near the bottom of the screen. This is called the baseline; it represents pure mobile phase passing through the flow cell over time. As the yellow analyte band passes through the flow cell, a stronger signal is sent to the computer. The line curves, first upward, and then downward, in proportion to the concentration of the yellow dye in the sample band. This creates a peak in the chromatogram. After the yellow band passes completely out of the detector cell, the signal level returns to the baseline; the flow cell now has, once again, only pure mobile phase in it. Since the yellow band moves fastest, eluting first from the column, it is the first peak drawn.

A little while later, the red band reaches the flow cell. The signal rises up from the baseline as the red band first enters the cell, and the peak representing the red band begins to be drawn. In this diagram, the red band has not fully passed through the flow cell. The diagram shows what the red band and red peak would look like if we stopped the process at this moment. Since most of the red band has passed through the cell, most of the peak has been drawn, as shown by the solid line. If we could restart, the red band would completely pass through

the flow cell and the red peak would be completed [dotted line]. The blue band, the most strongly retained, travels at the slowest rate and elutes after the red band. The dotted line shows you how the completed chromatogram would appear if we had let the run continue to its conclusion. It is interesting to note that the width of the blue peak will be the broadest because the width of the blue analyte band, while narrowest on the column, becomes the widest as it elutes from the column. This is because it moves more slowly through the chromatographic packing material bed and requires more time [and mobile phase volume] to be eluted completely. Since mobile phase is continuously flowing at a fixed rate, this means that the blue band widens and is more dilute. Since the detector responds in proportion to the concentration of the band, the blue peak is lower in height, but larger in width.

Types of HPLC:

1- Normal Phase HPLC:

This strategy isolates analytes on the premise of extremity. NP-HPLC utilizes polar stationary stage and non-polar portable stage. Subsequently, the stationary stage is generally silica and normal versatile stages are hexane, methylene chloride, chloroform, diethyl ether, and blends of these. Polar specimens are consequently held on the polar surface of the column pressing longer than less polar materials.

2. **Reverse Phase HPLC:**

The stationary stage is non polar (hydrophobic) in nature, while the versatile stage is a polar liquid, for example, blends of water and methanol or acetonitrile. It deals with the rule of hydrophobic collaborations thus the more non polar the material is, the more it will be held.

3**. Size-exclusion HPLC:**

The column is loaded with material having definitely controlled pore sizes, and the particles are isolated by its their atomic size. Bigger atoms are quickly washed through the column; littler atoms enter inside the permeable of the pressing particles and elute later.

4. Ion-Exchange HPLC:

The stationary stage has an ironically charged surface of inverse charge to the example particles. This strategy is utilized only with ionic or ionizable specimens. The more grounded the charge on the example, the more grounded it will be pulled in to the ionic surface and along these lines, the more it will take to elute. The portable stage is a fluid cradle, where both pH and ionic quality are utilized to control elution time.