Gas Chromatography

Gas chromatography is a term used to describe the group of analytical separation techniques used to analyze volatile substances in the gas phase. In gas chromatography, the components of a sample are dissolved in a solvent and vaporized in order to separate the analytes by distributing the sample between two phases: a stationary phase and a mobile phase. The mobile phase is a chemically inert gas that serves to carry the molecules of the analyte through the heated column. Gas chromatography is one of the sole forms of chromatography that does not utilize the mobile phase for interacting with the analyte. The stationary phase is either a solid adsorbant, termed gas-solid chromatography (GSC), or a liquid on an inert support, termed gas-liquid chromatography (GLC).

1. Introduction

In early 1900s, Gas chromatography (GC) was discovered by Mikhail Semenovich Tsvett as a separation technique to separate compounds. In organic chemistry, liquid-solid column chromatography is often used to separate organic compounds in solution. Among the various types of gas chromatography, gas-liquid chromatography is the method most commonly used to separate organic compounds. The combination of gas chromatography and mass spectrometry is an invaluable tool in the identification of molecules. A typical gas chromatograph consists of an injection port, a column, carrier gas flow control equipment, ovens and heaters for maintaining temperatures of the injection port and the column, an integrator chart recorder and a detector.

To separate the compounds in gas-liquid chromatography, a solution sample that contains organic compounds of interest is injected into the sample port where it will be vaporized. The vaporized samples that are injected are then carried by an inert gas, which is often used by helium or nitrogen. This inert gas goes through a glass column packed with silica that is coated with a liquid. Materials that are less soluble in the liquid will increase the result faster than the material with greater solubility. The purpose of this module is to provide a better understanding on its separation and measurement techniques and its application.

In GLC, the liquid stationary phase is adsorbed onto a solid inert packing or immobilized on the capillary tubing walls. The column is considered packed if the glass or metal column tubing is packed with small spherical inert supports. The liquid phase adsorbs onto the surface of these beads in a thin layer. In a capillary column, the tubing walls are coated with the stationary phase or an adsorbant layer, which is capable of supporting the liquid phase. However, the method of GSC, has limited application in the laboratory and is rarely used due to severe peak tailing and the semi-permanent retention of polar compounds within the column. Therefore, the method of gas-liquid chromatography is simply shortened to gas chromatography and will be referred to as

such here. The purpose of this module is to provide a better understanding on its separation and measurement techniques and its application.

2. Instrumentation

2.1 Sample Injection

A sample port is necessary for introducing the sample at the head of the column. Modern injection techniques often employ the use of heated sample ports through which the sample can be injected and vaporized in a near simultaneous fashion. A calibrated microsyringe is used to deliver a sample volume in the range of a few microliters through a rubber septum and into the vaporization chamber. Most separations require only a small fraction of the initial sample volume and a sample splitter is used to direct excess sample to waste. Commercial gas chromatographs often allow for both split and split less injections when alternating between packed columns and capillary columns. The vaporization chamber is typically heated 50 °C above the lowest boiling point of the sample and subsequently mixed with the carrier gas to transport the sample into the column.
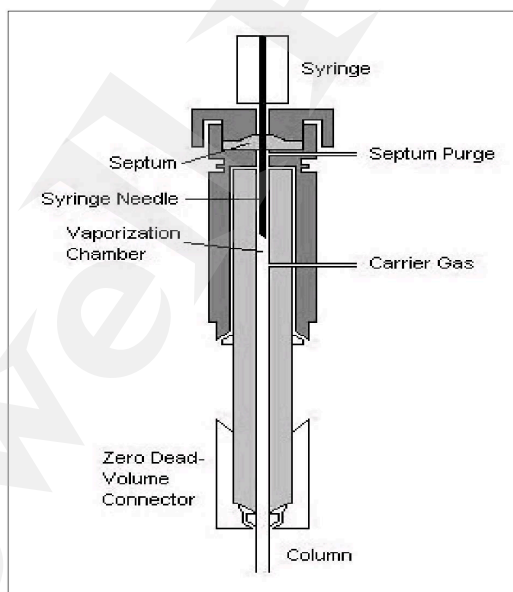

Figure 1: A cross-sectional view of a microflash vaporizer direct injector.

2.2 Carrier Gas

The carrier gas plays an important role, and varies in the GC used. Carrier gas must be dry, free of oxygen and chemically inert mobile-phase employed in gas chromatography. Helium is most commonly used because it is safer than, but comprable to hydrogen in efficiency, has a larger range of flow rates and is compatible with many detectors. Nitrogen, argon, and hydrogen are also used depending upon the desired performance and the detector being used. Both hydrogen and helium, which are commonly used on most traditional detectors such as Flame Ionization(FID), thermal conductivity (TCD) and Electron capture (ECD), provide a shorter

analysis time and lower elution temperatures of the sample due to higher flow rates and low molecular weight. For instance, hydrogen or helium as the carrier gas gives the highest sensitivity with TCD because the difference in thermal conductivity between the organic vapor and hydrogen/helium is greater than other carrier gas. Other detectors such as mass spectroscopy, uses nitrogen or argon which has a much better advantage than hydrogen or helium due to their higher molecular weights, in which improve vacuum pump efficiency.

All carrier gasses are available in pressurized tanks and pressure regulators, gages and flow meters are used to meticulously control the flow rate of the gas. Most gas supplies used should fall between 99.995% - 99.9995% purity range and contain a low levels (< 0.5 ppm) of oxygen and total hydrocarbons in the tank. The carrier gas system contains a molecular sieve to remove water and other impurities. Traps are another option to keep the system pure and optimum sensitive and removal traces of water and other contaminants. A two stage pressure regulation is required to use to minimize the pressure surges and to monitor the flow rate of the gas. To monitor the flow rate of the gas a flow or pressure regulator was also require onto both tank and chromatograph gas inlet. This applies different gas type will use different type of regulator. The carrier gas is preheated and filtered with a molecular sieve to remove impurities and water prior to being introduced to the vaporization chamber. A carrier gas is typically required in GC system to flow through the injector and push the gaseous components of the sample onto the GC column, which leads to the detector.

Gas Recommendations for Capillary Columns

Detector	Carrier gas	Preferred makeup gas	Second choice	Detector, anode purge, or reference gas
Electron Capture	Hydrogen	Argon/Methane	Nitrogen	Anode purge must be same as makeup
	Helium	Argon/Methane	Nitrogen	
	Nitrogen	Nitrogen	Argon/Methane	
	Argon/Methane	Argon/Methane	Nitrogen	
Flame Ionization	Hydrogen	Nitrogen	Helium	Hydrogen and air for detector
	Helium	Nitrogen	Helium	
	Nitrogen	Nitrogen	Helium	
Flame Photometric	Hydrogen	Nitrogen		Hydrogen and air for detector
	Helium	Nitrogen		
	Nitrogen	Nitrogen		
	Argon	Nitrogen		
Nitrogen-Phosphorus	Helium	Nitrogen	Helium**	Hydrogen and air for detector
	Nitrogen	Nitrogen	Helium**	
Thermal Conductivity	Hydrogen*	Must be same as carrier and reference gas	Must be same as carrier and reference gas	Reference must be same as carrier and makeup
	Helium			
	Nitrogen			

* When using hydrogen with a thermal conductivity detector, vent the detector exhaust to a fume hood or a dedicated exhaust to avoid buildup of hydrogen gas.

** Helium is not recommended as a makeup gas at flow rates > 5 mL/min. Flow rates above 5 mL/min shorten detector life.

Figure 2. Gas Recommendations for Capillary Columns

Gas Recommendations for Packed Columns

Detector	Carrier gas	Comments	Detector, anode purge, or reference gas
Electron Capture	Nitrogen	Maximum sensitivity	Nitrogen
	Argon/Methane	Maximum dynamic range	Argon/Methane
Flame Ionization	Nitrogen	Maximum sensitivity	Hydrogen and air for detector
	Helium	Acceptable alternative	
Flame Photometric	Hydrogen		Hydrogen and air for detector
	Helium		
	Nitrogen		
	Argon		
Nitrogen-Phosphorus	Helium	Optimum performance	Hydrogen and air for detector
	Nitrogen	Acceptable alternative	
Thermal Conductivity	Helium	General use	Reference must be same as carrier
	Hydrogen	Maximum sensitivity (Note A)	
	Nitrogen	Hydrogen detection (Note B)	
	Argon	Maximum hydrogen sensitivity (Note B)	

Note A: Slightly greater sensitivity than helium. Incompatible with some compounds.
Note B: For analysis of hydrogen or helium. Greatly reduces sensitivity for other compounds.

Figure 3. Gas Recommendations for Packed Columns

2.3 Column Oven

The thermostatted oven serves to control the temperature of the column within a few tenths of a degree to conduct precise work. The oven can be operated in two manners: isothermal programming or temperature programming. In isothermal programming, the temperature of the column is held constant throughout the entire separation. The optimum column temperature for isothermal operation is about the middle point of the boiling range of the sample. However, isothermal programming works best only if the boiling point range of the sample is narrow. If a low isothermal column temperature is used with a wide boiling point range, the low boiling fractions are well resolved but the high boiling fractions are slow to elute with extensive band broadening. If the temperature is increased closer to the boiling points of the higher boiling components, the higher boiling components elute as sharp peaks but the lower boiling components elute so quickly there is no separation.

In the temperature programming method, the column temperature is either increased continuously or in steps as the separation progresses. This method is well suited to separating a mixture with a broad boiling point range. The analysis begins at a low temperature to resolve the low boiling components and increases during the separation to resolve the less volatile, high

boiling components of the sample. Rates of 5-7 °C/minute are typical for temperature programming separations.
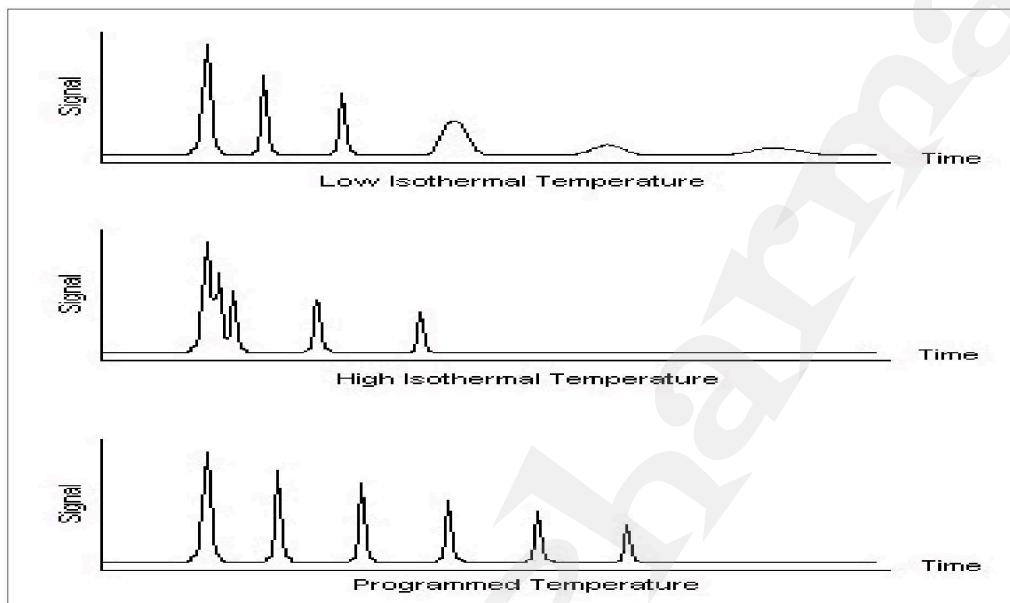

Figure 4. The effect of column temperature on the shape of the peaks.

2.4 Open Tubular Columns and Packed Columns

Open tubular columns, which are also known as capillary columns, come in two basic forms. The first is a wall-coated open tubular (WCOT) column and the second type is a support-coated open tubular (SCOT) column. WCOT columns are capillary tubes that have a thin layer of the stationary phase coated along the column walls. In SCOT columns, the column walls are first coated with a thin layer (about 30 micrometers thick) of adsorbent solid, such as diatomaceous earth, a material which consists of single-celled, sea-plant skeletons. The adsorbent solid is then treated with the liquid stationary phase. While SCOT columns are capable of holding a greater volume of stationary phase than a WCOT column due to its greater sample capacity, WCOT columns still have greater column efficiencies.

Most modern WCOT columns are made of glass, but T316 stainless steel, aluminum, copper and plastics have also been used. Each material has its own relative merits depending upon the application. Glass WCOT columns have the distinct advantage of chemical etching, which is usually achieved by gaseous or concentrated hydrochloric acid treatment. The etching process gives the glass a rough surface and allows the bonded stationary phase to adhere more tightly to the column surface.

One of the most popular types of capillary columns is a special WCOT column called the fused-silica wall-coated (FSWC) open tubular column. The walls of the fused-silica columns are drawn from purified silica containing minimal metal oxides. These columns are much thinner than glass columns, with diameters as small as 0.1 mm and lengths as long as 100 m. To protect the

column, a polyimide coating is applied to the outside of the tubing and bent into coils to fit inside the thermostatted oven of the gas chromatography unit. The FSWC columns are commercially available and currently replacing older columns due to increased chemical inertness, greater column efficiency and smaller sampling size requirements. It is possible to achieve up to 400,000 theoretical plates with a 100 m WCOT column, yet the world record for the largest number of theoretical plates is over 2 million plates for 1.3 km section of column.

Packed columns are made of a glass or metal tubing which is densely packed with a solid support like diatomaceous earth. Due to the difficulty of packing the tubing uniformly, these types of columns have a larger diameter than open tubular columns and have a limited range of length. As a result, packed columns can only achieve about 50% of the efficiency of a comparable WCOT column. Furthermore, the diatomaceous earth packing is deactivated over time due to the semi-permanent adsorption of impurities within the column. In contrast, FSWC open tubular columns are manufactured to be virtually free of these adsorption problems.

	Type of Column			
	FSWC	WCOT	SCOT	Packed
Length	10 to 1000 m	10 to 1000 m	10 to 100 m	1 to 6 m
Inner Diameter	0.1 to 0.3 mm	0.25 to 0.75 mm	0.5 mm	2 to 4 mm
Efficiency (plates/m)	2000 to 4000	1000 to 4000	600 to 1200	500 to 1000
Sample Size	10 to 75 ng	10 to 1000 ng	10 to 1000 ng	10 to 10^6 ng
Pressure	Low	Low	Low	High
Speed	Fast	Fast	Fast	Slow
Inertness	Best	Good	Fair	Poor

Figure 5. Properties of Gas chromatography columns.

Different types of columns can be applied for different fields. Depending on the type of sample, some GC columns are better than the others. For example, the FSWC column is designed especially for blood alcohol analysis. It produces fast run times with baseline resolution of key components in less than 3 minutes. Moreover, it displays enhanced resolutions of ethanol and acetone peaks, which helps with determining the BAC levels. This particular column is known as Zebron-BAC and it made with polyimide coating on the outside and the inner layer is made of fused silica and the inner diameter ranges from .18 mm to .25 mm. There are also many other Zebron brand columns designed for other purposes.

Another example of a Zebron GC column is known as the Zebron-inferno. Its outer layer is coated with a special type of polyimide that is designed to withstand high temperatures. It contains an extra layer inside. It can withstand up to 430 °C to be exact and it is designed to

provide true boiling point separation of hydrocarbons distillation methods. Moreover, it is also used for acidic and basic samples.

2.5 Detection Systems

The detector is the device located at the end of the column which provides a quantitative measurement of the components of the mixture as they elute in combination with the carrier gas. In theory, any property of the gaseous mixture that is different from the carrier gas can be used as a detection method. These detection properties fall into two categories: bulk properties and specific properties. Bulk properties, which are also known as general properties, are properties that both the carrier gas and analyte possess but to different degrees. Specific properties, such as detectors that measure nitrogen-phosphorous content, have limited applications but compensate for this by their increased sensitivity.

Each detector has two main parts that when used together they serve as transducers to convert the detected property changes into an electrical signal that is recorded as a chromatogram. The first part of the detector is the sensor which is placed as close to the column exit as possible in order to optimize detection. The second is the electronic equipment used to digitize the analog signal so that a computer may analyze the acquired chromatogram. The sooner the analog signal is converted into a digital signal, the greater the signal-to-noise ratio becomes, as analog signal are easily susceptible to many types of interferences.

An ideal GC detector is distinguished by several characteristics. The first requirement is adequate sensitivity to provide a high resolution signal for all components in the mixture. This is clearly an idealized statement as such a sample would approach zero volume and the detector would need infinite sensitivity to detect it. In modern instruments, the sensitivities of the detectors are in the range of 10^{-8} to 10^{-15} g of solute per second. Furthermore, the quantity of sample must be reproducible and many columns will distort peaks if enough samples are not injected. An ideal column will also be chemically inert and should not alter the sample in any way. Optimized columns will be able to withstand temperatures in the range of -200 °C to at least 400 °C. In addition, such a column would have a short linear response time that is independent of flow rate and extends for several orders of magnitude. Moreover, the detector should be reliable, predictable and easy to operate.

Table 2: Typical gas chromatography detectors and their detection limits.		
Type of Detector	**Applicable Samples**	**Detection Limit**

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Type of Detector	**Applicable Samples**	**Detection Limit**
Mass Spectrometer (MS)	Tunable for any sample	0.25 to 100 pg
Flame Ionization (FID)	Hydrocarbons	1 pg/s
Thermal Conductivity (TCD)	Universal	500 pg/ml
Electron-Capture (ECD)	Halogenated hydrocarbons	5 fg/s
Atomic Emission (AED)	Element-selective	1 pg
Chemiluminescence (CS)	Oxidizing reagent	Dark current of PMT
Photoionization (PID)	Vapor and gaseous Compounds	0.002 to 0.02 µg/L

Understandably, it is not possible for a detector meet all of these requirements. The next subsections will discuss some of the more common types of gas chromatography detectors and the relative advantages and/or disadvantages of each.

2.5.1   Mass Spectrometry Detectors

Mass Spectrometer (MS) detectors are most powerful of all gas chromatography detectors. In a GC/MS system, the mass spectrometer scans the masses continuously throughout the separation. When the sample exits the chromatography column, it is passed through a transfer line into the inlet of the mass spectrometer. The sample is then ionized and fragmented, typically by an electron-impact ion source. During this process, the sample is bombarded by energetic electrons which ionize the molecule by causing them to lose an electron due to electrostatic repulsion. Further bombardment causes the ions to fragment. The ions are then passed into a mass analyzer where the ions are sorted according to their m/z value, or mass-to-charge ratio. Most ions are only singly charged.

The Chromatogram will point out the retention times and the mass spectrometer will use the peaks to determine what kind of molecules are exist in the mixture. The figure below represents a typical mass spectrum of water with the absorption peaks at the appropriate m/z ratios.
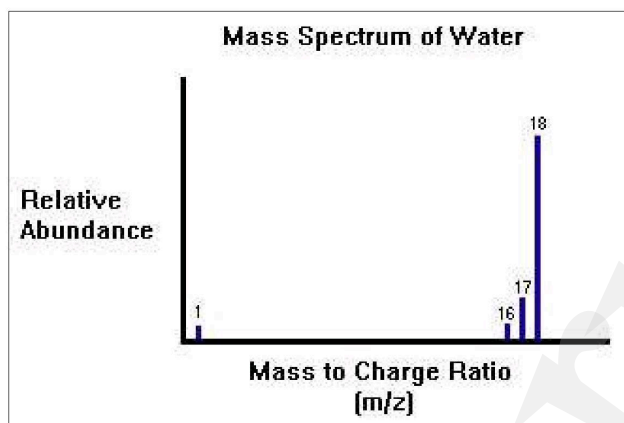
Figure 6. Mass Spectrum of Water

2.5.1.1 Instrumentation

One of the most common types of mass analyzer in GC/MS is the quadrupole ion-trap analyzer, which allows gaseous anions or cations to be held for long periods of time by electric and magnetic fields. A simple quadrupole ion-trap consists of a hollow ring electrode with two grounded end-cap electrodes as seen in figure #. Ions are allowed into the cavity through a grid in the upper end cap. A variable radio-frequency is applied to the ring electrode and ions with an appropriate m/z value orbit around the cavity. As the radio-frequency is increased linearly, ions of a stable m/z value are ejected by mass-selective ejection in order of mass. Ions that are too heavy or too light are destabilized and their charge is neutralized upon collision with the ring electrode wall. Emitted ions then strike an electron multiplier which converts the detected ions into an electrical signal. This electrical signal is then picked up by the computer through various programs. As an end result, a chromatogram is produced representing the m/z ratio versus the abundance of the sample.

GC/MS units are advantageous because they allow for the immediate determination of the mass of the analyte and can be used to identify the components of incomplete separations. They are rugged, easy to use and can analyze the sample almost as quickly as it is eluted. The disadvantages of mass spectrometry detectors are the tendency for samples to thermally degrade before detection and the end result of obliterating the entire sample by fragmentation.
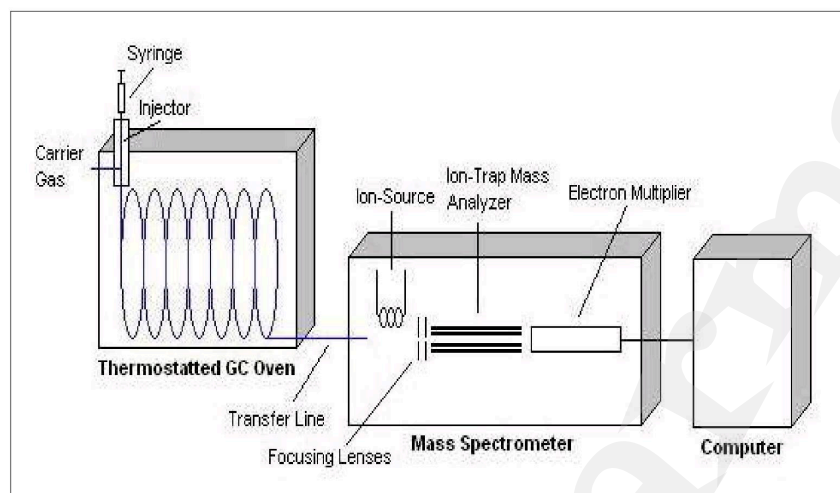
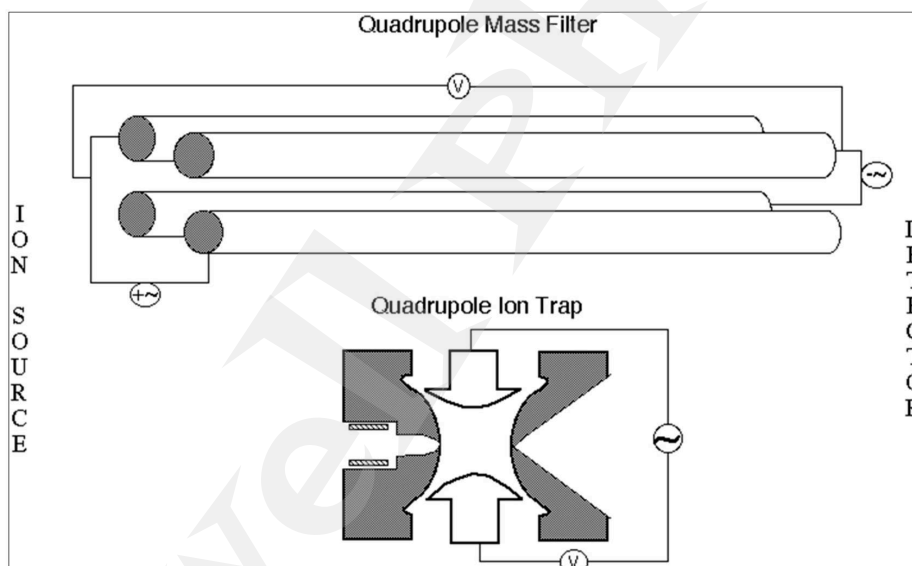**Figure 7**. Schematic of the GC/MS system.

Figure 8. Arrangement of the poles in Quadrupole and Ion Trap Mass spectrometers

2.5.2 Flame Ionization Detectors

Flame ionization detectors (FID) are the most generally applicable and most widely used detectors. In a FID, the sample is directed at an air-hydrogen flame after exiting the column. At the high temperature of the air-hydrogen flame, the sample undergoes pyrolysis, or chemical decomposition through intense heating. Pyrolized hydrocarbons release ions and electrons that carry current. A high-impedance picoammeter measures this current to monitor the sample's elution.

It is advantageous to use FID because the detector is unaffected by flow rate, noncombustible gases and water. These properties allow FID high sensitivity and low noise. The unit is both reliable and relatively easy to use. However, this technique does require flammable gas and also destroys the sample.
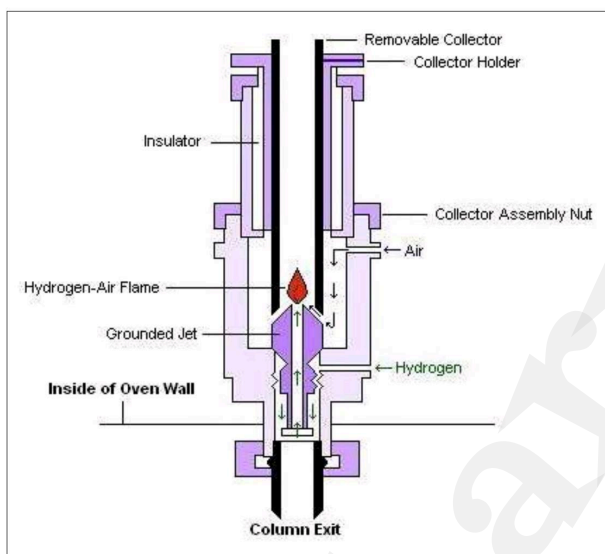
Figure 9. Schematic of a typical flame ionization detector.

2.5.3 Thermal Conductivity Detectors

Thermal conductivity detectors (TCD) were one the earliest detectors developed for use with gas chromatography. The TCD works by measuring the change in carrier gas thermal conductivity caused by the presence of the sample, which has a different thermal conductivity from that of the carrier gas. Their design is relatively simple, and consists of an electrically heated source that is maintained at constant power. The temperature of the source depends upon the thermal conductivities of the surrounding gases. The source is usually a thin wire made of platinum, gold. The resistance within the wire depends upon temperature, which is dependent upon the thermal conductivity of the gas.

TCDs usually employ two detectors, one of which is used as the reference for the carrier gas and the other which monitors the thermal conductivity of the carrier gas and sample mixture. Carrier gases such as helium and hydrogen has very high thermal conductivities so the addition of even a small amount of sample is readily detected.

The advantages of TCDs are the ease and simplicity of use, the devices' broad application to inorganic and organic compounds, and the ability of the analyze to be collected after separation and detection. The greatest drawback of the TCD is the low sensitivity of the instrument in relation to other detection methods, in addition to flow rate and concentration dependency.
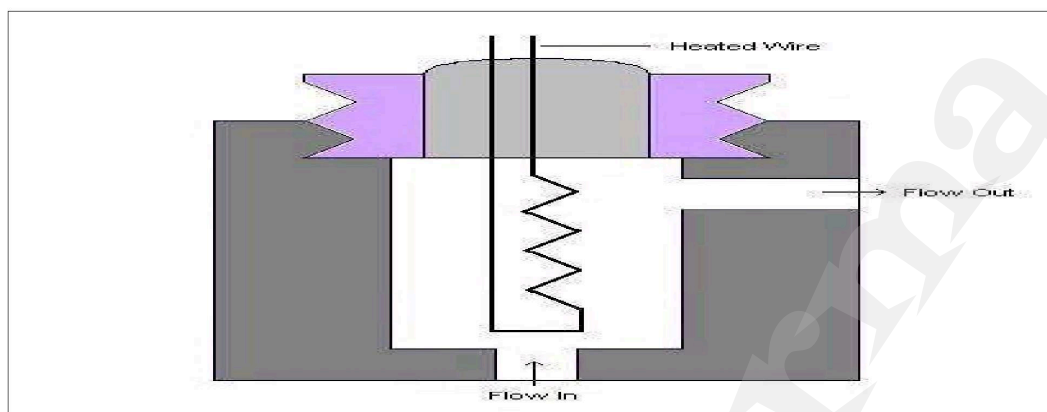
Figure 10. Schematic of thermal conductivity detection cell.

2.5.4 Electron-capture Detectors

Electron-capture detectors (ECD) are highly selective detectors commonly used for detecting environmental samples as the device selectively detects organic compounds with moieties such as halogens, peroxides, quinones and nitro groups and gives little to no response for all other compounds. Therefore, this method is best suited in applications where traces quantities of chemicals such as pesticides are to be detected and other chromatographic methods are unfeasible.

The simplest form of ECD involves gaseous electrons from a radioactive emitter in an electric field. As the analyte leaves the GC column, it is passed over this emitter, which typically consists of nickle-63 or tritium. The electrons from the emitter ionize the nitrogen carrier gas and cause it to release a burst of electrons. In the absence of organic compounds, a constant standing current is maintained between two electrodes. With the addition of organic compounds with electronegative functional groups, the current decreases significantly as the functional groups capture the electrons.

The advantages of ECDs are the high selectivity and sensitivity towards certain organic species with electronegative functional groups. However, the detector has a limited signal range and is potentially dangerous owing to its radioactivity. In addition, the signal-to-noise ratio is limited by radioactive decay and the presence of O2 within the detector.
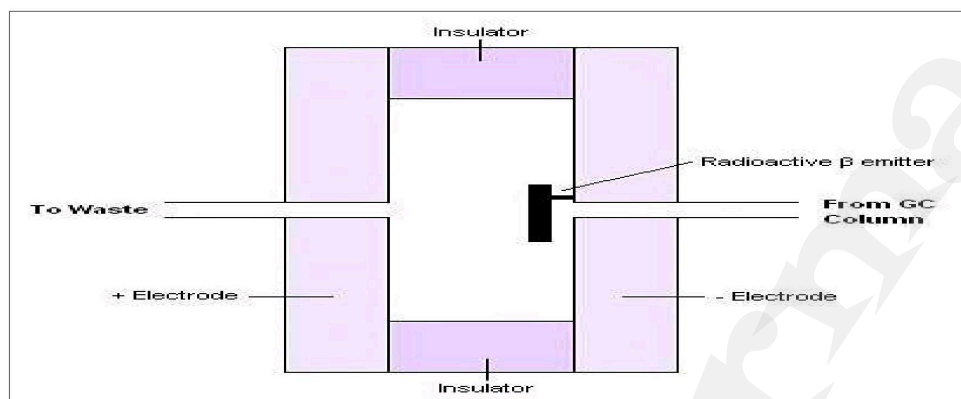
Figure 11. Schematic of an electron-capture detector.

2.5.5 Atomic Emission Detectors

Atomic emission detectors (AED), one of the newest additions to the gas chromatographer's arsenal, are element-selective detectors that utilize plasma, which is a partially ionized gas, to atomize all of the elements of a sample and excite their characteristic atomic emission spectra. AED is an extremely powerful alternative that has a wider applicability due to its based on the detection of atomic emissions. There are three ways of generating plasma: microwave-induced plasma (MIP), inductively coupled plasma (ICP) or direct current plasma (DCP). MIP is the most commonly employed form and is used with a position able diode array to simultaneously monitor the atomic emission spectra of several elements.

2.5.5.1 Instrumentation

The components of the Atomic emission detectors include 1) an interface for the incoming capillary GC column to induce plasma chamber,2) a microwave chamber, 3) a cooling system, 4) a diffraction grating that associated optics, and 5) a position adjustable photodiode array interfaced to a computer.
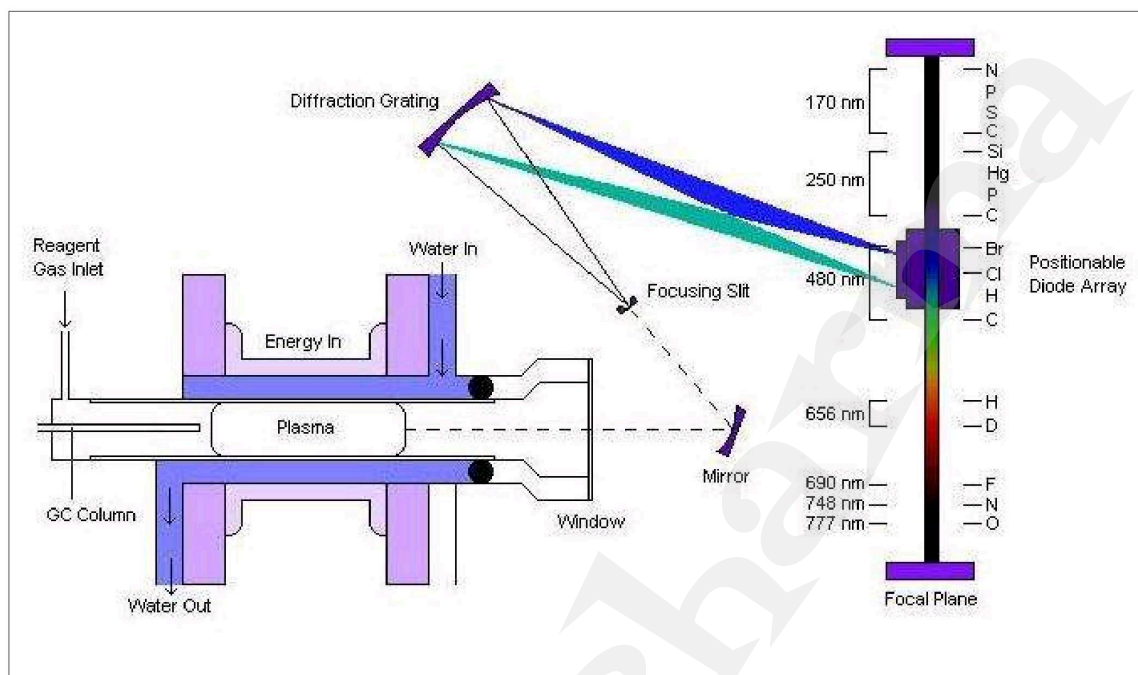
Figure 12. Schematic of atomic emission detector.

2.5.6 GC Chemiluminescence Detectors

Chemiluminescence spectroscopy (CS) is a process in which both qualitative and quantitative properties can be be determined using the optical emission from excited chemical species. It is very similar to AES, but the difference is that it utilizes the light emitted from the energized molecules rather than just excited molecules. Moreover, chemiluminescence can occur in either the solution or gas phase whereas AES is designed for gaseous phases. The light source for chemiluminescence comes from the reactions of the chemicals such that it produces light energy as a product. This light band is used instead of a separate source of light such as a light beam.

Like other methods, CS also has its limitations and the major limitation to the detection limits of CS concerns with the use of a photomultiplier tube (PMT). A PMT requires a dark current in it to detect the light emitted from the analyte.
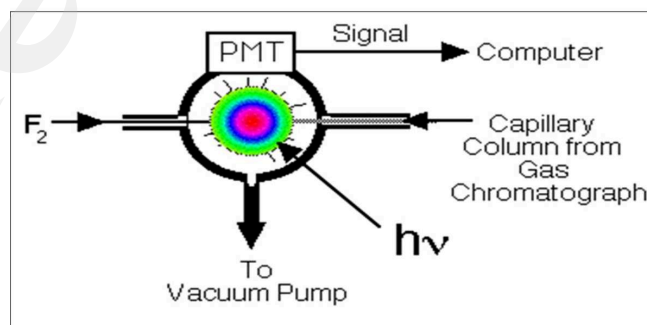

Figure 13. Schematic of a GC Chemiluminescence Detector

2.5.7 Photoionization Detectors

Another different kind of detector for GC is the photoionization detector which utilizes the properties of chemiluminescence spectroscopy. Photoionization detector (PID) is a portable

vapor and gas detector that has selective determination of aromatic hydrocarbons, organo-heteroatom, inorganic species and other organic compounds. PID comprise of an ultrviolet lamp to emit photons that are absorbed by the compounds in an ionization chamber exiting from a GC column. Small fraction of the analyte molecules are actually ionized, nondestructive, allowing confirmation analytical results through other detectors. In addition, PIDs are available in portable hand-held models and in a number of lamp configurations. Results are almost immediate. PID is used commonly to detect VOCs in soil, sediment, air and water, which is often used to detect contaminants in ambient air and soil. The disadvantage of PID is unable to detect certain hydrocarbon that has low molecular weight, such as methane and ethane.
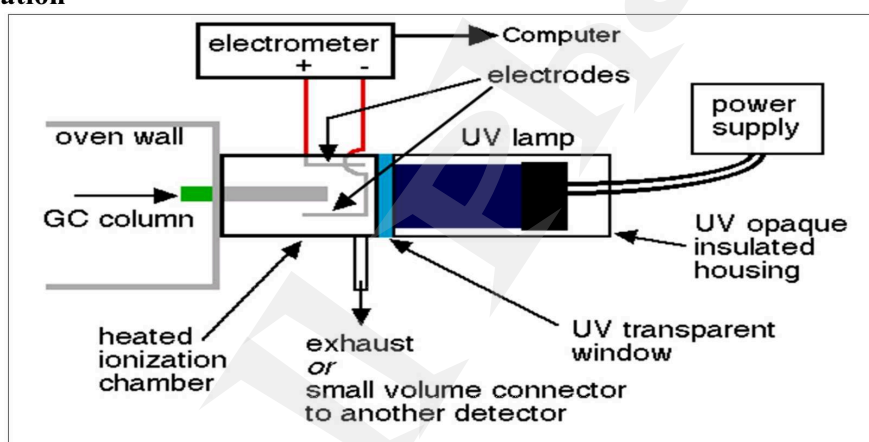
Instrumentation

Figure 14. Schematic of a photoionization detector

3. <u>**Derivatization**</u>

Derivatization is the process by which a compound is chemically changed, producing a new compound that has properties more amenable to a particular analytical method. Some samples analyzed by GC require derivatization in order to make them suitable for analysis. Compounds that have poor volatility, poor thermal stability, or that can be adsorbed in the injector will exhibit no reproducible peak areas, heights, and shapes. Other compounds that respond poorly on a specific detector may need to be "tagged" with a different functional group to improve detection. For example, tagging with chlorine can improve response on an ECD (electron capture detector). In addition to improving suitability and response, derivatization can improve resolution between co-eluting compounds and overlapping peaks.

3.1 Selection of Derivatization reagent

Derivatization reagent is the substance that is used to chemically modify a compound to produce

a new compound which has properties that are suitable for analysis in GC or LC. The following criteria must be used as guidelines in choosing a suitable derivatization reagent for GC analysis.

    i.   The reagent should produce more than 95 % complete derivatives.

    ii.   It should not cause any rearrangements or structural alterations of compounds during formation of the derivative.

    iii.   It should not contribute to loss of the sample during the reaction.

    iv.   It should produce a derivative that will not interact with the GC column.

    v.   It should produce a derivative that is stable with respect to time.

3.2 Objectives for derivatization

    i.   Improvement of resolution and reduce tailing of polar compounds which may contain – OH, –COOH, =NH, –NH_2, –SH, and other functional groups.

    ii.   Analysis of relatively nonvolatile compounds.

    iii.   Reduction of volatility of compounds prior to GC analysis.

    iv.   Improvement of analytical efficiency and hence increase detectability.

    v.   Stabilization of compounds for GC analysis.

3.3 Types of derivatization reactions

Derivatization reactions used for gas chromatography (GC) fall into three general reaction types namely; Alkylation of which the general process is esterification, Acylation and Silylation. Through these three processes, highly polar materials such as organic acids, amides, poly-hydroxy compounds, amino acids are rendered suitable for GC analysis by making them sufficiently volatile. These general processes are discussed below.

3.3.1. Alkylation

Alkylation is mostly used as the first step for further derivatizations or as a method of protection of certain active hydrogens in a sample molecule. It represents the replacement of active hydrogen by an aliphatic or aliphatic-aromatic (e.g., benzyl) group in process referred to as esterification. Equation 1 below shows the general reaction equation representing the esterification process.

$$RCOOH + PhCH_2X \rightarrow RCOOCH_2Ph + HX$$
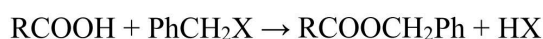
Equation 1: General reaction for esterification process

The principal chromatographic use of this reaction is the conversion of organic acids into esters, especially methyl esters that produce better chromatograms than the free acids. Alkylation reactions can also be used to prepare ethers, thioethers and thioesters, N- alkylamines, amides

and sulphonamides. In general, the products of alkylation are less polar than the starting materials because active hydrogen has been replaced by an alkyl group. The alkyl esters formed offer excellent stability and can be isolated and stored for extended periods if necessary. In esterification an acid reacts with an alcohol to form an ester. In the reaction, a catalyst more often an inorganic acid such as hydrochloric acid or thionyl chloride.

3.3.1.1 Derivatization reagents used in alkylation

Common derivatization reagents for the Alkylation type of reactions are Dialkylacetals, Diazoalkales, Pentafluorobenzyl bromide (PFBBr), Benzylbromide, Boron trifluoride (BF_3) in methanol or butanol and Tetrabutylammonium hydroxide (TBH) among others. Alkylation reagents can be used alone to form esters, ethers and amides or they can be used in conjunction with acylation or silylation reagents. The reaction conditions can vary from strongly acidic to strongly basic with both generating stable derivatives.

Examples

> **Dialkylacetals**

Dimethylformamide (DMF) is an example of dialkylacetals with a general formula $CH_3CH_3NCHOROR$ are used to esterify acids to their methyl esters. Dialkylacetals have a wider applicability for the derivatization of a number of functional groups containing reactive hydrogens. Because the principal reaction product is dialkylacetals (DMF), the isolation of the derivative is not required and the reaction mixture can be injected directly into the gas chromatograph. This reagent is an excellent first choice for derivatization of a compound for which there is no published method available. The reaction between N, N-dimethylformamide dimethylacetal and Carboxylic acid is as follows (Equation 2).

$$CH_3CH_3NCHOROR + R`COOH \rightarrow R`COOR + ROH + CH_3CH_3NCHO$$
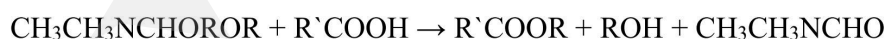
Equation 2: The reaction between N, N-dimethylformamide dimethylacetal and Carboxylic acid.

Although carboxylic acids, phenols, and thiols react quickly with DMF, to give the corresponding alkyl derivatives, hydroxyl groups are not readily methylated. During derivatization procedure, care should be taken because *N*, *N*-dimethylformamide dimethylacetals are moisture sensitive.

> **Tetrabutylammonium hydroxide (TBH)**

Derivatization of a carboxylic acid with tetrabutylammonium hydroxide (TBH) forms butyl ester, which will allow a longer retention times in a GC column. The reagent is most commonly used for low molecular weight acids and is especially suitable for low molecular

weight amines. Equation 3 represents the derivatization reaction for the conversion of carboxylic acid to alkyl esters using TBH.

$$[N (CH_3)_4]^+[OH]^- + RCOOH \rightarrow RCOOC_4H_9$$

Equation 3: Conversion of carboxylic acid to alkyl esters using TBH.

The following derivatization procedure can be used for flash alkylation which is suitable for biological fluids and thermally stable fatty acids analysis

3.3.2 Silylation

Silylation is the most prevalent derivatization method as it readily volatizes the sample and therefore very suitable for non-volatile samples for GC analysis. Silylation is the introduction of a silyl group into a molecule, usually in substitution for active hydrogen such as dimethylsilyl [$SiH(CH_3)_2$], t-butyldimethylsilyl [$Si (CH_3)_2C(CH_3)_3$] and chloromethyldimethylsilyl [$SiCH_2Cl(CH_3)_2$]. Replacement of active hydrogen by a silyl group reduces the polarity of the compound and reduces hydrogen bonding. Many hydroxyl and amino compounds regarded as nonvolatile or unstable at 200 – 300 °C have been successfully analyzed in GC after silylation. The silylated derivatives are more volatile and more stable and thus yielding narrow and symmetrical peaks.

3.3.2.1 Silylation reaction and mechanism

The silylation reaction is driven by a good leaving group, which means a leaving group with a low basicity, ability to stabilize a negative charge in the transitional state, and little or no back bonding between the leaving group and silicon atom (Knapp, 1979). The mechanism involves the replacement of the active hydrogens (in -OH, -COOH, -NH, -NH$_2$, and -SH groups) with a trimethylsilyl group.

Silylation then occurs through nucleophilic attack (SN_2), where the better the leaving group, the better the siliylation. This results to the production of a bimolecular transition state in the intermediate step of reaction mechanism. The general reaction for the formation of trialkylsilyl derivatives is shown by equation 4. The leaving group in the case of trimethylchlorosilane
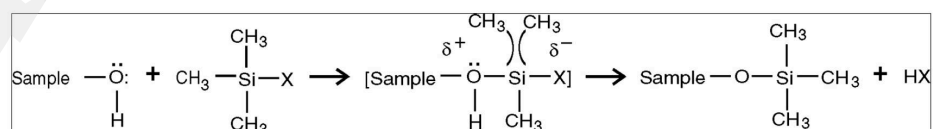
(TMCS) is the Cl atom.

Equation 4: Reaction mechanism for the formation of trialkylsilyl derivatives for trimethylchlorosilane, X = Cl

In silylation derivatisation, care must be taken to ensure that both sample and solvents are dry. Silyl reagents generally are moisture sensitive, and should be stored in tightly sealed containers and therefore the solvents used should be as pure and as little as possible.

3.3.2.2 Derivatization reagents used in Silylation

Reagents used for the silylation derivatization process include Hexamethyldisilzane (HMDS), Trimethylchlorosilane (TMCS), Trimethylsilylimidazole (TMSI), Bistrimethylsilylacetamide (BSA), Bistrimethylsilyltrifluoroacetamide (BSTFA), N-methyl- trimethylsilyltrifluoroacetamide (MSTFA), Trimethylsilyldiethylamine (TMS-DEA), N- methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and Halo-methylsilyl derivatization reagents.

Examples

> **Trimethylsilylimidazole (TMSI)**

Trimethylsilylimidazole (TMSI) is not a weak donor, but it is selective as it reacts with alcohols and phenols but not amines or amides (nitrogen groups). Since it is selective, it will target the hydroxyls in wet sugars and also derivatize the acid sites of amino acids. It will leave the amino group free for fluorinated derivatization. An example of reaction equation

using TMSI is shown below (Equation 5).

Equation 5: Silylation reaction using Trimethylsilylimidazole (TMSI): TMS = R, R` = Alk, Ar.

The derivatives produced are suitable for ECD analysis. Trimethylchlorosilane (TMCS) is also a weak donor. In addition, it produces hydrochloric acid as a byproduct with is acidic. It is therefore not commonly used. However, it is often found as a catalyst to increase TMS donor potential. An example of derivatization reaction using Trimethylchlorosilane (TMCS) is shown in equation 6.

Equation 6: Silylation reaction using Trimethylchlorosilane (TMCS): Y = O, S, NH, NR`, COO, R, R` = Al, Ar.

3.3.3 Acylation

Derivatization by acylation is a type of reaction in which an acyl group is introduced to an organic compound. In the case of a carboxylic acid, the reaction involves the introduction of the acyl group and the loss of the hydroxyl group. Compounds that contain active hydrogens (e.g., -OH, -SH and -NH) can be converted into esters, thioesters and amides, respectively, through acylation. Acylation is also a popular reaction for the production of volatile derivatives of highly polar and in volatile organic materials. Acylation also improves the stability of those compounds that are thermally labile by inserting protecting groups into the molecule. Acylation can render extremely polar materials such as sugars amenable to separation by GC and, consequently, are a useful alternative or complimentary to the silylation. Equation. 7 shows an example of an acylation is the reaction between acetic anhydride and an alcohol.

$$CH_3OCOCOCH_3 + HOR \xrightarrow{H^+} CH_3OCOR' + HOCOCH_3$$
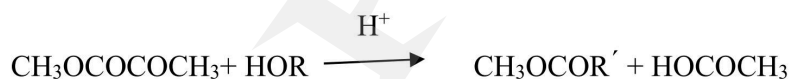
Equation 7: Acylation Reaction

- Benefits of acylation in GC analysis.

i. It improves analyte stability by protecting unstable groups.

ii. It can provide volatility on substances such as carbohydrates or amino acids, which have many polar groups that they are nonvolatile and normally decompose on heating.

iii. It assists in chromatographic separations which might not be possible with compounds that are not suitable for GC analysis.

iv. Compounds are detectable at very low levels with an electron capture detector (ECD).

3.3.3.1 Derivatization reagents used in acylation

Common reagents for the Alkylation process are Fluoracylimidazoles, Fluorinated Anhydrides, N-Methyl-bis(trifluoroacetamide) (MBTFA), Pentafluorobenzoyl Chloride (PFBCI) and Pentafluoropropanol (PFPOH). Acylating reagents readily target highly polar, multi-functional compounds, such as carbohydrates and amino acids. In addition, acylating reagents offer the distinct advantage of introducing electron-capturing groups and therefore enhancing detectability during analysis.

Examples

➢ **N-Methyl-bis(trifluoroacetamide) (MBTFA)**

N-Methyl-bis(trifluoroacetamide) (MBTFA) reagent reacts rapidly with primary and secondary amines, and also slowly with hydroxyl groups and thiols. Reaction conditions are mild with relatively inert and non acidic by-products and therefore do not damage the GC column. The general reaction is presented in equation 8;

$$F_3COCN (CH_3) OCCF_3 + H\text{-}Y\text{-}R \rightarrow F_3OC\text{-}Y\text{-}R + CH_3NHOCCF_3$$

Equation 8: Representative reaction of the derivatization of amines, hydroxyl groups and thiols using N-Methyl-bis(trifluoroacetamide) (MBTFA) reagent: Y = O, S, NH, NR´, R, R´ = Alk, Ar.

N-Methyl-N-bis(trifluoroacetamide is recommended for the analysis of sugars and as an acylation reaction is often used for amine drugs, such as stimulants, amino acids, and alcohols.

> **Pentafluorobenzoyl Chloride (PFBCl)**

Pentafluorobenzoyl chloride (PFBCl) is used in making derivatives of alcohols and secondary amines of which secondary amines are the most highly reactive, forming the most sensitive ECD derivatives of amine and phenol. Phenols are the most receptive site for this reagent. Pentafluorobenzoyl chloride (PFBCl) is suitable for functional groups that are sterically hindered. A base such as NaOH is often used to remove the HCl that is produced as byproduct. This derivatization procedure which is presented by the reaction below (equation 9) basically uses a pentafluorobenzoyl chloride (PFBCl) to provide rapid formation of the derivatives of amines and phenols.

$$C_6 F_5\text{-}OCCl + C_6H_5\text{-}OH \rightarrow C_6 F_5\text{-}OCO\text{-} C_6H_5 + HCl$$
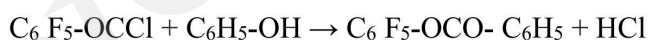
Equation 9: Formation of derivatives of amines and phenols using Pentafluorobenzoyl chloride (PFBCl).

3.3.4   Chiral Derivatization

Chiral derivatization involves reaction of an enatiomeric molecule with an enatiomerically pure chiral derivatizing agent (CDA) to form two diastereomeric derivatives that can be separated in this case using GC. A solution in which both enantiomers of a compound are present in equal amounts is called a racemic mixture. Diastereomers are stereoisomers (they have two or more stereo centers) that are not related as object and mirror image and are therefore not enantiomers. In other word, unlike enatiomers which are mirror images of each other and non-sumperimposable, diastereomers are not mirror images of each other and non-superimposable**.** Diastereomers can have different physical properties and reactivity.

Any molecule having asymmetric carbon is called as chiral molecule. Chirality of analyte molecules requires special consideration in their analysis and separation techniques. Scientists and other regulatory authorities are in the demand of data on concentrations and toxicity of the chiral pollutants, and therefore chiral derivatization is becoming an essential, urgent and demanding field. However the derivatization procedures are tedious and time consuming due to the different reaction rates of the individual enantiomers.

Generally, there are two ways of separating enantiomers by chromatography:

i. Separation on an optically active stationary phase

ii. Preparation of diastereomeric derivatives that can be separated on a non chiral stationary phase.

3.3.4.1 Gas chromatography chiral derivatization reagents

Gas Chromatography analysis of enantiomeric compounds on nonracemic or achiral stationary phases requires the use of enantiopure derivatization reagents. Enantiopure compounds refer to samples that contain molecules having one chirality within the limits of detection. These reagents generally target one specific functional group to produce diastereomers of each of the enantiomeric analytes in GC to produce chromatograms. Some of the most common Gass Chromatography chiral derivatization reagents are: (-) menthylchloroformate (MCF), (S)-(–)-N-(Trifluoroacetyl)-prolylchlorides (TPC), (–)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA).

Example

➢ **N-trifluoroacetyl-L-prolyl chloride (TPC)**

The reagent is used for optically active amines, most notably amphetamines as represented in the following reaction (equation 10) where N-Trifluoroacetyl-L-prolyl chloride couples with amines to form diastereomers which can be separated on GC columns as it increases
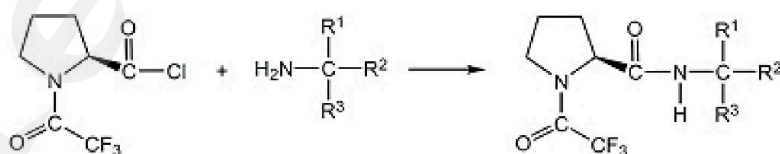
the sample volatility.

Equation 10: N-Trifluoroacetyl-L-prolyl chloride derivatization of amines

Limitations

1. Not suitable for detecting semi-volatile compounds

2. Only indicates if volatile organic compounds are presents.

3. High concentration so methane are required for higher performance.

4. Frequent calibration are required.

5. Units of parts per million range

6. Enviromental distraction, especially water vapor.

7. Strong electrical fieldsRapid variation in temperature at the detector and naturally occurring compounds may affect instrumental signal.

Applications:

Gas chromatography is a physical separation method in where volatile mixtures are separated. It can be used in many different fields such as pharmaceuticals, cosmetics and even environmental toxins. Since the samples have to be volatile, human breathe, blood, saliva and other secretions containing large amounts of organic volatiles can be easily analyzed using GC. Knowing the amount of which compound is in a given sample gives a huge advantage in studying the effects of human health and of the environment as well.

Air samples can be analyzed using GC. Most of the time, air quality control units use GC coupled with FID in order to determine the components of a given air sample. Although other detectors are useful as well, FID is the most appropriate because of its sensitivity and resolution and also because it can detect very small molecules as well. GC/MS is also another useful method which can determine the components of a given mixture using the retention times and the abundance of the samples. This method be applied to many pharmaceutical applications such as identifying the amount of chemicals in drugs. Moreover, cosmetic manufacturers also use this method to effectively measure how much of each chemical is used for their products.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. Introduction

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography) is a technique in <u>analytical chemistry</u> used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid <u>solvent</u> containing the sample mixture through a column filled with a solid <u>adsorbent material</u>. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

2. Principle

The purification takes place in a separation column between a stationary and a mobile phase. The stationary phase is a granular material with very small porous particles in a separation column.

The mobile phase on the other hand is a solvent or solvent mixture which is forced at high pressure through the separation column. Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe. Subsequently the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase. After leaving the column the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer. At the end of this operation a chromatogram in the HPLC software on the computer is obtained, which allows the identification and quantification of the different substances.

3. Types

There are following variants of HPLC, depending upon the phase system (stationary) in the process :

i. Normal Phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

ii. Reverse Phase HPLC

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

iii. Size-exclusion HPLC

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

iv. Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples.

The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

4.Instrumentation

HPLC instrumentation incorporates a pump, injector, column, finder and integrator or securing and show framework. The core of the framework is the column where division happens.
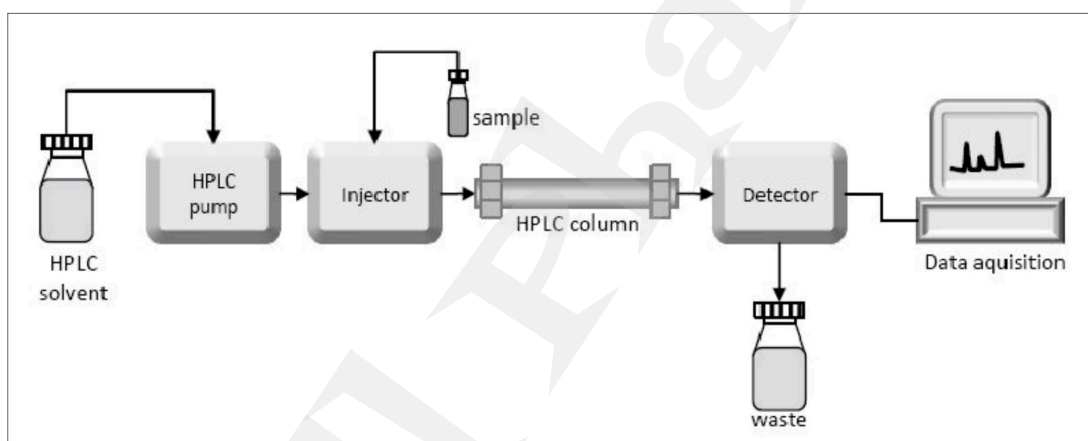

Figure.1 Schematic diagram of HPLC instrumentation

a. **Solvent Reservoir** : Mobile stage substance are contained in a glass reservoir. The versatile stage, or dissolvable, in HPLC is typically a blend of polar and non-polar liquid segments whose particular fixations are changed relying upon the arrangement of the specimen.

b. **Pump** : A pump suctions the versatile stage from the dissolvable reservoir and drives it through the framework's column and detector. Contingent upon various components including column measurements, molecule size of the stationary stage, the stream rate and sythesis of the versatile stage, working weights of up to 42000 kPa (around 6000 psi) can be created.

c. **Sample Injector** : The injector can be a solitary infusion or a mechanized infusion framework. An injector for a HPLC framework ought to give infusion of the liquid specimen inside the scope of 0.1–100 mL of volume with high reproducibility and under high weight (up to 4000 psi).

d. **Columns** : Columns are generally made of cleaned stainless steel, are in the vicinity of 50 and 300 mm long and have an inside distance across of in the vicinity of 2 and 5 mm. They are normally loaded with a stationary stage with a molecule size of 3–10 μm. Columns with interior distances across of under 2 mm are regularly alluded to as microbore HPLC columns. In a perfect

world the temperature of the portable stage and the column ought to be kept steady amid an examination.

e. **Detector** : The HPLC indicator, situated toward the finish of the column distinguish the analytes as they elute from the chromatographic column. Regularly utilized finders are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical indicators.

f. **Data Collection Devices** : Signals from the indicator might be gathered on outline recorders or electronic integrators that differ in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the identifier to every part and places it into a chromatograph that is anything but difficult to peruse and decipher.

g. Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymer membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line, it is more convenient and efficient. Many of new HPLC unit system contain a degasser.

h. Column Heater

The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep the consistent temperature conditions. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50 to 80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. Thus columns are generally kept inside the column oven (column heater).
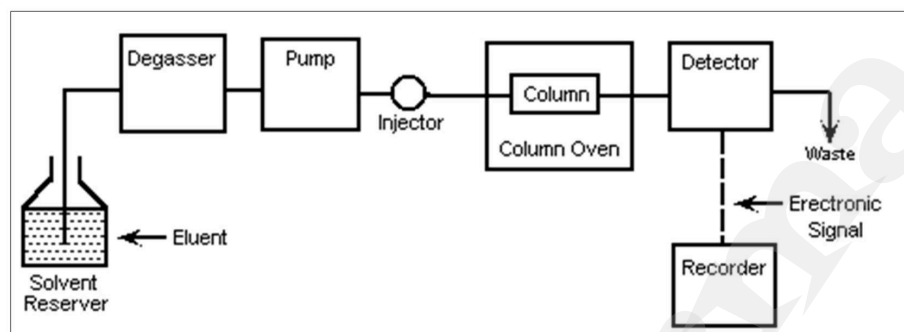
Figure 2. Components of HPLC system

4. **Applications**

- Water purification.
- Detection of impurities in pharmaceutical industries.
- Pre-concentration of trace components.
- Ligand-exchange chromatography.
- Ion-exchange chromatography of proteins.
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides.