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Gas Chromatography-Headspace (GC-HS) and its significance in Forensic Toxicology

Supriya Krishna[1], A. K. Jaiswal[2], Tisha Rosina Thomas[3], Millo Tabin[4], S. K. Gupta[5]

Author Affiliation: [1]Senior Scientific Assistant, [3]Student, Chemistry Division, LNJN National Institute of Criminology and Forensic Science, Rohini, Delhi, [2]Chemist, [4,5]Professor, and Head of Department, Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, New Delhi 110029, India.

Corresponding Author: A.K. Jaiswal, Chemist, Department of Forensic Medicine, All India Institute of Medical Sciences (AIIMS) New Delhi 110029, India.

E-mail: ashokjaiswal72@gmail.com

Abstract

Gas Chromatography (GC or GLC) is a commonly used analytic technique in any analytical laboratory for quality control as well as identification and quantitation of compounds in a mixture. High sensitivity, selectivity, resolution, speed, good accuracy and precision, wide dynamic concentration range, simple, economic and robust instrument design, and its ability to be interfaced with many established and emerging sampling and detection systems have made GC the instrument of choice in Forensic Science. A broad variety of organic samples can be separated and analysed as long as the compounds are sufficiently thermally stable and reasonably volatile. Gas chromatography can be used for the separation of gases, liquids and solids. Materials such as biological materials (tissues, blood, urine, etc), alcohol, fire debris, car paints, drugs, pesticides, plant material and fibres are the most common evidential materials analysed by forensic chemists/scientists and are characterized by a high degree of complexity. One of the major challenges is to ensure that the sample injected is truly the representative sample of the extract. Therefore, improvement in injection and sampling handling techniques has always been necessity. Inclusion of Headspace (HS) as a sample injection technique has improved and reduced the sampling error and sample loss during preparation. Headspace analysis is based on the principle that volatile components in avial, maintained at equilibrium, diffuse into the gas phase above the sample which is then extracted and introduced into the GC system for analysis. This ensures that the sample is the true representation of the test sample. In HS, sample injection volume can range anywhere between few µl to 1000µl. Some recent applications of GC-HS in Forensic Chemistry, including those in Forensic Toxicology, are presented in the article, which include alcohol estimation in drunken driving cases, estimation of drugs seized in bulk form, illicit drugs, estimation of pesticides in biological matrix and post-mortem volatiles in stored samples.

Keywords: Gas Chromatography Headspace; Applications; GC-HS; Forensic Toxicology etc.

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Introduction

Chromatography is a collective term for physicochemical techniques for separation of complex organic compounds in solid, liquid and gaseous state. Since it was first invented by Mikhail Tsvet in 1903, chromatography is being continuously modified and improved to meet the demands of ever expanding modern analytical chemistry[(1,2)]. Of the quasi-infinite possibilities in analytical chemistry, the technique finds its major applications in the field of Forensics for Alcohol analysis, Fire debris analysis, Metabolomics, Pharmaceutical industry, Polymer industry, Food industry for flavonoids, fragrances in perfumes and cosmetics[(3–8)].

In spite for various modifications in the instrumentation of chromatography the principle remains same. The technique is based on the principle of separation of an analyte as a consequence of partition between the mobile phase and stationary phase held on or inside a solid support[(9–11)]. The distribution of components between the two phases depends on the physicochemical properties of

adsorption, ionic interactions, diffusion, solubility or, in the case of affinity chromatography, specific interactions. Modifications in type of stationary phase, mobile phase and process of separation can divide chromatography into various techniques as illustrated in Fig. 1.

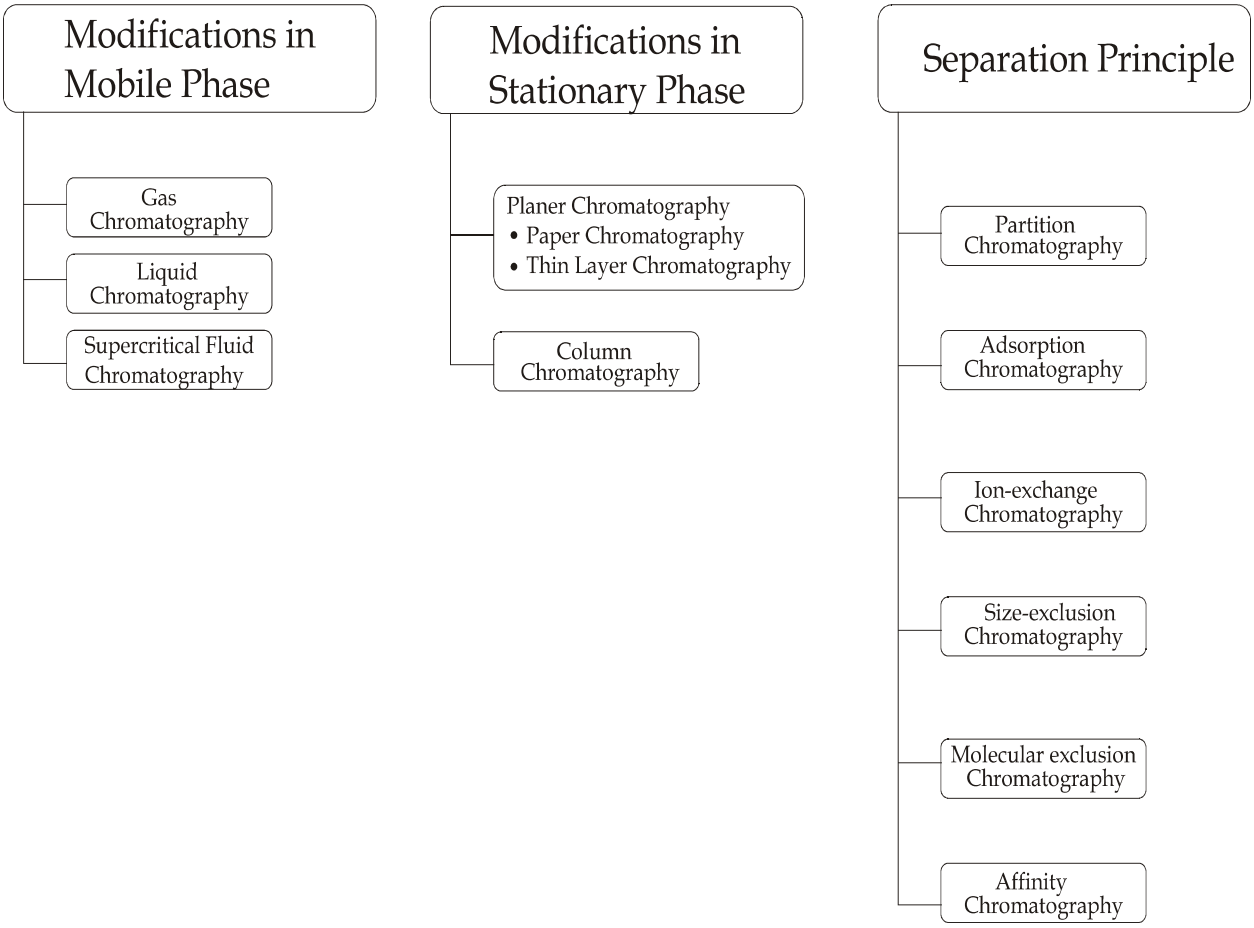

Fig. 1: Classification of chromatography according to modifications in stationary phase, mobile phase and process of separation.

Determination of volatile analytes in biological and non-biological samples is one of the most frequently done assay in a Forensic Toxicology Laboratories[12–14]. Routine analysis of volatiles has been done using titrimetric analysis, immunoassay, enzymatic reactions, chemical reactions, spectrophotometry and gas chromatography[15–19]. Due to various disadvantages in primitive techniques like lack of sensitivity, specificity, large reaction time, long sample preparation and improvement in the instrumentation of preexisting techniques. Sophisticated instruments like Gas Chromatography-Mass spectroscopy, NMR-Spectroscopy, Raman-spectroscopy, FTIR, Biosensors, Microdiffusion techniques have gained more acceptance nowadays[20–25].

Gas Chromatography is the most frequently used analytical technique for investigation of thermally stable organic volatile compounds or hydrocarbons within a matrix. GC is basically a separation technique in which the introduced volatile compounds get separated, fractionated by means of Selective Interaction (Partitioning) as a consequence of partition between a mobile gaseous phase and a stationary phase held inside a Stainless-Steel/ Quartz column. The components of a mixture possess different affinities for each phase, which causes the differential separation.

Samples in Forensic Toxicology can contain highly dense matrix with proteins, salts, fats, oils, drugs and other non-volatile material mixed with the target analyte that can remain in the GC system and result in poor analytical performance. Most samples need to be modified for the specific requirement of analysis of a particular analytical technique before injection. Therefore, laboratory analysts use extensive sample-preparation techniques to extract and concentrate the compounds of interest from this unwanted non-volatile matrix. Most of these cleanup procedures use some type of initial extraction procedure such as solvent extraction, solid-phase extraction, solid-phase micro extraction,

supercritical fluid extraction, distillation[26–28]. Such extraction and concentration techniques can become time consuming and costly depending on extend of extraction from sample. While the actual sample actual time to perform an analysis has been reduced with the improvement in instrumentation, sample preparation still is a time-consuming task. With the increase in sample load, more and more labs require automation, particularly in routine analysis.

Gas Chromatography-Headspace is the ideal choice for such operations with reduced cost and time of analysis. Static-Headspace sampling is an excellent technique for quantitative and qualitative analysis of volatile compounds that can be efficiently partitioned into the headspace gas volume from either solid, liquid or gaseous matrix. Direct Manual Injection and Headspace sampling are the chiefly used sample introduction techniques in GC but Headspace sampling has its advantage of automation, sensitivity, accuracy, less chances of alteration in sample preparation thereby reducing the sample error[29,30]. GC and GC-HS systems commercially available in many different models from different manufactures are illustrated in Fig. 2.

Fig. 2: GC/GC-HS from various manufactures A) PerkinElmer, B) Finnigan, C) Shimadzu, D) Chemito, E) Varian, F) ThermoFischer, G) Agilent, F) HP (Image source: Toxicology Lab, Deptt of FMT, AIIMS and Google images).

Principle of Gas Chromatography-Headspace (GC-HS)

Gas Chromatography-Headspace is a combination of two systems, a Headspace sampler for sample introduction and a Chromatography system for analyte separation and detection.

For analysis in GC, before getting injected in the inlet, the sample is changed to its volatile form. This is done by heating the sample in a sealed glass vial from the oven programmed at a stable temperature. At equilibrium this causes the volatiles from the sample matrix to get vaporized according to their boiling points and get concentrated in the neck region of the glass vial. The vapors are then extracted using an automated needle through the septa of the vial and introduced in the column for separation. Individual analytes then get separated according to the affinities for each phase in the column and get deferentially separated. Time taken within the sample injection and the emergence of individual peak is known as retention time, whereas the observed respective area/ height is propionate to the concentration. In order for correct separation and detection of the target analyte both the test and standard sample should be run under the same conditions of analysis.

Theory of Headspace

The term 'Headspace' is analytically associated with the vapour phase of a matrix, either solid or liquid sealed within a container. For analysis in gas chromatography, if the compound of interest is volatile mixed in solid or liquid matrix, the best way would be to examine the concentration of these analytes in the gas phase above the matrix in a closed container. This is done, either by taking the sample directly from the gas phase or trapping and concentrating the gas prior to analysis[31]. Headspace analysis reduces cost and time of analysis by directly sampling the volatile from the container in which the sample is placed to GC system.

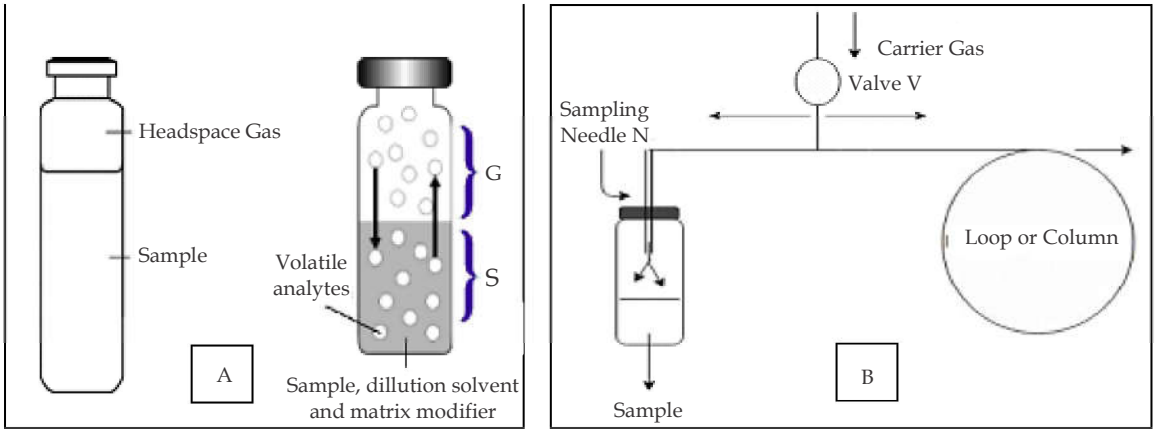

Fig. 3: A) Headspace vial and its components, B) Schematic diagram of Headspace analysis (Image Source: Analyticsshop.com).

The gas phase (G) is commonly referred to as the headspace and lies above the condensed sample phase in a sealed glass vial. The sample phase (S) contains the compound of interest (Fig. 3A). Once the sample phase is introduced into the vial and the vial is sealed, temperature provided to the sealed vial, diffuses the volatile components into the gas phase until the headspace has reached a state of equilibrium[32]. Inert carrier gas (Nitrogen/ Argon) enters the gas chromatograph through valve 'V' and branches before the column, part of the gas is directed to the sampling needle 'N' and rest to the column. When this sampling needle penetrates the septum, carrier gas flows into the vial and pressurizes it. Sample transfer is then done by closing this valve for a few seconds thus disconnecting the gas supply (Fig. 3B)[33]. The loop then disconnects from the valve to get connected to the 'Transfer line' from which the vapours within the vial are transferred to the column.The vial remains sealed inside the chamber until an aliquot is withdrawn for analysis, thus guaranteeing sample integrity.

Theory of Gas Chromatography

Gas chromatography is one of the most widely used techniques for analyzing hydrocarbon mixtures that can be vaporized without decomposition. It utilises a gaseous inert mobile phase to transport sample components through either packed columns or hollow capillary columns containing a polymeric liquid stationary phase. 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[10–15]. Some of the advantages of chromatography are the Dynamic range of measurement, the detection of a wide range of components in mixtures and standards, and the repeatability of the measurements. Like for all other column chromatographic techniques, a mobile and a stationary phase incorporated in a column are required for this technique. The mobile phase (carrier gas) is comprised of any of the inert gases i.e., Helium, Argon, or Nitrogen. The stationary phase consists of a packed column in which the packing or solid support itself acts as stationary phase, or is coated with the liquid stationary phase (high boiling polymer). Most analytical gas chromatographs use capillary columns, where the stationary phase coats the walls of a small-diameter tube directly (i.e. 0.25µm film in a 0.32mm tube)[34].

The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase and mobile phase. The stronger the interaction is, the longer the compound interacts with the stationary phase, and the more time it takes to migrate through the column or elute. The most common type of sample introduction injection port consists of a rubber septum through which a syringe needle is inserted to inject the sample (5 µl-25 µl). The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture to ensure that the whole sample will be vaporized. Since the partitioning behaviour is dependent on temperature as well as the different interaction of each component with the stationary phase coated on the column, the column is usually contained in a thermostat-controlled oven. Starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components accomplishes the separation of components with a wide range of boiling points. As the components exit the column they pass through a detector that generates a response that is registered as a deflection in the baseline in form of peaks[35]. Peak height and peak area are used to identify the compound quantitatively.

Instrumentation of Gas Chromatography-Headspace

Gas Chromatograph is a combination of carrier gas system, a sampling system, a separation system, a detection system and a data recording system (Fig. 4). These parts of a basic chromatograph have remained unchanged all through the years, with technological advancement only in design, material and methodologies.

Fig. 4: Parts of a basic Chromatographic system.

a. Carrier Gas System

The carrier gas system consists of carrier gas source, its purification panel and gas flow control. The purification panel of gases is composed of Hydrocarbon traps, Oxy traps and Moisture traps for trapping impurities in gas before introduction in the instrument as shown in Fig. 5. Helium, Nitrogen, and Argon are inert gases and frequently used as mobile phase in Gas Chromatography[36]. Use of these carrier gas in a methodology, is dependent upon type of detector used, for example detectors like Discharge Ionization Detection (DID) requires Helium as an carrier gas[37].

When analysing a volatile or gaseous sample, matrix is purged with these inert gases as they do not show in the response of detector. Nature of gas used is also significant in type of analytes, for example an analysis with poor resolution of peaks would be better analysed with a slow velocity gas like Helium, compared to Nitrogen. Also, safety and availability of gases are another factor deciding the use of gas, for example Argon and Helium are more costly than Nitrogen.

The flow rates of carrier gas effect the separation of analytes same ways as temperature. Higherflow rate increases the run time but decreases the resolution of analytes. Selection of flow rate is therefore dependent upon the level of separation and the length of analysis in the column[38].

b. Sampling System

Sampling and sample preparation majorly impact the integrity of GC analysis of forensic samples, especially when dealing with trace and ultra- trace levels of the target analyte(s) present in various complex matrices (e.g., biological, environmental,

Fig. 5: Gas traps for introduction of Nitrogen, Zero Air and Hydrogen in GC System (Image Source: Toxicology Lab, Deptt of FMT, AIIMS).

fire debris, and explosive residues). In addition, in the majority of cases, the volume of available samples to the forensic investigators is limited. Therefore, a valid sampling and sample preparation strategy should be adopted prior to beginning the analytical process in order to ensure that there is minimum sample loss and theanalysed samples are truly representative of the evidence matrix. Due to the complex and incompatible nature of the sample matrix where the analyte(s) of interest are present, most often, forensic samples cannot be introduced directly into the GC inlet. This incompatibility stems from two factors. First, the complex sample matrix, if introduced directly into the GC inlet without employing any sample treatment/cleanup procedure, may exert a detrimental impact on the performance of the GC by contaminating the inlet with residue, as well as by compromising the sensitive stationary phase of the GC column. Second, if the concentration of the target analyte in the sample matrix is very low so that it may fall below the detection limit of the GC, no usable chromatographic data would be generated (noise). Since every forensic case is unique, standardization of the sampling and sample preparation techniques for the forensic samples is necessary and often dependent upon the knowledge, experience, published literature and judgment of the chemist.

Sample preparation techniques frequently employed in processing forensic samples prior to GC analysis include Solvent Extraction, Solid-Phase Extraction (SPE), Purge and Trap, Liquid-Liquid Extraction (LLE), Supercritical Fluid Extraction (SFE), Steam Distillation, Accelerated Solvent Extraction (ASE), Microwave-Assisted Extraction (MAE), Solid-Phase Micro Extraction (SPME), Liquid-Phase Micro Extraction (LPME), Stir Bar Sorptive Extraction (SBSE), Solid-Phase Dynamic Extraction (SPDE), etc.[26,27,39].

Samples to be run in Gas Chromatography can be changed to gaseous or liquid state by dissolving them in appropriate volatile solvent. These can then be introduced in the inlet by a microliter volume syringe needle through a self-sealing septum consisting of thermally stable silicon rubber. The discrepancies associated with manual sampling in GC has led to higher sample injection techniques like Auto Liquid Sampling and Headspace Sampling as shown in Fig. 6.

Manual Injection

Manual injection is the biggest cause in variation of quantification when multiple injections and operators are compared. For manual injection, liquid sample is aspirated into the syringe by

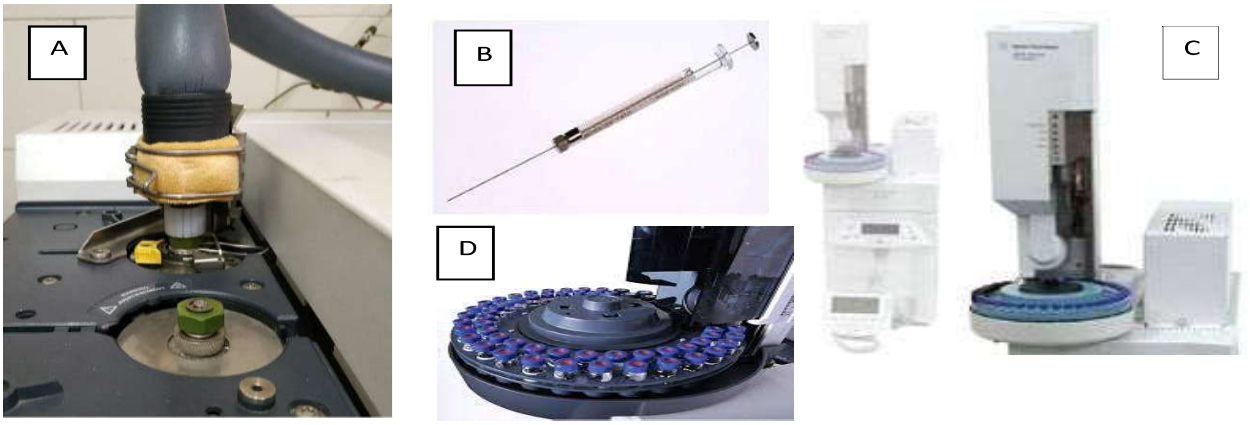

Fig. 6: Types of injection mode in GC systems A) Manual and Transfer line, B) 25µl Microliter Syringe, C) Auto Liquid Sampler, D) Carousel for autosampler vials (Image source: Toxicology Lab, Deptt of FMT, AIIMS and Google images).

withdrawing the plunger and ensuring no air bubbles in the cavity. Needle is immediately inserted in the inlet and plunger is pushed to its full depth. Needle is withdrawn after few seconds of injection.

Syringe injections are inherent to a number of problems causing reduced sample repeatability. Vaporisation of sample in needle before plunger is depressed, is the major problem in manual injection. Disproportionation of sample injection can occur when plunger is not fully depressed and some volume of sample is retained in the cavity of syringe[40]. This may cause considerable error in quantification. Syringe handling should be consistent in sample requiring low volumes of analysis or higher level of precision.

Autosampler

The autosampler provides the means to introduce a fixed volume sample automatically into the inlets, this technique is more effective and more reliable when compared by doing by hand. Automatic insertion provides better reproducibility and time-optimization. Different kinds of autosamplers exist. Autosamplers can be used anywhere when attached with GC system like forensics, environmental science, clinical setup, pharmaceutical and food and beverage industry.

Headspace Sampler

Allows introduction of volatile compounds from virtually any matrix directly into GC or GC/MS instrument. This is done by heating the sample in a sealed glass vial stored at equilibrium from the oven programmed at a stable temperature. At equilibrium this causes the volatiles from the sample matrix to get vaporized according to their boiling points and get concentrated in the neck region of the glass vial. The vapors are then extracted using an automated needle through the septa of the vial and introduced in the column for separation. Individual analytes then get separated according to the affinities for each phase in the column and get deferentially separated.

Methods of Headspace Sampling

Following three types of sample injection (Syringe injection, balanced pressure, and pressurized loop) are commonly used in Static Headspace.

1. Syringe Injection

It is the most commonly used and reproducible sample injection method of headspace sampling. Here the syringe is heated and agitated in oven for a predefined period of time. The heated syringe then removes an aliquot of the headspace and directly injects it directly into the GC (Fig. 7). The syringe

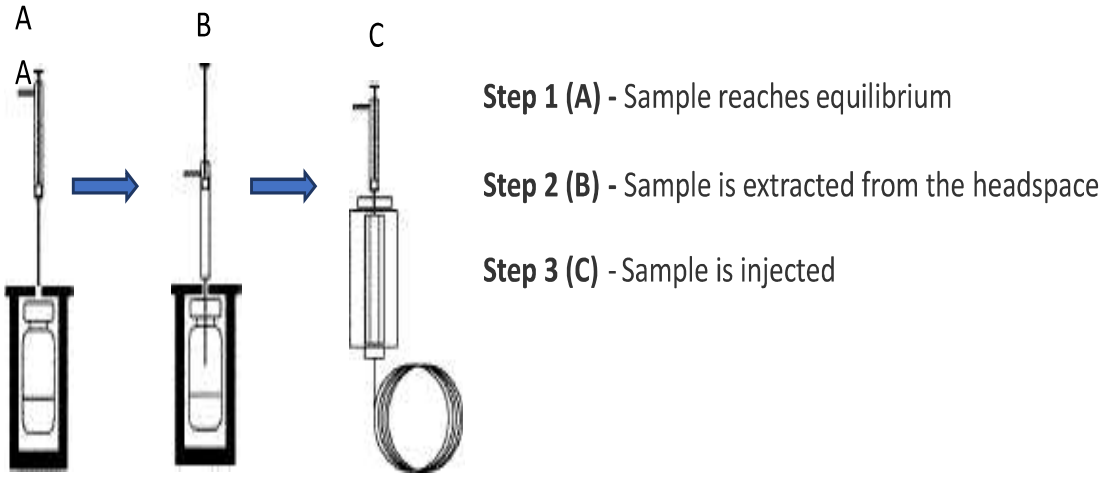

Fig. 7: Syringe Injection Technique (Image source: Analyticsshop.com).

must be heated few degrees above the temperature of the oven to avoid the risk of condensation and hence carry-over from one sample to the next. After injection, the syringe is flushed with nitrogen or carrier gas. This type of system having following advantages

1. Very high level of reproducibility
2. Low carry-over
3. Fast transfer of sample to GC/GLC
4. Precise control of sample syringe for sample size and injection speed
5. Easy to clean syringe
6. GC injection port is always free for manual samples
7. Many syringe auto samplers can be retrofitted onto existing GC systems.

2. *Balanced Pressure*

This technique uses a seamless injection directly from the vial into the carrier gas stream without moving parts other than a valve and a needle (Fig. 8). The balanced pressure technique, like other techniques, uses an incubation oven to thermostat the vial so the sample reaches equilibrium in a closed environment. During these initial steps, a needle is inserted into the vial and is then pressurized with a carried gas. After the vial is pressurized and equilibrium has been reached, the valve is switched on for a specific amount of time to redirect the sample into the transfer line and onto the column. In this technique the absolute volume of the sample injected is unknown. This technique can be quite accurate but can also suffer from disadvantages such as:

1. These negative aspects include sample carry-over
2. The injection port is always occupied and therefore not available for manual use.
3. Quantification becomes difficult.

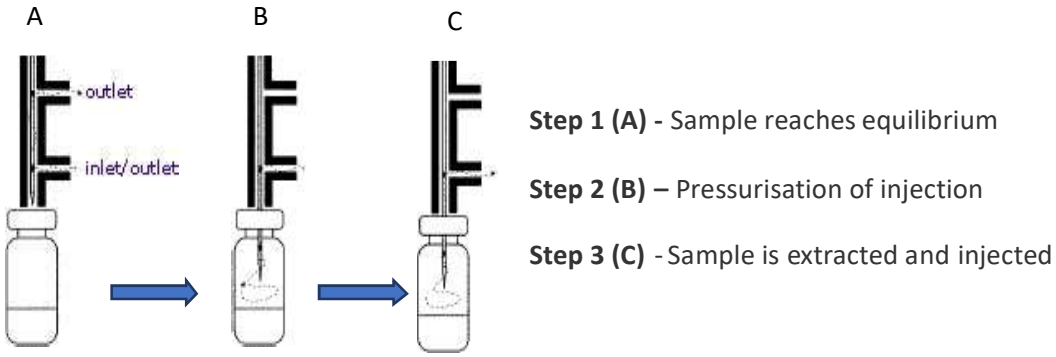

Fig. 8: Balanced Pressure Technique (Image source: Analticsshop.com).

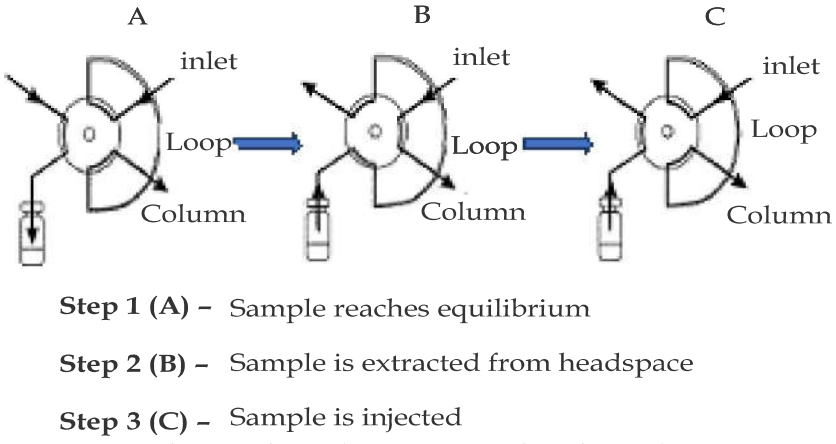

Fig. 9: Pressurised Loop Technique (Image source: Analyticsshop.com).

3. Pressurized loop technique

The pressurized loop system uses a known amount of sample. This technique typically uses a six-port valve, which thermostats and pressurizes the vial as in the previously described sample injection techniques. After pressurization, the valve is turnedand the loop is filled with the sample. Once the loop has been filled, the valve is turned again to redirect the gas flow and flush the sample into the transfer line leading to the column (Fig. 9). This type of system allows high temperatures to be used but it also suffers from the same disadvantages as the balanced pressure system such as sample carry over and the injection port is always occupied.

c. Column

A column is the heart of Gas Chromatography, because the components of the mixture are separated in it by the virtue of different interaction with the column packing. The column is chosen according to the polarity of the sample for maximum separation. The rate at which compounds move through the column depends on the nature and strength of the interaction between the analyte and the stationary phase. The column contains the stationary phase coated on an inert solid support. GC columns are of two types-Packed and Capillary[34].

Packed Column

Packed columns are usually made of Stainless Steel or Copper Tubing. Diameter of glass tube or metal is ¼" or 0.25 in with lengths ranging from 5-50feet. Short length columns are straight and installed vertically in the thermostat whereas longer columns are U-shaped but columns with over 1meter length are coiled.

Capillary Column

Capillary columns are also known as Open Tubular Column which are generally fabricated form Stainless Steel or Quartz. Its diameter is 1/16 inches or less with length ranging up to 200-300 mts.

As the analytes are carried to the column from the injection port they interact with the stationary phase and are retained. Components that interact more strongly with the stationary phase spend proportionally less time in the mobile phase and therefore move through the column more slowly. Other variables that affect Retention time are column temperature and carrier gas flow rate. Long elution times in experimentation should be avoided as they not only waste valuable resources but broadening of the peaks and loss of resolution will become evident when the elution times are too long. Thus the optimum conditions are those that result in complete separation of the peaks in the shortest possible time[41].

d. Detector

After separation analytes elute from the column, they interact with the detector. The detector converts this interaction into an electronic signal that is sent to the data system for representation in a readable form. The magnitude of the signal (mA) is plotted versus time (min), from the time of injection and a chromatogram is generated. Some detectors respond to any analyte eluting from the column while others respond only to analytes with specific structures, functional groups or atoms. Detectors that exhibit enhanced response to specific types of analytes are called Selective Detectors.

Variety of detectors may be employed for the detection, quantification, and/or identification of the analyte(s) which include Flame Ionization Detector (FID), Nitrogen Phosphorus Detector (NPD), Sulfur and Nitrogen Chemiluminescence Detector, Flame Photometric Detector (FPD), Atomic Emission Detector (AED), Thermal Energy Analyzer (TEA), Electron Capture Detector (ECD), Ion Mobility Mass Spectrometry (IMMS), Time-of-Flight Mass Spectrometry (TOFMS), and Isotope Ratio Mass Spectrometry (IRMS). However, the most popular is the Mass Spectrometer (MS) attached to GC as it offers both identification and quantification of an unknown substance with high confidence. In some cases, MS in tandem with another MS is also used as the detector.

General requirements of any detector are: high sensitivity; physically suitable; capable of operating up to maximum column temperature; ease of operation; no response to undesirable compounds; linear response exceeding to high concentrations. Based on these physical properties there are several detectors available[33,42,43].

1. Flame Ionisation Detector (FID)

FID is the most commonly used detector with main use for the detection of hydrocarbons or carbon containing compounds. It uses an air/ hydrogen flame to pyrolyze the effluent sample. The pyrolysis of the carbon containing compounds in the flame creates ions. A voltage is applied across the flame and the resulting flow of ions is detected

as a current (mA). Sensitivity of this type detector can range between 0.1-01ng.

2. Electron Capture Detector (ECD)

ECD detector are more suited for polyhalogenated organic compounds. It uses a beta emitter such as radioactive Tritium or Nickel and uses it to ionise the carrier gas. Fast beta particles generated by the radioactive source collide with the molecules of the carrier gas. Electronegative compounds capture electrons generated resulting in a reduction in the current. The amount of current loss is indirectly measured and a signal generated is displayed. Sensitivity of this type of detector can range between 0.1-10pg for halogenated compounds, 1-100pg for nitrates and 0.1-1ng for carbonyls.

3. Thermal Conductivity Detector (TCD)

TCD detectors are based on change in the thermal conductivity of the gas stream. It is universal detector with the detection of air, hydrogen, carbon monoxide, nitrogen, sulphur dioxide, inorganic gases and many other compounds. The difference in the thermal conductivity between the column effluent flow and the reference flow of the carrier gasproduces a voltage signal proportional to this difference. The signal is proportionate to the concentration of the sample components. The sensitivity of this detector can range from 5-20ng.

4. Flame Photometric Detector (FPD)

FPD detectors are based upon the luminous emission from a hydrogen rich flame in the presence of compounds containing either Sulphur (394nm) or Phosphorus (526nm). It consists of a hydrogen air burner and a photomultiplier. The detector is very specific with the detection based on type of optical filter used. The sensitivity of the detector can range from 10-100pg for Sulphur containing compounds and 1-10pg for Phosphorus containing compounds.

5. Photo Ionisation Detector (PID)

Typical photoionization detectors measure organic volatiles and other gases. Organic compounds eluting into a cell are bombarded with high energy photons emitted from a lamp. Compounds with ionization potentials below the photon energy are ionized. The resulting ions are attracted to an electrode, measured, and a signal is generated. The PID is used mostly to detect VOCs in soil, sediment, air and water. It is often used to detect contaminants in ambient air and soil during drilling activities and during spills to identify potential problems.

6. Electrolytic Conductivity Detector (ELCD)

ELCD Detectors are selective to Halogens, Sulphur and Nitrogen containing compounds. Compounds are mixed with a reaction gas (Hydrogen) and passed through a high temperature reaction tube. Specific reaction products are created which mix with a solvent and pass through an electrolytic conductivity cell. The change in the electrolytic conductivity of the solvent is measured and a signal is generated. Reaction tube temperature and solvent determine which types of compounds are detected. The sensitivity of the detectors can range from 5-10pg for halogens, 10-20pg for sulphur containing compounds and 10-20pg for nitrogen containing compounds.

7. Helium Ionisation Detector (HID)[44]

HID Detectors are a universal detector responding to all molecules except Neon. Detector uses Helium as both carrier gas and the ionization gas. HID uses ion detector which uses a radioactive source, typically β-emitters to create metastable helium species. The metastable Helium species have an energy of up to 19.8eV. These metastable helium species can then ionize all compounds with the exception of neon which has a bigger ionization potential of 21.56eV. As components elute from the column they collide with the metastable helium ions, which then ionize the individual components. The ions produce an electric current, which is the signal output of the detector. The greater the concentration of the component, the more ions are produced, and the greater the current. The drawback to HIDs are that they contain a radioactive source. Discharge ionization detectors have generally supplanted them.

8. Argon Ionisation Detector (AI)[43,45]

AI Detectors are based upon the production of metastable Argon atoms which are used to ionise the sample, which is held in a carrier gas. The produced electrons are focused towards the collector electrode and produced current is measured.

9. Mass Spectrophotometer (MS)

Mass Spectrophotometer can be used to detect the compound even in the absence of a certified standard. Complex mixtures can be separated

using an LC or GC system attached or simply injected in the inlet of MS. Ionised samples are bombarded with electrons (EI) or gas molecules (CI). Compounds fragment into characteristic charged ions or fragments. The resulting ions are focused and accelerated into a mass filter. The mass filter selectively allows all ions of a specific mass, as decided by the software, to pass through to the electron multiplier. All of the ions of the specific mass are detected. The mass filter then allows the next mass to pass through while excluding all others. The mass filter scans stepwise through the designated range of masses several times per second. The total number of ions are counted for each scan. The abundance or number of ions per scan is plotted versus time to obtain the chromatogram. A mass spectrum is obtained for each scan which plots the various ion masses versus their abundance or number. The detector is maintained under constant vacuum. The detector can be sensitive within a arrange of 1-10ng for full scan, 1-10pg for selective scan.

Different Accessories used in Operation of GC-HS

a. Headspace Vials

It is a small container, typically cylindrical and made of glass, headspace vials are available in 6, 10, 20 and 22 ml sizes (Fig. 10A).

b. Crimp

It is a kind of metallic cap used to provide consistently secure seals to the sample vials, along with the septa (Fig. 10B).

c. Septa

It is a circular membrane, generally white in colour, used to cap the sample vial to prevent any form of leakage (Fig. 10C). Most commonly used material for septa is Polytetrafluoroethylene (PTFE).

d. Crimper

A crimper is a tool to conjoin septa and crimp, using compressive force to constrict the edges around the neck of vial. This helps in proper sealing of the

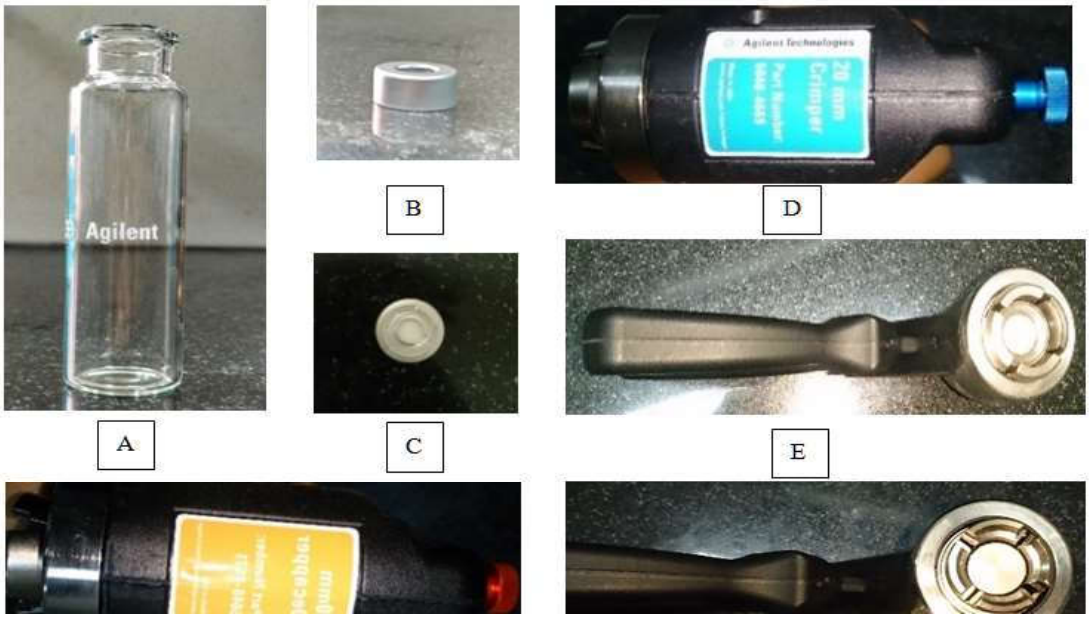

Fig. 10: Accessories of Gas Chromatography Headspace: A) 20ml GC-HS Vial, B) Metallic Crimp, C) PTFE Septa, D) Top View Crimper, E) Bottom view Crimper, F) Top View Decrimper, G) Bottom view Decrimper (Image source: Toxicology Lab, Deptt of FMT, AIIMS).

crimp on the sample vial (Figure 10D, E). They are generally colour coded with blue knobs and label.

e. Gas Traps

- Gas traps are external devices attached to the GC systems to remove the detrimental impurities from the carrier and the detector gases.
- Gas Clean Filter System or gas traps delivers clean gases, reducing the risks of column damage, sensitivity loss, and instrument downtime.
- Inserting a Gas Clean Filter System in the gas line immediately before the instrument inlet greatly reduces the level of impurities, thus improving trace analysis.
- The most commonly used traps in the GC systems are Moisture, Oxygen, and Hydrocarbon traps; however sometimes nitrogen and hydrogen traps are also used.
- Reducing impurity level can prolong column life and may improve sensitivity. The effectiveness of the traps depends on the initial quality of the gas.
- Little enhancement by traps to the GC system is obtained by traps when using very high purity gases (e.g., ultra-high purity or similar grades) while obvious improvement is obtained with lower grades of gas. Traps may provide some protection if there is a leak at or around the gas cylinder.

Different Consumables used for mantainence of GC-HS

a. FID Jet

The most common detector used in Gas Chromatography is the Flame Ionisation Detector (FID) is connected through FID jet. It measures the concentration of organic substances passing through the gas stream (Fig. 11A). After certain analysis it should be replaced by new one.

b. Column Inlet Nut

Column inlet nut is a very tiny installation part of the GC column, which should be very precisely placed for accurate and reproducible results. Manufacturers offers a selection of capillary column nuts for GC fittings to facilitate good column installation (Fig. 11B). Column nuts help prevent leakage as it strongly tightens the inlet and outlet portions with the column.

c. Autosampler Syringe (if injection is done without HS)

1 mL, 2.5 mL and 5 mL syringes are available for headspace. Syringes are consumables and need to be replaced frequently (Fig. 11C). In a gold standard autosampler syringe, the upper portion of the tapered needle offers the strength of a 23-gauge, while the lower portion at 26 s-gauge enables use with split/splitless or on-column injections with 0.53 mm id columns.

d. Inlet Septa

The general-purpose inlet septa are made from an enhanced injection-moulded silicone rubber material (Fig. 11D). The septa material, dark red or grey in colour, is specified to withstand over 200 automatic injections at an injection port temperature of 350°C.

e. Capillary Column

Capillary columns (Fig. 11E) show a higher degree of resolution because of its longer column, about 80-100 feet, and a narrow width of about 250 µm. Capillary columns are made of purified silicate glass, and have the inner surface coated with the stationary phase. Sufficient number of capillary columns should be in hand for different applications

f. Ferrule

Ferrule is a part of the GC column, which maintains a leak-free connection between the column and the injector. It is available in a wide range of materials and configurations (Fig. 11F). For instance, graphite ferrules can withstand temperatures as high as 450°C.

g. GC Inlet Liner

An injection port liner is used to make the connection between sample introduction and the GC column (Fig. 11G). Four primary injection techniques are used in GC; split, splitless, direct, and on-column.

Fig. 11: Different consumables used formentainence of GC-HS: A) FID Jet, B) Column Inlet Nut, C) Inlet Septa, D) Inlet Liner, E) Capillary Column, F) Ferrule (Image source: Google images).

Inlet liners are selected based on the injection technique being used to ensure optimal sample transfer to the column.

f. O-Ring

O–rings are used to seal the liners in the inlet (Fig. 11H). They are easy to use and remove, and help in eliminating out-gassing of contaminants. Graphite O-rings can be used when operating inlet temperatures exceed 350°C.

System Optimization for Gas Chromatography-Headspace Analysis

Chromatographic performance of a sample is greatly influenced by how it is introduced into the analytical column. Variables that affect sample preparation and transfer of sample from the headspace unit to the analytical column must be optimized to obtain reproducible and efficient separations. Different steps involved for system optimization are as follows.

a. Preparation of Sample

Samples for HS-GC must be prepared in such a manner as to maximize the concentration of the volatile sample components in the headspace while minimizing the unwanted co-extracts from other compounds in the sample matrix. Few points about sample preparation are as follows:

- Water vapor from the sample matrix also can cause problems by condensing in the transfer line. Incomplete or inefficient transfer of high molecular weight compounds or water vapor from sample matrices can deteriorate the column by producing adsorptive areas in the transfer line or injection port that can lead to split peaks, or irreproducible responses or retention time.
- To minimize matrix problems and prevent water condensation from aqueous samples, use a higher transfer line temperature (~125°C–150°C).
- High concentration can produce ghost peaks in subsequent analyses due to carryover of sample from previous injections.

- Sample carryover can be minimized by using higher transfer line and injection port temperatures, but some samples may need to be diluted and reanalyzed to obtain reliable results.

b. Selection of Sample Vial

Sample vial should be selected to match the type and size of the sample being analyzed. Few points about selection of sample vial are:

- Vials that are not properly cleaned prior to packaging or that absorb contaminants during shipping can produce unknown chromatographic peaks or ghost peaks. Ghost peak that are result of vial contamination can be identified by running method blanks and zero standards during the system calibration sequence.
- The septa used to seal the headspace of the vials also can be the source of the contaminants, which can bleed into the headspace of the vials during equilibration.
- Vials for sample and standard should be same.

c. Sample Vial Heater and Mixer

Once the sample is placed inside a clean, dry, sterile vial and the vial is sealed with septa, volatile compounds from the sample will partition into the headspace until the state of equilibrium is reached. Few points about sample vial heater and mixer are:

- Temperature, time, and mixing can improve the transfer of volatile analytes from the sample into the headspace of the vial.
- Sufficient time must be built into the sample cycle in order to achieve a constant state of equilibrium

d. Sampling

There are several techniques used to transfer samples from the vial to GC. Few points about sampling are:

- When using a gas-tight syringe for sampling, heat the syringe to a temperature comparable to the sample vial temperature. This minimizes pressure differences and condensation problems. Ensure the septum is well maintained to decrease the possibility of leak.
- For balanced-pressure sampling instruments, analyst should consider the inertness and efficiency of the components that make the sample pathway inside the auto sampler. If sensitive compounds are being analyzed, an inert pathway should pathway should be used to decrease possible adsorption.
- Analyst should ensure that balanced-pressure instruments are leak free and operate with the least amount of dead volume in the sample flow path. This will help optimal peak shape and sensitivity.
- When using pressure-loop sampling instrument, inert sample pathways and low dead volume systems will yield the best chromatographic performance.
- If low response or broad peaks are observed, it may be necessary to increase the sample vial pressure to ensure that the sample loop is completely filled with the headspace sample.
- If there are extraneous peaks present due to carryover of matrix contaminants, increase the sample valve temperature to prevent sample carryover,condensation and contamination.

e. Transfer Line

After the headspace sample is withdrawn from the vial, it is ready to be transferred to the GC/GLC. In balanced-pressure and pressure loop systems a short piece of tubing called transfer line is used to transfer the sample from the autosampler to the GC/GLC.

- The internal diameter of the transfer line should be chosen depending upon the internal diameter of the analytical column, the column flow rate and the flow rate delivered from the autosampler.
- Transfer line should be set depending on the analyst of interest and the sample matrix.
- A typical transfer line temperature ranges from 80°C to 125°C. To minimize matrix problems and prevent water condensation from aqueous samples, use a higher transfer line temperature (~125°C–150°C).

f. Injection Port Interface

The quality of the connection of the transfer line to the analytical column greatly affects the analyte separation. In most cases, the transfer line has a smaller internal diameter than the injection port liner and the vaporized headspace sample carrying the compounds of interest will be diluted into a larger volume of carrier gas when the sample elutes from the transfer line into the inlet liner. This can lead to broader peaks, tailing peaks, lower

sensitivity, and loss of resolution. Few points about injection port selection are:

- Using injection port liners that have smaller internal diameters and lower buffer volumes will help maintain a narrow bandwidth as samples move from the end of the transfer line to the head of the analytical column.
- If the band-broadening due to excess dead volume in the system is still a problem, peak shape may be improved by refocusing sample analytes at the analytical column head.
- Highly volatile compounds can be trapped at the column head and refocused into a narrow bandwidth by reducing the initial oven temperature below the boiling point of the compound of interest.

g. Derivatization Technique

Derivatization is another technique that can be used to increase sensitivity, chromatographic performance and enable detection for specific noncompatible compounds. Few points about derivatization technique are:

- Compounds such as acids, alcohols and amines are difficult to analyze because of the presence of reactive hydrogen. When attempting to analyze these types of compounds, they can react with the surface of the injection port or the analytical column and result in reduced detector performance in shape of tailing peaks and low response. In addition, they may be highly soluble in the sample phase, causing very poor partitioning into the headspace and low response. Derivatization of these compounds can improve their volatility, as well as reduce the potential for surface adsorption once they enter the GC system.
- Common derivatization techniques used in reaction headspace/GC are esterification, acetylation, silylation, and alkylation.
- Derivatization reagents, as well as the by-products from derivatization reactions, may be volatile and can partition into the headspace along with derivatization compounds. These extra volatile compounds may pose problems by eluting with similar retention times as the compounds of interest, causing either partial or complete co-elutions.
- Derivatization reactions are typically run at elevated temperatures than usual vial heating. Pressures inside the sample vial then may exceed the pressure handling capabilities of the vial or the septa. Specially designed caps are available that allow excess pressure to be vented during derivatization reactions. Use of the correct and compatible vial, cap and septa is important.

Different Factors which Affect the Sensitivity of GC-HS

a. Influence of the Sample and Temperature on Headspace Sensitivity

- The sample volume (Vs) is included in the phase ratio (β) but its influence on the headspace sensitivity is not independent of the partition coefficient (K).
- The latter can vary widely from practically zero in the case of gas sample up to several thousands, where the applicability of HS-GC ends.
- The phase ratio (β) and thus the influence of the sample volume does not generally span such a wide range. For example, 1mL sample in a 10mL vial has a phase ratio of 9, while with a sample volume of 5mL the phase ratio decreases to 1.
- This causes an increase in the resulting gas concentration, and thus on the resulting peak area, depends mainly on the partition coefficient (K>100) e.g. ethanol in water a change in the phase ratio from 1 to 5 will barely influence the headspace sensitivity in contrast where the partition coefficient is very small the sensitivity increases in proportion to the sample volume .
- The vapor pressure of a compound increases exponentially with temperature.
- However, there is a dependence on the partition coefficient. In case of non-volatile compound ($K \rightarrow \infty$) a higher temperature will not alter its non-volatility.
- In the case of a highly volatile compound ($K \rightarrow 0$ at room temperature) the temperature will not affect the headspace sensitivity either, because in this case nearly all the compounds are already present in the gaseous phase.

b. Sensitivity enhancement by Matrix modification

The partition coefficient can be altered by modifying the sample matrix.

- A common technique is the use of the salting-out effect. For aqueous samples with high

partition coefficient (ethanol in water) the addition of salt may enhance the sensitivity by up to a factor of 10.

- The result depends upon the value of the partition coefficient.
- In the case of a highly volatile compound (K→0) where nearly all of the analyte is already present in the gas phase, the sensitivity will not improve.
- A similar effect is achieved with a sample containing a non-polar volatile compound dissolved in water miscible organic solvent such as dimethylacetamide, dimethylformamide, etc.
- If water is added to this solution, the solubility of the non-polar compound will decrease and its volatility will increase.
- Common salts such as Sodium Sulphate, Sodium chloride, Sodium citrate, Potassium carbonate, Ammonium Sulphate, and Ammonium chloride decreases matrix effect.

c. Sensitivity Enhancement by modifying the volatile analyte

- Polar compounds particularly those with active hydrogen such as alcohols, phenols, acids, amines etc. usually have low volatility as a result of intermolecular interaction with the polar matrix through hydrogen bond formation.
- However, the reactivity of the active hydrogen can be used to prepare less polar derivatives with better volatility and lower solubility.
- Simple derivatization (such as esterification, transesterification, acetylation, etc.) reactions are preferred which are carried out in the headspace vial during the equilibrium time.
- An advantage of GC-HS is that the reaction products are less polar and more volatile thus shifting the equilibrium of the chemical reaction towards completeness.
- Sensitivity is increased when partition coefficient (K) is minimized.
- Sensitivity is increased when phase ratio (β) is minimized.
- Lower K and β results in higher concentration of volatile compounds in gas phase and better sensitivity

d. Headspace Sample

- In addition to working with the partition coefficient, phase ratio, derivatization techniques sensitivity can also be improved by simply increasing the size of the headspace sample that is withdrawn from the sample vial and transferred to the GC.
- Increasing the sample size also means that the amount of time it takes to transfer the sample to the column will increase in proportion to the column volumetric flow rate
- Sample size can be increased only to the point that increases in peak width, caused by longer sample transfer times, will not affect chromatographic separations.
- Larger sample sizes and longer transfer times can be offset by using cryogenic cooling and sample refocusing at the head of the column.

Do's and Don'ts while operating GC-HS

a. *Do's*

- Before starting the instrument, verify the entire gas cylinder. If the primary gas pressure is less than 3psi, replace it.
- Purity of carrier gas should be maintained to prevent degradation of chromatographic hardware.
- Once the system power is on, leave for 1hour to stabilize the baseline if the system is left off for 2 days.
- To minimize matrix problems and prevent water condensation from aqueous samples, use a higher transfer line temperature (~125°C–150°C).
- Inject standards and samples in order from low to high concentrations to help minimize carryover.
- For high concentration samples in a sequence of samples, run a blank after the suspected samples to reduce carryover contamination.
- Handle standards and method blanks the same way samples are handled to make any vial or sample preparation problems easier to identify.
- Always use pre-cleaned vials for sample preparation and storage.
- Septa with a PTFE face should be used to seal the headspace vial to eliminate bleed from the rubber portion of the septa.

- Built sufficient time into the sample cycle to achieve constant state of equilibrium.
- Adjust the temperature of the sample to change the solubility of the analyte as well as to drive the equilibrium in towards the gaseous phase.
- Shaking or vibrating the vial containing high viscosity sample matrices during heating can assist in achieving equilibrium faster.
- Heat the syringe to a temperature comparable to the sample vial temperature to minimise pressure differences, when using gas tight syringes.
- Flush the syringe after each injection to prevent carryover from the inside of the syringe.
- Ensure the septum of the GC injection port is well maintained to decrease the possibility of a leak.
- Increase the oven temperature after the samples are completely transferred to the column to increase the movement of compounds inside the column.
- Use injection port liners of small internal diameters and lower buffer volumes to maintain a narrow bandwidth.
- Use indicating traps closest to the GC to determine when to change the traps that are upstream.
- Indicating traps are not intended to be the primary oxygen removal trap, but should be used in conjunction with a high capacity non-indicating oxygen trap.
- Immediately change the expired oxygen traps to avoid gas contamination, in addition to failing to remove oxygen.
- During cartridge replacement, check valves and close off the system to the atmosphere, further minimizing the entry of contaminants.
- Replace split vent traps approximately every six months.
- GC instrument maintenance should include checking fittings and connections with a gas leak detector.
- Use an on-column syringe when injecting into an on-column inlet so that the injector, syringe and column are not damaged.
- To prevent stationary phase decomposition, the oven and inlet should be at room temperature when not in use and when changing the septum.
- After running the samples, condition the system before shut down.
- Before shutting down the system the inlet, detector and oven should be cooled and temperature condition to 500°C.

b. Don'ts

- Constant exposure of capillary columns to oxygen and moisture should be avoided especially at high temperatures as it may produce rapid and severe column damage.
- Improper handling or installation of plumbing should be avoided as moisture introduced by this can be a common cause of column stationary phase degradation.
- Do not use sample matrices containing high molecular weight compounds to avoid incomplete or inefficient transfer into the GC injection port.
- Transfer line temperature should not be kept low as water from the sample matrix can cause problems by recondensing in the transfer line.
- Avoid using high-concentrated samples lest they produce ghost peaks in subsequent analyses due to carryover of sample from previous injections.
- Do not use unclean or unpacked vials.
- Septa with PTFE face should not be reused.
- Do not use transfer line having smaller internal diameter than the injection port liner to avoid broader peaks, tailing peaks, lower sensitivityand loss of resolution.
- No need to use a large buffer volume in the liner to allow for sample expansion because headspace samples are already in the gaseous state.
- Excess sample analytes should not be used to avoid band-broadening.
- It is not recommended that regulator materials and choice of tubing be interchanged.
- Non-metallic types of tubing such as polyethylene and Teflon are not recommended for GC applications due to their gas permeability and difficulty in cleaning.
- Unclean or improperly cleaned tubing can lead to contamination of the system with disastrous results.
- Never open the GC door if oven temperature is more than 100°C or during running conditions.

- Do not inject air into the vials to prevent the vacuum. This often damages the cap seal.
- Avoid cleaning agents that are alkaline, contain phosphatesor are strongly acidic for syringes.

Application of Gas Chromatography-Headspace in Forensic Toxicology

Forensic science defines scientific principles, tools, and methodologies to resolve legal issues and disputes. Forensic chemists analyse a wide variety of forensic samples, extracting and interpreting information from the chemical and analytical data that may potentially have to withstand rigorous challenge when presented in court. As such, it is imperative that any analytical methodology developed for solving forensic problems should meet, at a bare minimum, the required standard set forth by the scientific community of uniformity and conformity.

Among all analytical instruments currently being used in routine forensic analyses as well as in forensic research, Gas Chromatography-Headspace (GC-HS) is one of the most widely used analytical tool. High sensitivity, selectivity, resolution, speed,accuracy and precision, wide dynamic concentration range, simple and robust instrument design, online and offline monitoring of the equipment and its ability to be interfaced with many established and emerging detection systems have made GC the instrument of choice in many facets of forensic science. As such, new GC instruments (hardware) along with their operating systems (software) are so simple and user friendly that even a novice operator with proper training and induction can operate it with confidence.

Due to the inherent advantages of GC-HS, applications of this reliable analytical instrument in forensic science isa necessary presence. Major application areas in forensic science include bulk seized drug analysis, drug screening from biological specimens, alcohol quantification in drunken driving cases, methanol estimation in illicit alcohol, postmortem toxicology, trace evidence analysis, explosive analysis, analysis of ignitable liquid residues from fire debris, noxious gases analysis in postmortem samples etc.

a. Analysis of Ethyl Alcohol in Driving Under Influence cases from Biological matrix

Ethanol in alcoholic drinks is one of the most widely abused licit drug all over the world. The age limit fixed by the government for the legal consumption of alcohol in India is 21 years. The statutory limit is 30mg% of alcohol in blood under the Motor Vehicle Act, (1988, amended) Section-185, if found driving under influence[46]. Alcohol intoxication associates mainly with road traffic accidents, unruly behaviour, Drug Facilitated Sexual Assault, homicides and suicides[47–50]. Therefore, the estimation of alcohol in blood is very important in medicolegal cases and GC-HS provides a sophisticated system for qualitative and quantitative analysis of alcohol[48,51–53]. In forensic laboratories Static Gas Chromatography-Headspace has become the instrument of choice for this purpose. GC-HS combines both qualitative and quantitative analysis based on detector response of peak height and peak area. Use of Headspace is preferred over manual injection over the convenience of minimised matrix

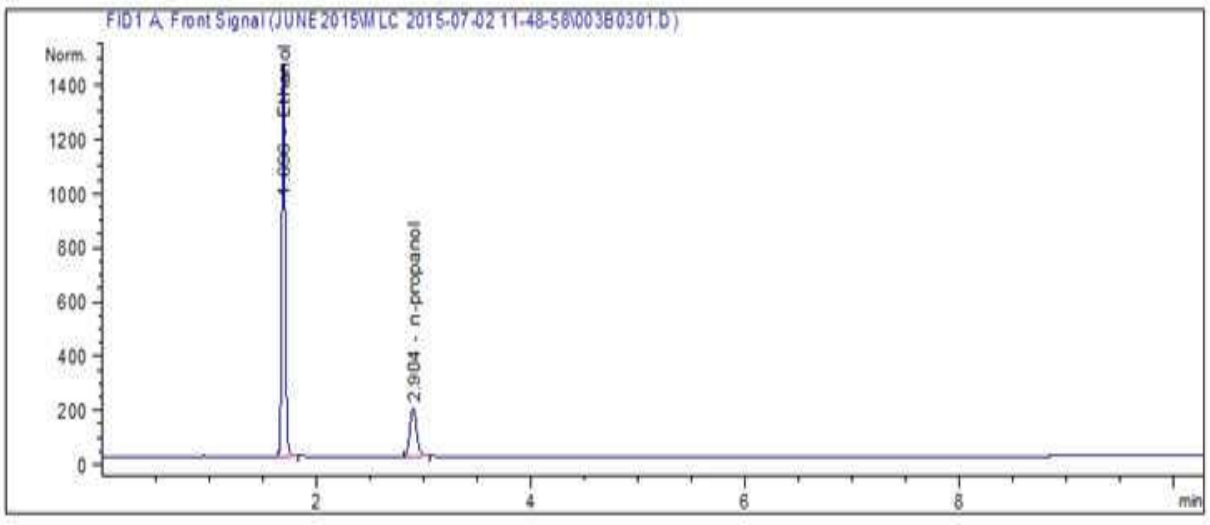

Fig. 12: Chromatogram showing ethanol and internal standard n-propanol in the matrix (Image source: Toxicology lab, Deptt of FMT, AIIMS).

artefacts, contaminants and minimal sample preparation to avoid loss of volatile analyte. Samples such as Blood, Serum, Vitreous Humour, Bile, Muscle, Gastric Content, cerebrospinal fluid, brain tissue can be easily and routinely analysed without any major sample preparation[17,54–56].

Analysis by one such method, developed and validated in the department laboratory is shown in Fig. 12. The methodology is valid for identification of ethanol in biological matrices like blood, vitreous humour with addition of n-propanol as an Internal Standard for quantification[57].

b. Analysis of Methyl Alcohol from Biological and Non-Biological Matrix

Methanol is commonly known as Wood alcohol, wood-naphtha, carbinol, or methylated spirit. It is widely encountered in day-to-day life as a solvent for extraction, antifreeze, fuel, and denaturant to render alcohol (industrial ethanol) unfit for consumption. Methanol is an alcohol, chemically, which is toxic to humans on consumption. Cases related to methanol analysis can be received in an forensic laboratory relating to illicit alcohol, poisoning due to consumption. Majority of cases of poisoning are accidental in nature which happen due to unintentional drinking of industrial ethanol, drinking illicit alcohol or malicious intent of poisoning[58–59]. Blood alcohol testing is one of the most accurate methods for measuring alcohol toxicity in clinical and forensic setting both, as the testing presents the physiopathological chemistry of compound and its metabolites in body. The analysis of blood and other body fluids for alcohol is most commonly performed using "Headspace-Gas Chromatography" due to its simplicity in operation and the number of matrices that can be analysed with one instrument with one method[13,14,30].

Analysis by one such method, developed and validated in the department laboratory is shown in Fig. 13. The methodology is valid for identification of methanol in whole blood with addition of Acetonitrile as an Internal Standard for quantification[60].

c. Analysis of Inhalants

In India, surprisingly a large number of children, young adults and adolescents partake in substance abuse[61,62]. Items such as paints, glues, correction fluid, thinners, nail polish removers, laboratory

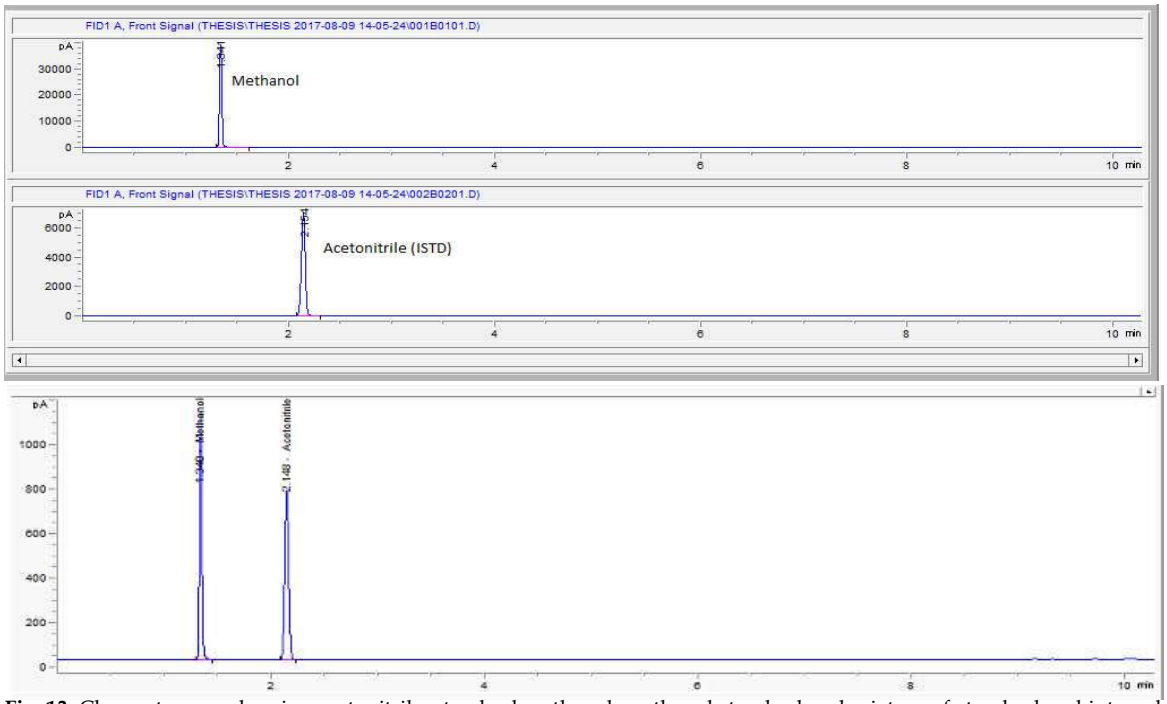

Fig. 13: Chromatogram showing acetonitrile, standard methanol, methanol standard and mixture of standard and internal standard in the matrix (Image source: Toxicology lab, Deptt of FMT, AIIMS).

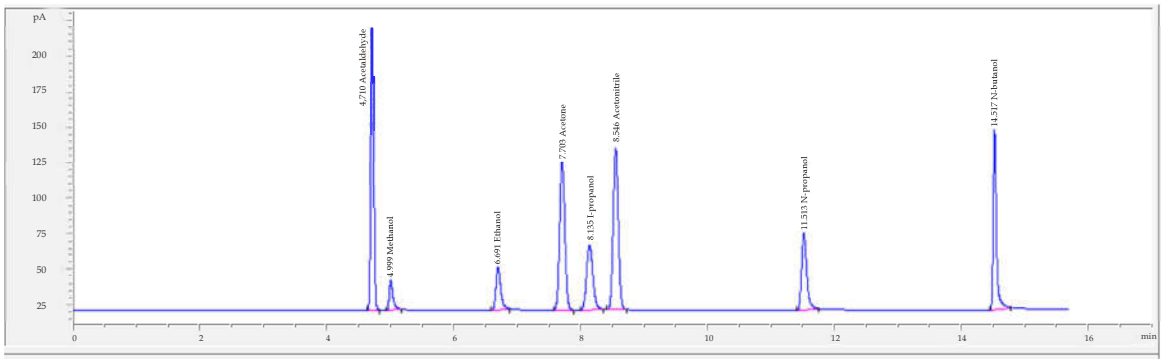

Fig. 14: Chromatogram showing Acetaldehyde, Methanol, Ethanol, Acetone, N-propanol, Acetonitrile, I-propanol and N-butanol separated in the matrix, (Image source: Toxicology lab, Deptt of FMT, AIIMS).

solvents, polish, varnish are the most frequently reported substances of abuse in this age group[63–65]. The common term for this type of substances is 'Inhalants'. Some of the main reasons, for such a large-scale addiction of inhalants is the cheap and easy availability in the near surroundings; legal alternative to other products of abuse and easy concealment as a routine product. Administration of inhalants can be achieved through numerous methods, such as direct inhalation of compressed air duster products through bags or breathing through solvent-soaked rags/handkerchief, and may be referred as huffing, sniffing, snorting, bagging, or spraying depending on the method of administration.

Chemically these compounds are Volatile Organic Compound(s), falling into several chemical groups such as Hydrocarbons, Oxygenated compounds and Halogenated compounds. These chemicals possess low to moderate molecular weights and low boiling points, allowing them to be vaporized and inhaled in their gaseous state at room temperature. The abuse of inhalants produces euphoric and psychoactive effects occasionally resulting in severe toxicity or death[66]. Although these types of cases are not frequently encountered in forensic toxicology casework yet seems like a limited but persistent case type.

Analysis of abuse by inhalants can be detected by direct detection of the parent compound in blood (antemortem) or tissues (postmortem), such as the heart, liver, kidney, and brain, but also on the detection of metabolites, particularly in urine. VOCs in biological matrices can beeasily determined by Gas Chromatography with FID/ ECD after extracting the compounds with static and dynamic Headspace techniques, or even with pulse-heating and solvent extraction methods[67,68].

d. Analysis of Pesticides from Poisoning and Residue cases

Analysis of pesticide in parent and in residue form in a routine analysis depending on the location and types of cases received in forensics laboratories. Out of all categories of pesticides, Organophosphorus pesticides are still widely used and reported. Following metabolism in the organism these compounds cause many cases of acute accidental or suicidal poisonings by the inhibition of cholinesterase activity via phosphorylation by the oxygen analogue. A rapid identification of the causal pesticide would provide very useful information to clinicians for making treatment decisions in emergencies.

Poisoning by organophosphorus pesticides can occur in various situations, India being an agrarian culture its use is widely reported in commercial and domestic agriculture which makes it the most commonly encountered poison in suicide and homicidal cases. Conventional methods for the determination of such compounds require special sample cleanup such as liquid–liquid extraction, Solid Phase Extraction[69,70]. These methods although traditional and most preferred in the routine analysis are labor-intensive, time-consuming, need large amounts of organic solvents, results in less recovery[26–39]. Even with the most careful cleanup it is nearly impossible to achieve absolute clean samples, thus often impurity peaks are found in chromatograms that can sometimes cover the real peaks leading to loos of information in qualitative and quantitative analysis. With the development of methodologies for Headspace-SPME method no matrix compounds are carried onto the GC column, thus limiting sample errors[71,72].

Literature review of the techniques chiefly being used for the analysis of some organophosphorus pesticides in urine, blood, plasma and other biological matrices have reported methodologies like Gas Chromatography-Flame Photometric Detector (GC-FPD); Gas Chromatography- Flame Ionization Detector (GC-FID); GC-Nitrogen Phosphorus Detector (GC-NPD); and GC-Mass Spectrometry (GC-MS)[72–74].

e. Analysis of Volatile Organic Compounds in Stored Biological Matrix

The decomposition process in human body begins immediately after death and may continue for years depending upon the environmental conditions and surroundings in which the body is placed. Formation of volatile organic compounds (VOCs) is an integral part of the decomposition process. In cases of postmortem sampling of biological fluids, in absence of a preservative and cordial temperature conditions, the samples remain in the state of continues putrefaction or decomposition[75,76]. During the course of decomposition, different macromolecules (proteins, carbohydrates, and lipids) breakdown to produce a variety of VOCs. For example, carbohydrates produce oxygen-rich compounds, including alcohols, aldehydes, ketones, acids, esters, and ethers; proteins yield nitrogen, sulphur, and phosphorous containing compounds; lipids break down into hydrocarbons, nitrogen, phosphorous, and oxygenated containing compounds. Estimation of these volatiles became important in scenarios where quantity of these volatiles prior to death is significant.

Quantification of low carbon chain volatiles in samples stored for ethanol estimation is one such condition[77–80]. Estimation of volatile profile during storage can prove to be an storage artefact and misinterpret the results. GC-HS/FID is the most frequently used technique for estimation of volatiles in stored samples for ethanol estimation[48,51-81].

Analysis by one such method, developed and validated in the department laboratory is shown in Fig. 14. The methodology is valid for identification of volatiles like, ethanol, methanol, acetone, acetaldehyde, n-propanol, iso-propanol, n-butanol in biological matrices ranging from blood, vitreous humour, urine, cerebrospinal fluid with addition of Acetonitrile as an Internal Standard for quantification[82].

f. Analysis of Carbon Monoxide Gas from Biological Matrix

Carbon Monoxide (CO) is a deadly, colorless, odorless, nonirritating and tastelessgas that is a product of the incomplete combustionof carbon containing materials. When inhaled, CO is readily absorbed from the alveoli in the lungs into the bloodstream to form a reversible complex with hemoglobin known as Carboxyhemoglobin (COHb) due to affinity of CO being 220 times greater than oxygen. Presence of COHb in place of Oxyhemoglobin in blood reduces the oxygen carrying capacity to the tissues, causing tissue hypoxia. Levels as high as >40% of COHb in blood have been reported to be fatal, with levels between 10%-40% indicative of exposure. Common sources of Carbon Monoxide poisoning include house fires, furnaces or heaters in enclosed spaces, wood-burning stoves, motor vehicleexhaust, and propane-fueled equipment[83].

Although clinical and forensic laboratories predominantly use aspectrophotometric method and colour test for the determination of carboxyhemoglobin (COHb) in blood. Use of GC is being preferred due to its inherent capacity to differentiate between Carbon Monoxide and other putrefactive byproducts like methemoglobin and sulfhemoglobin in postmortem samples. The analysis by GC-HS requires blood sample to be mixed with a cell lyser such as acid or ferrocyanide potassium in a glass vial. This releases carbon monoxide from cells, the gas thus released can be analyzed as a routine procedure in lab[7–84]. The commonest procedure is after headspace injection and gas chromatographic separation, the CO is reduced by a nickel catalyst to methane, which can then detected by using FID[85,86]. Although use of GC-HS for determination of carbon monoxide seems like a specific method with high accuracy and sensitivity problems such as time-consuming sample and standard preparation, expensive has prevented routine use of GC-HS for analysis.

g. Analysis of Cyanide from Biological Matrix in Postmortem Cases

Cyanide is a powerful chemical poison exhibiting cellular asphyxiation following administration. Cases can be received in a forensic laboratory following voluntary ingestion (suicide) of salt of cyanide (KCN, NaCN) or by involuntary inhalation (fire, accidental exposure) of Hydrogen Cyanide.

Cyanide ingestion causes asphyxiation by bonding to the cytochrome C oxidase, a mitochondrial enzyme responsible for respiration and the oxygen carrying hemoglobin forming cyanohemoglobin (CNHb). Blood concentration upto 2-3µg/ml of blood are considered lethal.

Laboratories employ traditional methods of colour tests and spectrophotometric detection preceded by distillation or microdiffusion pretreatment[87]. Technique of Gas Chromatography with headspace sampling provides a faster analysis, higher sensitivity, specific detection and higher rate of recovery[12–88,89]. The procedure of analysis involves sealing a sample in headspace glass vial and addition of internal standard and an acid through the septum, this releases cyanide in the gas phase where detection can then be done using highly specific detectors such as Nitrogen Phosphorus Detector (NPD)[90]. A method describing the determination of cyanide inblood by HS-GC with Electron Capture Detector (ECD) has also been reported. This method involves transformation of cyanide into cyanogen chloride by reacting the hydrogen cyanide with chloramine-T on a stick of filter paper in the space above the blood in the headspace vial[91,92].

h. Analysis of Phosphine from Biological Matrix in Postmortem Cases

Aluminium Phosphide is an inorganic poison if ingested and routinely used otherwise as a rodenticide, insecticide and fumigant for stored cereal grains. When in contact with moisture, water or acid, it releases a colorless, lethal phosphine (PH_3) gas. India has reported phosphine as one of the major agricultural poison. Phosphine can be administered in body by two major routes of administration, first, from oral route by direct injection of tablets or powders containing Aluminium Phosphide and second, by inhaling vapors of phosphine gas in a closed environment[93]. Cases received in forensic laboratories can range from suicidal (ingestion of tablets), homicidal (mainly children) and accidental (inhalation of fumes)[94,95]. Phosphine on absorption in body is rapidly metabolized into phosphite and hypophosphite. Its high lethality is attributed to inhibition of cytochrome C oxidase and oxidative phosphorylation and its lack of specific antidote making it one of the potent poisons.

Phosphine poisoning can be easily detected qualitatively by simple chemical and calorimetric tests in biological (viscera, gastric lavage, urine) and non-biological (liquid, tablets, food material, water) samples[96,97]. In biological samples stored for a certain duration, loss of volatile by evaporation or breakdown by-products can lead to false negative or positive results. Papers as early as 1983, have reported use of Gas Chromatography-Headspace with Nitrogen Phosphorus Detector (NPD), for detection of phosphine from postmortem collected samples[98]. Authors have coupled GC systems with Mass Spectrophotometry (MS) for confirmatory analysis. Sampling has been improved by immediately collecting biological samples like blood, urine, kidney, adrenals, brain and heart directly into the headspace glass vial and analyzing the sample immediately or storing then in HS vials for later analysis. For sample preparation, acid is added to the mixture through septa of the vial, sample is vortexed and vial used in Headspace[99–101].

Conclusion

In analytical chemistry, Gas Chromatography is one of the most frequently used and the most indispensable technique available for testing of thermally stable compounds in vapour form without degradation. Analysis if variety of organic compounds can be facilitated by knowledge about the chemistry of sample introduction, column and capacity of detector. The instrument can analyse single compounds as well as separate and quantify mixtures. Using the variety of detectors available specific to a specific category of compounds, the instrument can simultaneously detect and quantify the analyte. For fingerprint identification of a compound, simply the standard and sample need to be need in tandem against the same conditions of analysis, appearance of peak on same Rt can opion the unknown against known standard.

Ever since Gas Chromatography was commercially available, its application has gradually increased by embracing new growths and directions. In addition to its numerous advantageous features, the basic principle and the theory of GC has been well studied and understood since its inception more than half a century ago. Its use can be readily understood from breadth of its applications. The range of material which can be analysed by chromatographic methods is essentially unlimited with applications found in varied fields of Forensic Science, Food and Agriculture,

Pharmaceuticals, Biological and Clinical chemistry, Environmental toxicology, Polymer industry and many others.

Forensic science, like any other discipline of analytical chemistry, is heavily dependent on Gas Chromatography, a glimpse of its vide application as discussed in the paper. A continuous influx of new column chemistries, sample injection methods, sample extraction assemblies, development of highprecision thermal and pneumatic controlling systems, advancement in control electronics, and a large variety of detection systems have positioned gas chromatography as a formidable foe to other competing analytical instruments. Although the applications of Gas Chromatography are limited to volatile and semi volatile organic compounds, rapid development in derivatization chemistry and sample extraction has allowed for new organic compounds to be analysed by GC, extending its horizon.

Use of Static Headspace-Gas Chromatography as a persistent and mature technique of choice in forensic laboratories and can be demonstrated by the presence in majority of laboratories worldwide. Along the various applications, sample preparation with minimum modification, linear calibration curve, repeatable results, excellent validation parameters, no carryover and minimum handling of hazardous samples/standards/extracts are some of the unanimous reasons of worldwide acceptance of Headspace sampling technique. Capabilities of Headspace has been improved with the use of SPME for sample extraction, leading to application within a wide variety of compounds. The introduction of two-dimensional Gas Chromatography, Gas Chromatography-Isotope Ratio Mass Spectrometry, and Fast Gas Chromatography has contributed significantly to forensic applications by extracting additional information from the collected sample that can aid forensic scientists. It can be speculated with high confidence that Gas Chromatography-Headspace will remain a strong attribute in analytics in the forensic field and will continue to offer new and unique attributes to solve more challenging forensic problems with the growth in advancement of instruments and the samples to be tested.

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