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Instrumental Methods in Bioprospecting: Gas Liquid Chromatography

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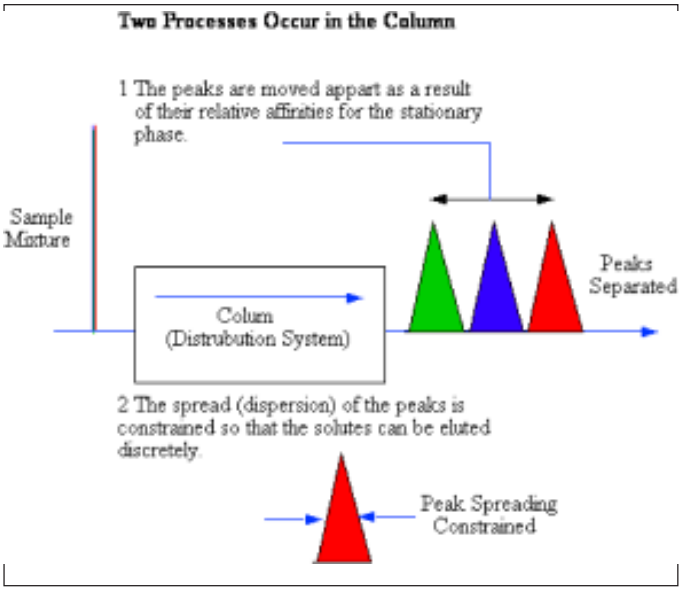
Chromatography: An Overview

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. Nevertheless, to a limited extent, it is also used for preparative purposes, particularly for the isolation of relatively small amounts of materials that have comparatively high intrinsic value. In a single step process it can separate a mixture into its individual components and simultaneously provide an quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two entantiomers to a multi component mixture containing widely differing chemical species. The first scientist to recognize chromatography as an efficient method of separation was the Russian botanist Tswett, who used a simple form of liquid-solid chromatography to separate a number of plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography for this type of separation (color writing). Although color has little to do with modern chromatography, the name has persisted and, despite its irrelevance, is still used for all separation techniques that employ the essential requisites for a chromatographic separation, viz. a mobile phase and a stationary phase. Today, chromatography is an extremely versatile technique; it can separate gases, and volatile substances by gas chromatography (GC), in-volatile chemicals and materials of extremely high molecular weight (including biopolymers) by liquid chromatography (LC). Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; *ipso facto* a separation is achieved.

In practice, the distribution system, (that part of the chromatographic apparatus where the solutes are distributed between the phases) can take the form of a column such as a tube packed with particulate matter on which the stationary phase is bonded or coated. The mobile phase (which may be a gas or a liquid) passes under pressure through the column to elute the sample. The column form may also be a long, small-diameter open tube that has the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system may take the form of a plate (usually glass) the surface of which is loaded with particulate matter to which the stationary

phase is coated or bonded. The mobile phase (a liquid) is arranged to percolate up the plate (usually by surface tension forces) to elute the sample. The sample is injected into the mobile phase stream just before the front of the columns. The column is designed to allow two processes to take place that will produce the separation. Firstly, as a result of different forces between each molecular type and the stationary phase, each solute is retained to a different extent and, thus, the more weakly held will elute first and the more strongly held elute last. The process is diagramatically depicted below.



Classification of Chromatography

As all chromatographic separations are carried out using a *mobile* and a *stationary* phase, the primary classification of chromatography is based on the physical nature of the *mobile* phase. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of chromatography, namely, gas chromatography (GC) and liquid chromatography (LC).

Table 1 The Classification of Chromatography

Mobile phase	Stationary phase
Gas	Liquid
Gas Chromatography (GC)	Liquid Gas-liquid chromatography (GLC) SolidGas Solid Chromatography (GSC)
LiquidLiquid chromatography (LC)	LiquidLiquid –liquid chromatography (LLC) SolidLiquid solid chromatography (LSC)

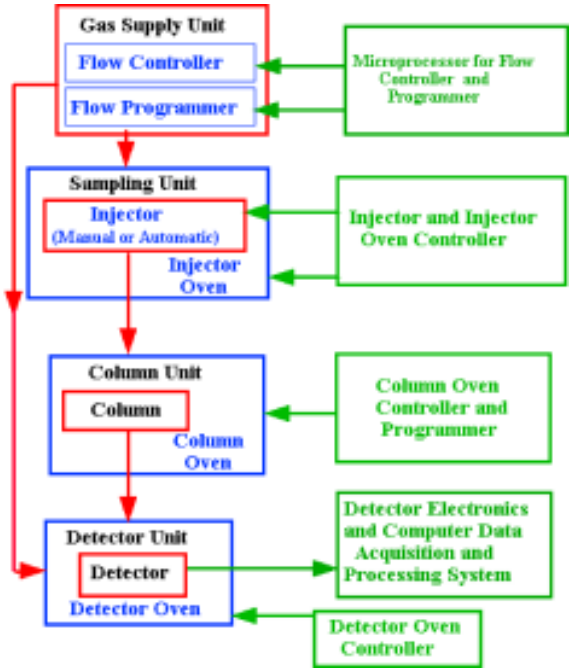
The stationary phase can also take two forms, solid and liquid, which provides two subgroups of GC and LC, namely; gas–solid chromatography (GSC) and gas–liquid chromatography (GLC), together with liquid solid chromatography (LSC) and liquid chromatography (LLC). The different forms of chromatography are summarized in Table 1. Most thin layer chromatography techniques are considered liquid-solid systems although the solute normally interacts with a liquid-like surface coating on the adsorbent or support or, in some cases an actual liquid coating.

Gas Liquid Chromatography

Gas-liquid chromatography (GLC) was in invented by James and Martin and is a chromatography separation technique in which the mobile phase is a gas (usually helium or nitrogen) and the stationary phase is a liquid. In the original columns used by James and Martin, the liquid stationary phase was adsorbed on the surface of an inert support such as Celite (a diatomateous earth) or calcined Celite (a form of brick dust). The support was usually deactivated before use by acid treatment and

subsequent reaction with hexamethyldisilazane. The technique was extensively used for the separation of a wide range of volatile substances including fatty acids.

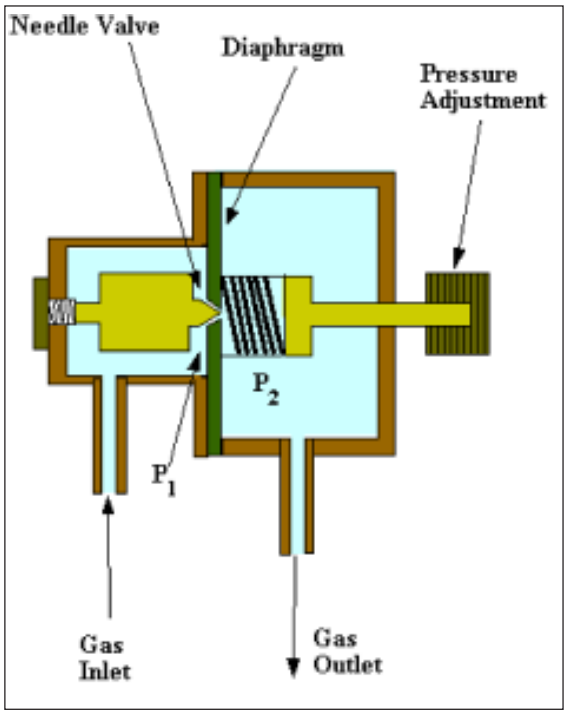
The modern gas chromatograph is a fairly complex instrument mostly computer controlled. The samples are mechanically injected, the analytical results are automatically calculated and the results printed out, together with the pertinent operating conditions in a standard format. However, the instrument has evolved over many years although the majority of the added devices and techniques were suggested or describe in the first three international symposia on gas chromatography held in 1956, 1958 and 1960. The layout of the modern gas chromatograph is shown as a block diagram:



The different components of GLC

Gas supplies

Gases (carrier gas-N_2 or He; and fuel gas-air and H_2) for use with the gas liquid chromatography were originally all obtained from gas cylinders fitted with reducing valves that are set to supply the gas to the instrument at the recommended pressure defined by the manufacturers. The reducing valves on the gas tanks are examples of simple pressure controllers and the flow controllers that are used for detector and column flow control often involve devices based on the same principles. The pressure controller consists essentially of two chambers separated by a diaphragm, in the center of which is a needle valve that is actuated by the diaphragm. The diaphragm is held down by a spring that is adjustable so that the pressure in the second chamber, and thus the outlet flow, can be set at any chosen value. When gas enters the lower chamber, the pressure on the lower part of the diaphragm acts against the spring setting, and opens the valve. Gas then passes into the upper chamber and pressure is built up in the upper chamber to the value that has been set at which time the diaphragm moves downward closing the valve. If the pressure falls in the upper cylinder, the



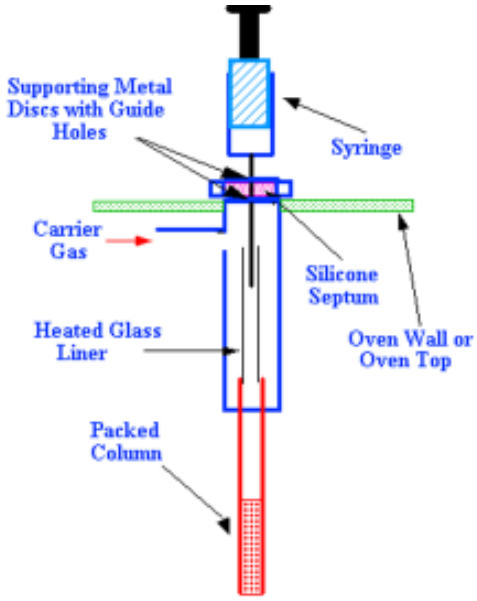
Pressure controller

diaphragm again moves upward due to the pressure in the lower chamber, which opens the valve and the pressure in the upper chamber is brought back to its set value.

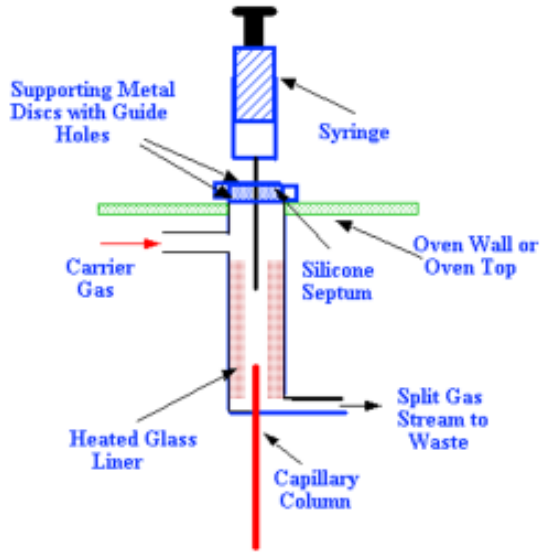
Injectors

The sample is injected by a hypodermic syringe, through a silicone rubber septum directly into the column packing or into a flash heater. An example of a septum injection system used for packed columns is shown in following figure. The silicone septum is compressed between metal surfaces in such a manner that a hypodermic needle can pierce it, but when it is withdrawn the hole is closed as a result of the septum compression and there is no gas leak. The glass liner prevents the sample coming in contact with the heated metal wall and thus, reduces the chance of thermal decomposition. The glass liner can be fitted with a separate heater and the volatilization temperature can, thus, be controlled. By using a syringe with a long needle, the tip can be made to penetrate past the liner and discharge its contents directly into the column packing. This procedure is called 'on-column injection' and, as it reduces peak dispersion on injection and thus, provides higher column efficiencies, is often the preferred procedure.

The basic difference between the two types of injection systems is that the capillary column now projects into the glass liner and a portion of the carrier gas sweeps past the column inlet to waste. As the sample passes the column opening, a small fraction is split off and flows directly into the capillary column, *ipso facto* this device is called a split injector. The split ratio is changed by regulating the portion of the carrier gas that flows to waste which is achieved by an adjustable flow resistance in the waste flow line. This device is only used for small diameter capillary columns where the charge size is critical. Consequently, quantitative analyses carried out using the high efficiency small diameter capillary columns may have limited accuracy and precision, depending on the nature of the sample.



Packed Column Injector



Split Injection System

GLC Columns

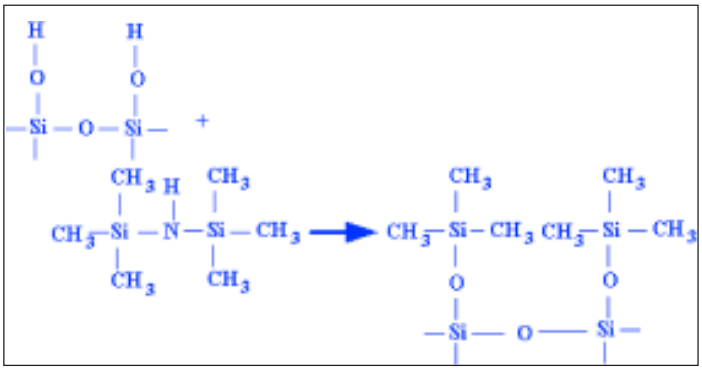
There are two types of columns in common use in GC and they are the conventional packed column and the open tubular column. The former are usually 2 to 4 mm I.D. and 1-4 m long and, packed with a suitable adsorbent, are mostly used for gas analysis. As a result of the simpler injection procedure and the more precise sampling method, the packed column tends to give greater quantitative accuracy and precision. However, despite its problems with sample injection, the open tubular column is seen as the 'state of the art' column and is by far the most popular column system in general use. The length of open tubular or capillary columns range from about 10-100 m and can have internal diameters from 100-500 ìm. The stationary phase is coated on the internal wall of the column as a film 0.2-1 ìm thick.

The Packed GC Column

Packed columns are usually constructed from stainless steel or Pyrex glass. Pyrex glass is favored when thermally labile materials are being separated such as essential oils and flavor components. Longer columns can be U-shaped but columns more than a meter long are usually coiled. Glass columns are sometimes treated with an appropriate silanizing reagent to eliminate the surface hydroxyl groups which can be catalytically active or produce asymmetric peaks.

Supports for GLC

There have been a number of materials used as supports for packed GC columns including, Celite (a proprietary form of a diatomaceous earth), fire-brick (calcined Celite), fire-brick coated with metallic silver or gold, glass beads, Teflon chips and polymer beads. Today however, the vast majority of contemporary packed GLC columns are filled with materials that are either based on of Celtic or polystyrene beads as a support. There are two processes used to modify Celite. One was to crush, blend and press the Celite into the form of a brick and then calcine it at a temperature of about 900ÚC. Under these conditions some of the silica is changed into cristobalite and traces of iron and other heavy metals interact with the silica causing the material to become pink in color. This material is sold under the trade name of Chromosorb P. The second process involves mixing the Celite with sodium carbonate and fluxing the material at 900ÚC. This causes the structure of the Celite to be disrupted and the fragments adhere to one another by means of glass formed from the silica and the sodium carbonate. As the original Celite structure is disrupted, the material exhibits a wide range of pore sizes which differs significantly from the material that was calcined in the absence of sodium carbonate. This materials is sold under the name of Chromosorb W together with two similar materials called Chromosorb G and Chromosorb S. The residual deleterious adsorptive properties of the support are due to silanol groups on the surface and these can be removed by silanization. The support is treated with hexamethyldisilazane which replaces the hydrogen of the silanol group with a trimethylsilyl radical. The reaction proceeds as follows,



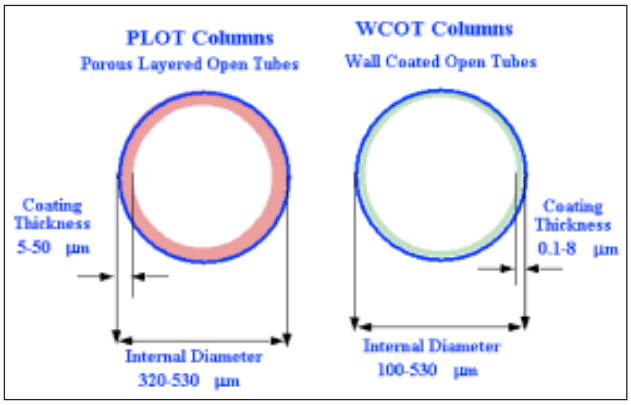
In this way the strongly polar silanol groups are methylated and assume dispersive characteristics that do not produce peak tailing. Although the major contributors to adsorption by the support are the silanol groups, a residual adsorption results from the presence of trace quantities of heavy metals such as iron, which can be largely removed by acid washing prior to silanization.

The Capillary or Open Tubular Column

Capillary columns are fabricated from stainless steel. Metal columns provide the high efficiencies expected from open tubular columns and were used for the analysis of petroleum, fatty acids and fuel oils, etc. Metal columns, however, have some disadvantages as although easily coated with dispersive stationary phases (*e.g*., squalane, Apiezon grease etc.) they are not so easily coated with the more polar stationary phases such as CARBOWAX®. In addition, hot metal surfaces can cause decomposition or molecular rearrangement of many thermally labile materials such as the terpenes contained in essential oils. Metal can also react directly with some materials by chelation and adsorb polar material which results in asymmetric and tailing peaks. Nevertheless, metal columns are rugged, easy to handle and easy to remove and replace in the chromatograph consequently, their use has persisted in many application areas despite the introduction of fused silica columns.

Open Tubular Column Types

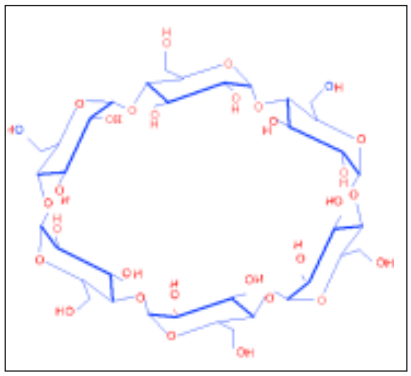
Open Tubular columns are broadly split into two classes, *the wall coated open tubular columns* or WCOT Columns (which have already been described and are by far the mot popular,) and the *porous layer open tubes* or PLOT Columns. The two types of column are shown diagramatically in the following figure. The external diameter of PLOT columns range from 320-530 ìm with a porous layer that can be 5-50 ìm thick.



Open Tubular Column Types

Chiral stationary phases

Modern organic chemistry and pharmaceutical research are becoming increasingly interested in methods of asymmetric syntheses. This enthusiasm has been provoked by the differing physiological activity that has been shown to exist between the geometric isomers of pharmaceutically active compounds. A tragic example being the drug Thalidomide, which was made available as a racemic mixture of N-phthalylglutamic acid imide. The important physiological activity resides in the R-(+)-isomer and it was not found, until too late, that the S-enantiomer was probably tetratogenic and caused serious fetal malformations. The separation and identification of isomers can, clearly, be very important and chromatography can be very effective in the resolution of such mixtures. The use of GC for the separation of asymmetric isomers is not as common as LC, but nevertheless there some very effective optically active stationary phases that



The Structure of Cyclodextrin

can be used in GC for the separation of enantiomers. Some of the more useful GC stationary phases are based on cyclodextrins already described. The columns are usually 30-60 m long 0.25 mm I.D. and have an operating temperature range of 30ÚC to 250ÚC. In order to employ the cyclodextrins as stationary phases for GC the permethylated cyclodextrins are often embedded in a siloxane matrix (e.g. 35% phenyl-65% methyl polysiloxane) which is deposited on the walls of fused quartz capillary tubes.

Derivatization of the base cyclodextrin structure can introduce groups to which only one enantiomer can interact, while the other(s) are partially or wholly entropically hindered from interaction. This increases the differential interaction between the enantiomers and the stationary phase, thus, increasing the separation ratio and hence the resolution.

Column oven and accessories

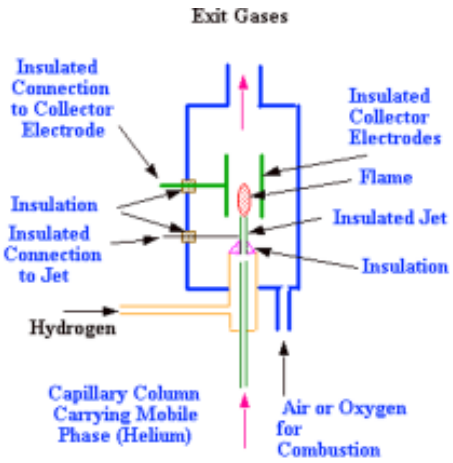
The column oven should operate over a fairly wide temperature range (*e.g.* from 5ÚC to 400ÚC). In practice, however, the maximum oven temperature needed is usually less than 250ÚC, particularly when synthetic stationary phases are being used, as many of them tend to be unstable and either decompose or volatilize at higher temperatures. Similarly, initial temperatures below 50ÚC are also rarely needed. The oven usually has air circulation driven by a powerful fan to ensure an even temperature throughout the oven. The temperature programmer (hardware and software) usually has a range of linear gradients from 0.5ÚC/min. to about 20ÚC/min. Some programmers include nonlinear programs such as logarithmic and exponential, but most GC analyses can be effectively accomplished using linear programs only. The program rate can be changed at any time in the chromatographic development or intermittent isothermal periods can be inserted where necessary in the program. The temperature programming limits are usually the same as those of the oven (*viz*. 5ÚC to 400ÚC).

GC detectors

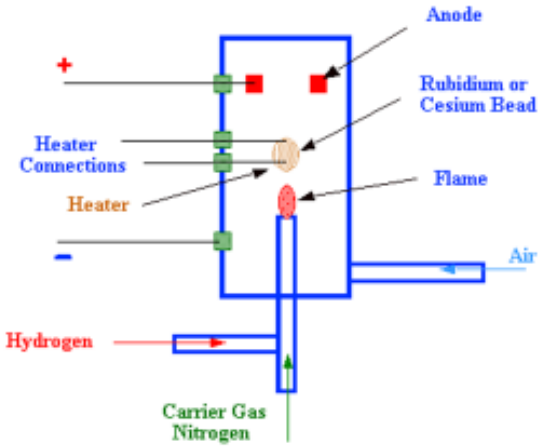
A large number of GC detectors have been developed and made commercially available. The detectors with the highest sensitivity tend to be specific and sense specific types of sample (*e.g*., halogenated substances by the electron capture detector). The detectors with a catholic response are the most popular and the majority of GC separations are monitored by the flame ionization detector (FID). The most commonly used specific detectors are the nitrogen phosphorus detector (NPD) and the electron capture detector (ECD).

Flame Ionization Detector

The FID detector employs hydrogen as the combustion gas which is mixed with the column eluent (helium, nitrogen or other appropriate gas) and burnt at a small jet situated inside a cylindrical electrode. A potential of a few hundred volts is applied between the jet and the electrode and when a carbon containing solute is burnt in the jet, the electron/ion pairs that are formed are collected at the jet and cylindrical electrode. The current is amplified and fed to a recorder or to the A/D converter of a computer data acquisition system. During the process of oxidation, oxidized or partially oxidized fragments of the solute are formed in the flame which is thought to generate electrons by thermionic emission. The background current (ions and electrons from the hydrogen flame alone) is very small (1-2 x 10^{-12} amperes) and consequently, the noise level is also commensurably small (about 10^{-14} amperes).



Flame Ionization Detector



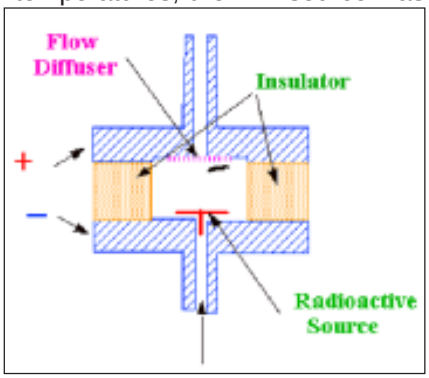
Nitrogen Phosphorus Detector

Nitrogen Phosphorus Detector

The nitrogen phosphorus detector (NPD) is a highly sensitive but specific detector and evolved directly from the FID. It gives a strong response to organic compounds containing nitrogen and/or phosphorus. Although it appears to function in a very similar manner to the FID, in fact, it operates on an entirely different principle. The actual NPD sensor is a rubidium or cesium bead contained inside a small heater coil. A potential is applied between the bead and the anode. The heated alkali bead emits electrons by thermionic emission which is collected at the anode and thus produces an ion current. When a solute containing nitrogen or phosphorus is eluted, the partially combusted nitrogen and phosphorus materials are adsorbed on the surface of the bead. This adsorbed material reduces the work function of the surface and, as consequence, the emission of electrons is increased which raises the anode current. The sensitivity of the NPD is about 10^{-12} g/ml for phosphorus and 10^{-11} g/ml for nitrogen).

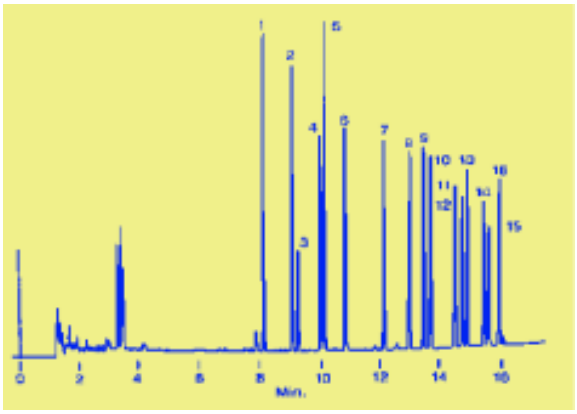
Electron Capture Detector

The electron capture detector contains a low energy â-ray source which is used to produce electrons for capturing by appropriate atoms. Although tritium adsorbed into a silver foil has been used as the â particle source, it is relatively unstable at high temperatures, the Ni^{63} source was found to be preferable. The detector can be used in two modes, either with a constant potential applied across the cell (the DC mode) or with a pulsed potential across the cell (the pulsed mode). In the DC mode, hydrogen or nitrogen can be used as the carrier gas and a small potential (usually only a few volts) is applied across the cell that is just sufficient to collect all the electrons available and provide a small standing current. If an electron capturing molecule (for example a molecule containing a halogen atom which has only seven electrons in its outer shell) enters the cell, the electrons are captured by the molecule and the molecules become charged. The mobility of the



Electron Capture Detector

captured electrons is much smaller than the free electrons and the electrode current falls dramatically. In the inactive period of the wave form, electrons having thermal energy only will attached themselves readily to any electron capturing molecules present in the cell with the consequent production of negatively charged ions. The negative ions quickly recombine with the positive ions (produced simultaneously with the electrons by the â particles) and thus become *unavailable* for collection. Consequently the standing current measured during the potential pulse will be reduced.



The basic electron capture detector consists of a small chamber one or two ml in volume enclosing two metal electrodes. The electrodes may be concentric cylinders or metal discs separated by an insulator. The cell contains the radioactive source, electrically connected to the entrance conduit and to the negative side of the power supply. A gauze "diffuser" is connected to the cell exit and to the positive side of the power supply. The output from the sensor is processed by suitable electronics and the output passed to either a potentiometric recorder of a computer data acquisition system. The electron capture detector is very sensitive, probably the most sensitive GC detector available (*ca*. 10^{-13} g/ml) and is widely used in the analysis of halogenated compounds.

1	2	3	4 Heptachlor
5	6 Aldrin	7 Heptachlor Epox.	8 Endosulphan
9 p,p'-DDE	10 Dieldrin	11 Endrin	12 p,p'-DDD
13 Endosulphan 11	14 p,p'-DDt	15 Endin Aldehyde	16Endosulp. Sulf.

Analysis of chlorinated insecticides

Data acquisition and processing

Originally, analytical results were calculated from measurements made directly on the chromatogram provided by the chart recorder. The output from the detector (which is only rarely the direct output from the detector sensor) is usually in millivolts and is suitable for direct connection to a potentiometric recorder. The output from the detector usually passes directly to a scaling amplifier that modifies the signal to a range that is appropriate for the analog-to-digital (A/D) converter. The output can alternatively pass to a potentiometric recorder and produce the chromatogram in real time. The computer system can also produce a real time chromatogram but, to do so, the data must be processed and the chromatogram presented on the printer.

Quantitative analysis

There are three important stages in a GC analysis,

1. The preparation of the sample.

2. The development of the separation and the production of the chromatogram

3. The processing of the data and the presentation of the results.

Each stage is equally important and if not carried out correctly the results will be neither precise nor accurate. Sample preparation can be very simple involving no more that diluting a known weight of sample with mobile phase or be much more complex including an extraction procedure followed by derivatization and then dilution. Liquid extraction is a clumsy procedure, particularly when used on the micro scale which is often necessary in sample preparation. An alternative procedure is solid phase extraction. The procedure is relatively simple and involves the use of a short tube packed with an appropriate adsorbent such as silica, reversed phase silica or, for some applications, macro porous polymer beads. The adsorbent must be capable of removing the substances of interest from the liquid medium.

Derivatization

GC samples are usually derivatized to render highly polar materials sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement. Examples of such materials that need to be derivatized are the organic acids, amides, poly hydroxy compounds, amino acids etc. In order to render such materials more volatile, they are either esterified, silanated or acetylated using one of a number of different methods of derivatization. Acids can be esterified by treating them with an appropriate alcohol using an inorganic acid to catalyze the reaction. Hydrochloric acid was popular for this purpose because it's strength was adequate and any excess could be easily removed. Other catalysts that have been found effective are trifluoroacetic acid, dichloroacetic acid, benzene sulphonic acid, *p*-toluene sulphonic acids and suphuryl and thionyl chlorides. A volatile acid is recommended such as hydrochloric acid or thionyl chloride. However, the derivative must be must be sufficiently involatile not to allow loss when removing the excess alcohol and where appropriate the catalyst itself. The Lewis acid boron trifluoride or the equivalent reagent boron trichloride is also very useful for forming ester derivatives. Boron trifluoride is supplied as a 14% solution in methanol. Boron trifluoride catalyzed reactions are very fast and can be complete in a few minutes. The esters can be extracted with *n*-hexane with vigorous shaking. Another popular esterifying reagent is diazomethane. Diazomethane is a yellow gas but is used in the form of an ethereal solution. Its reacts with an organic acid in the following manner,

$$R—COOH + CH_2N_2 \longrightarrow R—COO–CH_3 + N_2$$

When the reaction is complete, the yellow color persists and thus the reagent acts as its own indicator.

Suggested Reading

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