Swayam Course - Analytical Techniques

Week: 5, Module11 - Gas Chromatography

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Learning outcomes: After completing this chapter, the student shall be able to:

a. **Describe the principle of gas chromatography**

b. **List the various components of gas chromatographic system**

c. **Describe the process of gas chromatography**

d. **List the different types of detectors used**

e. **List different applications of gas chromatography**

1. Introduction: chromatographic techniques are used widely across scientific disciplines and in industry for multiple applications, some of which may even be overlapping. Broadly classified into planar and column chromatography, the general principle involves the separation of components (i.e. analytes of interest) of a mixture (of widely varying constitution e.g. peptides, lipids, pigments etc.) based on their relative solubility in, and hence movement through "phases" i.e. mobile and stationary, used in the technique. Further, chromatography is divided into gas and liquid based on the nature of the mobile phase. When the mobile phase is liquid then the technique is referred to as "liquid chromatography" and when the mobile phase is gaseous then as "gas chromatography" (GC). The concept was introduced and first experiments on gas chromatography were done by the Austrian chemist, Erika Cremer, who used a solid stationary phase. This technique, was put into extensive use by Martin and Synge in the 1940s , using gas-liquid chromatography as an analytical tool who demonstrated its utility by separating a mixture of organic acids and amines using GC. The work of Martin and Synge also heralded the use of GC in the petroleum industry. Martin and Synge were awarded the Nobel prize for chemistry for their work in partition chromatography and development of GC.

Gas chromatography is a highly sensitive technique that is routinely used in industrial laboratories for quantification of compounds, quality control and in forensic laboratories for detection of many chemicals that are present in extremely small amounts.

The mobile phase in gas chromatography is an inert gas that does not interact with the analytes in the mixture to be separated. If the gas phase moves over a solid stationary phase, then it is called "gas solid chromatography". This type of gas chromatography is most commonly used for separation of low molecular weight gaseous species. If the gas moves over a non-volatile liquid phase coated over a support, then it is referred to as "gas-liquid chromatography". Gas-liquid chromatography is much more widely used than gas-solid chromatography.

2. Principle of gas chromatography

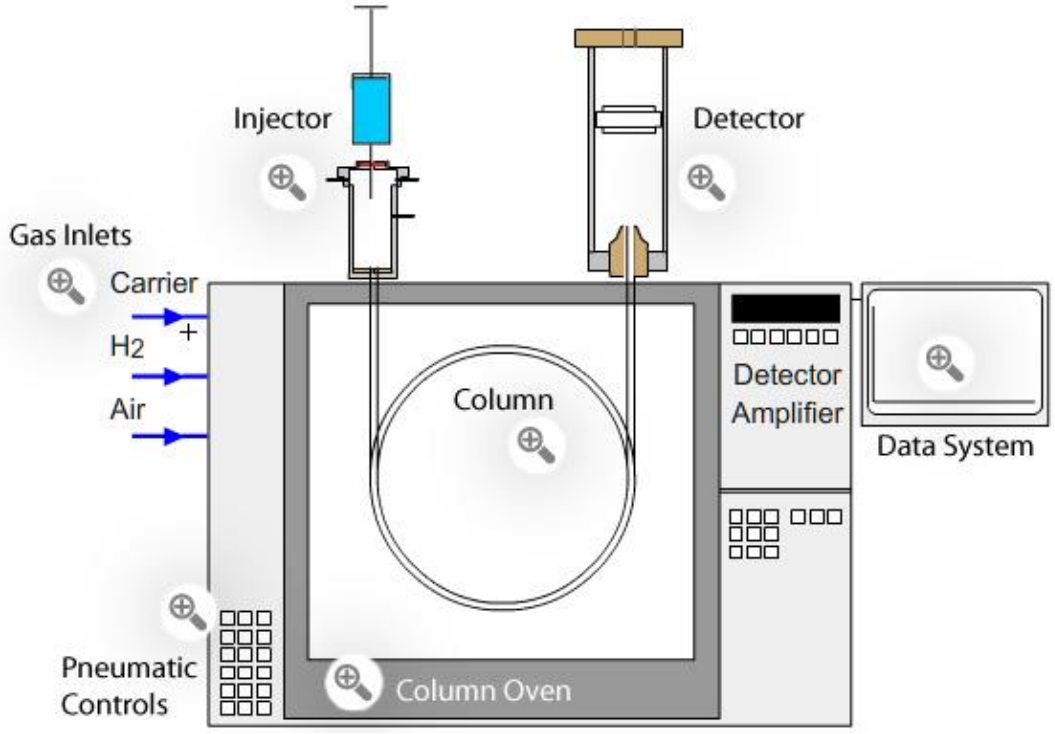
The underlying premise of separation using GC is based on the presence of analytes that are volatile (or modified i.e. *derivatized* to be volatile) at the temperatures at the point of injection. These analytes are typically dissolved in a solvent, and small amounts (typically in microliters) are injected into the gas chromatograph through an inlet. The separation will depend how strongly the compounds in the injected sample interact with the stationary phase. A stronger interaction means a longer interaction with the stationary phase and hence a longer time to migrate through the column and exit the column.

Each of the components of the sample leaves the column at a certain time called its "*retention time*". The exit of analytes from the column is perceived by a detector and converted into electrical signals for each of the analytes exiting the column which are visible as peaks on the recorded output of the process. This output is called the "*chromatogram*". There are many kinds of detectors(D) used in gas chromatography e.g. electron capture (ECD), flame ionization (FID), thermal coupled (TCD), Nitrogen-Phosphorus (NPD) and mass spectrometer (MS).

3. The gas chromatographic system:

Overview: the system consists of an injection port, an oven containing the column which may operate isothermally or with temperature programming, an exit port and a detector that detects the analytes coming out of the column. In addition, gas cylinders for maintaining the flow of the mobile phase i.e. gas, are also connected to the oven containing the column.

Figure 1a: **Schematic diagram of a gas chromatographic system**


Injector
Detector
Gas Inlets
Carrier
H2
+
Air
Column
Detector
Amplifier
Data System
Pneumatic
Controls
Column Oven

Components:

4.1. Carrier gas: is the medium in which the sample mixture is carried along the column, and flows out. The carrier gas should be inert, should allow a sufficient detector response and from a practical perspective, also be low-cost. The gases usually selected as carrier gases are helium or nitrogen although some other gases have also been used. Certain types of detectors such as the Flame Ionization Detector also need a source of combustion for the detector to function (i.e. the flame to be lighted). The gas used for this is hydrogen.

4.2: **The injection port** is where the sample is introduced into the column (i.e. the column head). In modern gas chromatographs, the port is usually heated so that sample injection and vaporization can take place simultaneously. The sample volumes typically injected are in the range of a few microliters through a rubber septum and into the vaporization chamber. Depending on whether the whole sample is needed (uncommon) for analysis or only a small amount is needed, the injection can be split less or split by means of a splitter that directs excess sample to a waste collector.

The chief considerations on sample introduction to the gas chromatograph include sample volatility, appropriate analyte concentration and care in the physical process of injecting the sample to prevent degradation during separation[1].

4.3. Column:

4.3.1. **Column types:** The column is the heart of the gas chromatography- based separation process. There are hundreds of types of columns available with varying chemical composition and lengths. They are broadly categorized into "packed" and "capillary". In the initial decades of analytical gas chromatography, **packed columns** were used, in which a glass or metal (mostly stainless steel but also aluminium, copper, polytetrafluorethylene) column tubing is packed with small spherical inert supports (e.g. diatomaceous earth). The liquid phase adsorbs onto the surface of these beads in a thin layer. Packed columns have higher sample capacity[2]. However, packed columns are usually are less efficient and have lower resolution as compared to capillary columns. They are often used for preparative work and gas analysis. Their inner diameters range from 1.5 mm to 6 mm. A category of micro-packed columns are also available. They are micro pore tubes having inner diameters ranging from 0.3-1 mm and at lengths varying from 1 to 15 m, packed with particles 0.007-0.3 mm in diameter.

The other kind of columns, called "**capillary columns**" are much more commonly used nowadays and consist of a tubing, the walls of which are coated with the stationary phase or an adsorbent layer, which supports the non-volatile liquid phase. Stationary phase thickness is typically 0.25 microns. Capillary columns have a higher efficiency and hence better peak separation than packed columns. Capillary columns are categorized into "The Wall Coated Open Tubular (WCOT)" columns, "Support Coated Open Tubular" (SCOT) and the "Porous Layer Open Tubular (PLOT)" columns. A **wall-coated open tubular column** (WCOT) has a thin layer of stationary phase, typically 0.25 μm thick, coated on the capillary's inner wall. A **porous-layer open tubular column** (PLOT), has a porous solid support such as alumina, silica gel, and molecular sieves attached to the capillary's inner wall. A **support-coated open tubular column** (SCOT) is a PLOT column that includes a liquid stationary phase. Capillary columns have inner diameters ranging from 0.10 to 0.20 mm and lengths including 10 m, 15m, 30m, 50 m, up to 15 m

[1]The sample must be injected into the minimum possible volume of mobile phase. Precolumn band broadening can be caused by injecting the sample into a moving stream of mobile phase and injecting a liquid sample instead of a gaseous sample. The design of a gas chromatograph's injector is important to minimize these problems.

[2] The amount of sample that can be applied to a column without overloading.

depending on the inner diameter. A very efficient, popular type of WCOT column is the fused-silica wall-coated (FSWC) open tubular column that is chemically very inert and requires very small sample sizes.

The columns are typically packed or coated with solid material which could perform the role of adsorbent (Gas Solid chromatography), in which case the separation takes place by adsorption, or the solid material could be support for stationary phase (which is a non-volatile liquid), as in Gas Liquid Chromatography, coated on a granular material, in which case the separation takes place by absorption.

4.3.2. Stationary phases: A wide range of solid and liquid stationary materials have evolved for use over time, numbering into hundreds. These are sometimes naturally occurring materials such as Kieselguhr (diatomaceous earth based, that contain polysilicic acid as hydrated amorphous silica with a porous structure and varying amounts of metal oxides of Fe, Al, Mg, Ca, Na, K) or synthetically created polymers such as Squalane, teflon. If a liquid is chosen as the stationary phase, it should dissolve all the components of the samples differentially, be practically non-volatile at the temperature of the column, be chemically inert and have high thermal stability. The liquid may be non-polar (e.g. silicone oils methylsilicone type), polar (polyethylene glycol, silicone oils with cyanopropyl groups) or of intermediate polarity (e.g. phenyl methyl silicone phase, dinonyl phthalate). The organic compounds are also empirically classified based on their chromatographic separation from Class I (very polar) to Class V (non-polar).

A few examples of commonly used gas-liquid chromatography stationary phases and their applications is listed below[3]:

stationary phase	polarity	temperature limit (^{o}C)	representative applications
Squalene	nonpolar	150	low-boiling aliphatics hydrocarbons
Apezion L	nonpolar	300	amides, fatty acid methyl esters, terpenoids
Polydimethyl siloxane	slightly polar	300–350	alkaloids, amino acid derivatives, drugs, pesticides, phenols, steroids
Phenylmethyl polysiloxane (50% phenyl, 50% methyl)	moderately polar	375	alkaloids, drugs, pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls

stationary phase	polarity	temperature limit (^{o}C)	representative applications
Trifluoropropylmethyl polysiloxane (50% trifluoropropyl, 50% methyl)	moderately polar	275	alkaloids, amino acid derivatives, drugs, halogenated compounds, ketones
Cyanopropylphenylmethyl polysiloxane (50% cyanopropyl, 50% phenylmethyl)	polar	275	nitriles, pesticides, steroids
Polyethylene glycol	polar	225	aldehydes, esters, ethers, phenols

The suitability of the column packing/coating material for analysis of the sample mixture will depend on the chemical (and occasionally, structural) nature of the analytes in the mixture so as to allow an interaction between the stationary phase so that retention times vary as per the respective degrees of interaction between the analyte and stationary phase. If the boiling points of the components of a mixture's are significantly different, then the choice of stationary phase is less critical. However, if two solutes have similar boiling points, then the critical factor determining separation is the selective interaction of the stationary phase with one of the solutes. In such a scenario, if the analyte is polar, the stationary phase should be polar and if the analyte is non-polar, then a non-polar/less-polar stationary phase should be chosen. Since compounds vary in their polarity in the same mixture, a compromise is often made while choosing the stationary phase.

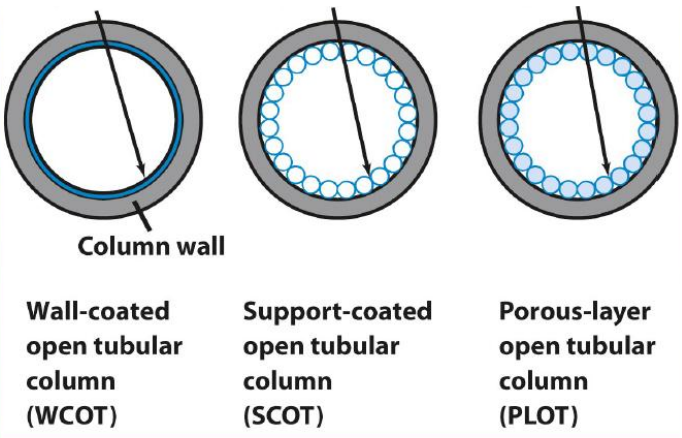
The stationary phases on the GC columns need to be conditioned and activated by allowing the mobile phase to flow over the column for a few hours or over a few days before the samples for analysis are applied onto the column for separation. Also, while using the columns, care must be taken to understand the robustness of the column coatings and supports with regard to its thermal stability and the effect of certain corrosive chemicals that may poison the column (e.g. oxygen, chromic acid, potassium hydroxide, perfluoroacids). To minimize changes of such damage, a guard column can be installed ahead of the main column. Sometimes trimming a few feet of the initial part of the column can remove the damaged section.

Liquid stationary phases also have a tendency to bleed. In order to maintain stability of columns, precautions during sample preparation/injection should be taken. Stationary phase modifications are also done [4]. Avoiding injection of dirty samples, using high-temperature low-bleed septa

[4] Maintaining the temperature limits defined for different kinds of stationary phase minimizes the chance of bleeding. Stability of capillary columns can be increased by bonding or cross-linking stationary phases. A bonded stationary phase is chemically attached to the capillary's silica surface. Cross-linking links together separate polymer chains after the stationary phase is in the capillary column.

and split injection when possible, replacing the inlet liner when it becomes contaminated, keeping column inlet section above the ferrules clean , and keeping the carrier gas free of oxygen and leaks are some precautions to minimize bleed. Column bleed is also increased with thicker stationary phase films and larger column inner diameters.

Fig 2: Types of GC columns and their stationary phases:



The resolution of a mixture into its individual components, depends on multiple factors as discussed above. A sample related factor is derivatization. Derivatization in GC analysis is a procedure that primarily modifies an analyte's functionality in order to enable chromatographic separations. They enhance the suitability of the chemical compunds of interest for analysis, the volatility and efficiency of the chromatographic process.

Derivatization allows highly polar materials to be sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition. This requires an understanding of the chemistry of the analytes, different derivatizing reagents, and the detailed functionality of Gas Chromatography including compatible detector types. Compounds with functional groups having active hydrogens such as -SH, -OH, -NH and -COOH are of primary concern as these functional groups tend to form intermolecular hydrogen bonds which affect the inherent volatility of compounds containing them, their tendency to interact with column packing materials and their thermal stability. Three general types of derivatization reactions are usually carried out, namely Alkylation, Acylation and Silylation. These enable highly polar materials such as organic acids, amides, poly-hydroxy compounds, amino acids to be rendered suitable for GC analysis by increasing their volatility.

4.4 Detectors:

Analysis: The quantitative analysis of a given component is based upon evaluating the chromatographic peak, which is triangle-shaped when columns with filling are used; its surface is measured and divided by the total surface, in different percentages for different types of detectors. For capillary columns with good resolution, the signal takes the shape of straight lines and calculating the composition of the mixture is done in the order of succession, dividing each individual line by the total number of lines and using an adequate calibration curve, drawn upon determinations of known compounds

Mixture components can be identified by comparison using chromatographic standards – that are pure substances or known mixtures of components. In these cases, universal detectors may be used. For unknown mixtures, mass spectrometry is recommended.

In gas chromatography, the area under the peak is proportional to the concentration of the analyte.

In order to get a quantitative estimate, a calibration curve that relates the detector's response to the concentration of analyte is required. Careful procedures to ensure identical injection volumes for every standard and sample, can allow accurate and precise results using an external standardization. Internal standards are recommended for adjusting for any inter-assay variability

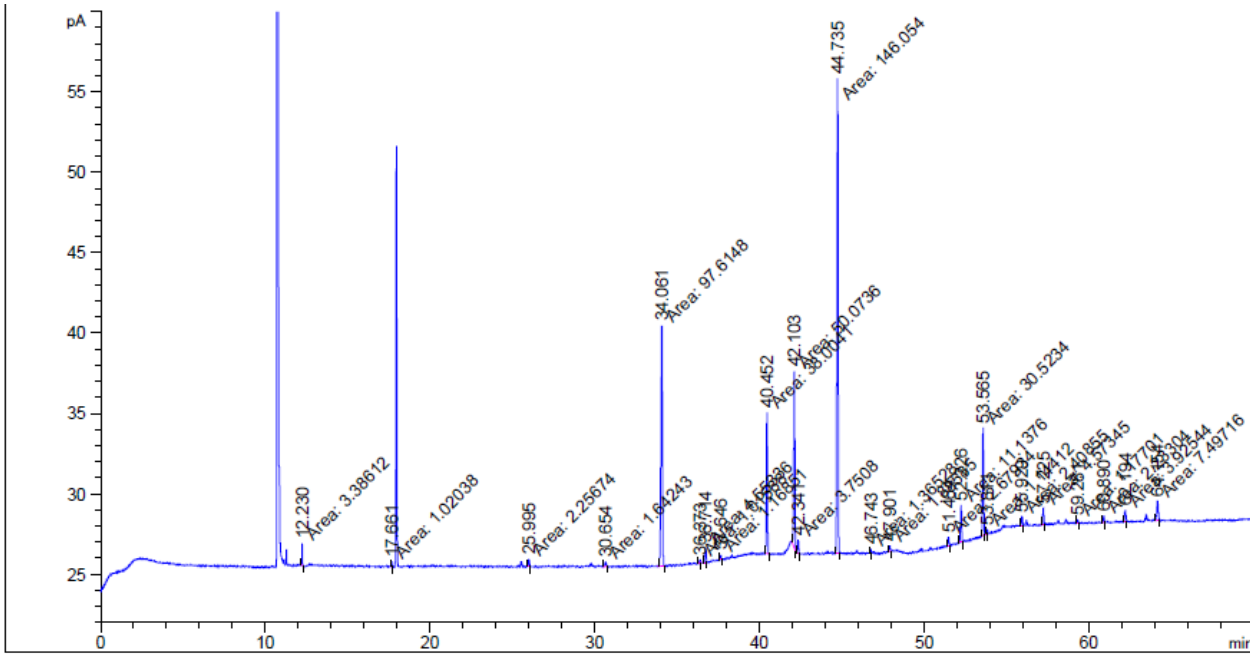


Figure 3: Representative chromatogram of fatty acid profile in serum (the numbers represent retention times; peak areas are also given. The peak areas are determined by integration by the instrument's inbuilt computer software or by an electronic integrating recorder):

Detectors: A wide range of detectors are available that are linked to gas-chromatography systems and are of varying sensitivities and application. Some of these are able to detect a wide range of samples down to picogram limits. The key attributes of a detector are sensitivity [which is indicated by the limit of detection [5] (LOD)], selectivity[6] and dynamic range[7]. A detector may respond to the concentration of an

[5] This is the minimum quantity of material than can be distinguished from background

[6] the ratio of the amount of a compound that does not contain the selected property that generates the same signal as a compound with the selected functionality

analyte passing through it or to the mass of the analyte passing through it. The former is called *concentration-sensitive* detector and the latter, *mass sensitive detector*. One of the detector types linked to the GC instrument, the *thermal conductivity detector* (TCD), is the most commonly used c*oncentration-sensitive detector*.

As the thermal conductivity of the mobile phase is affected by all solutes, the thermal conductivity detector is a universal detector. The TCD also has the advantage of a linear response over a concentration range spanning 10^4–10^5 orders of magnitude. The detector is non-destructive, allowing us to isolate analytes using a post-detector cold trap. However, a poor detection limit of the TCD detector for many analytes is a significant disadvantage.

The Flame ionization detector is one of the most commonly used detectors since most carbon atoms (except those in carbonyl and carboxylic groups) generate a signal, making FID an almost universal detector for organic compounds. However, FID is a destructive detector.

One example of a selective detector is the **electron capture detector**. The detector consists of a β-emitter, such as ^{63}Ni. The ECD is highly selective toward solutes with electronegative functional groups, such as halogens and nitro groups. It has a very good detection limit but its linear range extends over only about two orders of magnitude.

A mass spectrometer as the detector has the advantage of providing excellent detection limits, typically 25 fg to 100 pg, with a linear range of 10^5 orders of magnitude. The principle of the mass spectrometric detector is recording and analyzing the mass spectrum of the column's eluent, so the mass spectrum for any time increment can be analyzed. This is a great advantage for GC–MS since the mass spectrum to help identify a mixture's components.

The list below describes the most common types of detectors used in gas chromatography:

Type of detector	**Type of response**	**Response characteristic**	**Destructive**	**LOD**
Flame Ionization Detector	Universal to C*	Mass	Yes	1 pg/s
Thermal Conductivity Detector	Universal	Concentration	No	500 pg/ml
Electron Capture Detector	Selective	Concentration	No	5 fg/s
Nitrogen Phosphorous Detector	Selective	Mass	Yes	1 pg N/s
Flame Photometric Detector	Selective	Mass	Yes	0.1-1pg P or S /s
Mass Spectrometry	Both	Mass	Yes	.25 to 100 pg
Atomic Emission Detector	Both	Mass	Yes	0.1 ng-1 pg/s

*Carbon.

5. Applications of gas chromatography:

Gas chromatography has many applications, both quantitative and qualitative. These include:

[7] the usable (operating) range over which the detector will generate a changing signal as the amount of analyte changes

a. Environmental analysis: pesticides' analysis in water/vegetables, vehicle emissions; organic pollutants in soil and water: volatile organic compounds (VOCs); polycyclic aromatic hydrocarbons (PAHs)

b. Clinical medicine: blood alcohol, drugs (nicotine, opioids)

c. Forensic medicine: explosives; alcohol; drugs

d. Consumer goods quality control: perfumes, alcoholic drinks

e. Food analysis: fatty acid composition, flavor components of edible products like spices

f. Petrochemicals: petrol composition; solvent purity.

6. **Factors to be considered during GC method development to optimize time of separation and optimum resolution[8]:**

- **Carrier gas flow rate**: an increase in flow rate shortens retention times but may decrease resolution, because of peak broadening.
- **Temperature program heating rates**: very high column temperatures lead to loss of resolution; optimum separation and retention time are usually achieved with temperature gradients.
- **Column length, column diameter**: A longer column and thinner diameter improve resolution.
- **Thickness of the stationary phase**: Standard thickness in capillary columns is 0.25 microns; thicker stationary phases increase sample capacity and retention time as well as improve resolution of early peaks.
- **Sample volumes**: the sensitivity of capillary column based gas chromatography also limits the sample volume that can be applied to the column; overloading the column can lead to tailing of peaks and poor resolution.

A modified gas chromatography technique, called "**Fast Gas chromatography**" is able to shorten analysis time without loss of resolution. The process typically uses shorter length columns e.g. 20m, with smaller internal diameters (i.d.) to shorten analysis times with good resolution.

7. **Illustrative methodology of gas chromatography using the example of analysis of fatty acids in serum**: this method involves the conversion of fatty acids into their methyl esters so that they can be volatilized, extraction of the derivatized esters into an organic phase and analysis on a polar gas chromatographic capillary column.

PROTOCOL : Fatty Acid analysis in serum:

<u>Step 1: Sample preparation and derivatization</u>

Glassware Required:

- Borosilicate glass tubes with Teflon-lined screw capes

[8] Resolution is a measure of how well two elution peaks can be differentiated in a chromatographic separation.

Reagents Required:

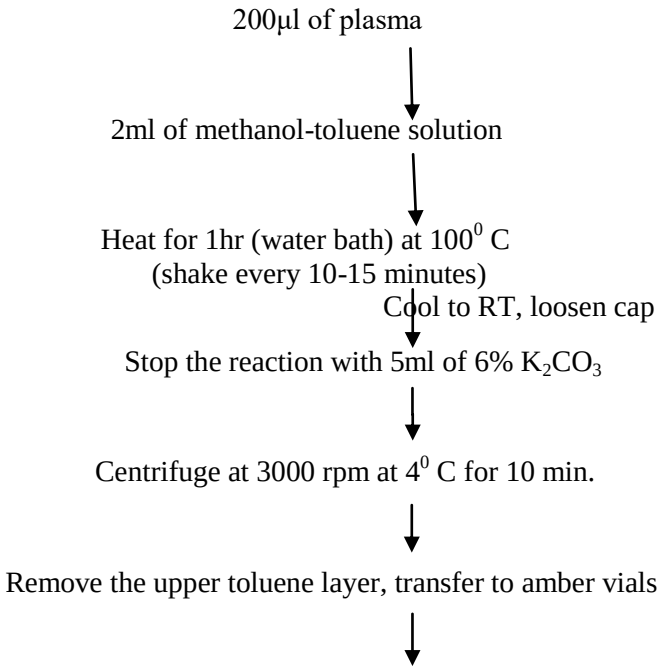
- Acetyl chloride
- HPLC grade methanol, chloroform, and toluene.
- Internal standard : undecanoate(C_{11}) FAME: 1 mg /ml of C_{11} in $CHCl_3$ (Chloroform HPLC grade)
- 2[6]di-tert-butyl*p*-cresol(Butylated Hydroxytoluene (BHT)]: 5mg of BHT/100 ml of methanol (HPLC grade)
- Potassium carbonate (K_2CO_3) : 6mg % in distilled water
- Nitrogen gas

PROCEDURE:

All glassware should be rinsed with chloroform-methanol 2:1 (v/v) and dried under nitrogen before starting the experiment.

Prepare a fresh solution containing methanol-toluene solution in the ratio of 4:1 methanol and toluene. Add internal standard(25 µl) and BHT(2.5 µl) per sample followed by acetyl chloride (e.g. for 20 samples, 40ml methanol + 10 ml toluene + 500µl C_{11} + 50µl BHT + 2.5 ml acetyl chloride - mix acetyl chloride dropwise while keeping tube on low speed vortex to the other components! **IMPORTANT: CRITICAL AND HAZARDOUS STEP. Wear gloves and mask.**)

BRIEF METHOD FLOW DIAGRAM:



Step 2: Gas chromatographic analysis: For the GC analysis a gas chromatograph equipped with a hydrogen flame ionization detector, split mode injector and the capillary column SP- 2560 (100 m x 0.25 mm; biscyanopropyl siloxane stationary phase) will be used.

The operating conditions will be: (temperatures, in ^{0} C): injector, 225; detector, 285; initial temp, 100; ramp, 3 ^{0} C/min; final temp, 240; hold 15 min. The split ratio will be set at: 200:1.

Reconstitute sample using 2-3 µl isooctane. Take 1 µl of each FAME for GC analysis. A mixed FAME standard solution will be used to optimize the running protocol and identify the fatty acids before testing the FAMEs prepared from the samples. Individual isomers will be identified by comparing with known retention times relative to the internal standard, C11.0. Specific isomers are also identified by running the unmodified FAME mix/sample followed by one spiked with the individual FAME isomer of interest.

Precautions: The reagents used must be HPLC grade and glassware used must be clean and free of detergent. Ideally, use of plastic ware should be avoided or it should be ensured that it is inert to organic reagents used in the protocol. Also, all reactions are to be run in triplicates for ensuring data validity when running for research purposes etc. The gas used for combustion, i.e. hydrogen is highly inflammable and care should be taken to maintain appropriate pressures when operating the gas flow valves.