CHROMATOGRAPHIC TECHNIQUES

The term chromatography embraces a family of closely related separation methods based on Tswett and Days experiments described in 1903 – 1906. The importance of chromatography lies primarily in its use as an analytical separations tool although preparative applications have gained prominence as a production tool since last two decades. It serves as a means of resolution of mixtures and for the isolation and partial description of the components whose presence may be known or suspected.

In chromatography, the components to be separated are distributed between a stationary bed of large surface area and a fluid that percolates through or along the stationary bed. Mass transfer between the mobile phase and the stationary phase occurs either because of the molecules of the mixture are absorbed on particle surfaces or absorbed into particle pores or they partition into pools of liquid held on surfaces or within the pores. This process is known as sorption.

Separation of the components in a sample is based on the fact that the rate of travel of an individual solute molecule through a column or thin layer of adsorbant is directly related to the partition of that molecule between the mobile and stationary phase. The partition coefficient of each component determines how much of it is in each phase at any time and how long it remains in that phase. If selective retardation differences prevail, each component can travel through a column or along the stationary phase at a rate dependant upon the sorption characteristics.

After some time interval they will be distributed in space over the stationary phase and subsequently emerge out of the stationary phase as a single components. Several techniques result depending upon the choice of stationary and mobile phases . Thus we can have

MOBILE	STATIONARY PHASE	TECHNIQUE
Liquid	Liquid	Partition chromatography
Gas	Liquid	Gas - Liquid chromatography
Gas	Solid	Gas - Solid chromatography
Liquid	Ion exchange resin	Ion exchange chromatography
Liquid	Molecular sieves	Ion Exclusion / Gel permeation
Liquid	Thin layer of Silica/Alumina	Thin layer chromatography
Liquid	Paper	Paper chromatography

The chromatographic behavior of a solute can be described either by its retardation factor(R) or by retention volume (V_R). By varying sorbent-solvent combinations and operating parameters, the degree of retardation can be varied over a wide range from nearly total retention to a state of free migration. Let us define some technical terms related to chromatography.

1. Partition coefficient

$$K_d = C_s / C_m$$

where C_s & C_m are the concentration of the solute in the stationary phase and mobile phase.

2. Retardation factor

$$R = \frac{t_m}{t_m + t_s}$$

t_m and t_s time spent by the molecule in mobile and stationary phases.

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the mobile phase}}$$

In paper chromatography and thin layer chromatography R is greater than R_f by about 15%.

$$\frac{R}{1-R} = \frac{C_m V_m}{C_s V_s}$$

where C_m and V_m are the volumes of mobile and stationary phases.

$$R = \frac{V_m}{V_m + K_d V_s} = \frac{1}{1 + K_d(V_s/V_m)}$$

3. Retention volume

In column elution methods, it is the amount of mobile phase which has left the column at the instant the maximum of the solute zone emerges from the column. At its peak , maximum 50% solute has eluted in retention volume V_R and 50% is retained in mobile phase and stationary phase. Thus

$$V_R C_m = V_m C_m + V_s C_s$$

$$V_R = V_m + k_d V_s$$

Since $V_R = t_R$ .Fc (time x volume rate of flow of mobile phase)

and $V_R = L.Fc/R\nu$
where L is the length of the column and ν is the linear flow rate of the mobile phase.

$$V_R = L \,.\, Fc\,(V_m + K_d.V_s)/\nu \,.V_m$$

The ratio [LFc/ν] is the volume of mobile phase. The first term is called the bed void or free column volume. It represents the retention volume of a substance that is unadsorbed or insoluble in stationary phase (for $K_d = 0$).

4. Column capacity

$k = C_s V_s / C_m V_m$

The volumetric phase ratio β in a column is designated as V_m/V_s.

Thus $k = k_d / \beta$

$R = 1/(1-k)$

$k = (V_R - \nu V_m) / V_m$

5. Temperature effects

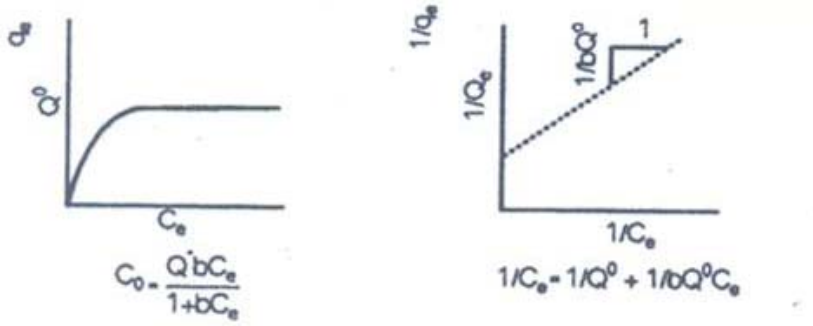
Temperature effects are very important parameters in the operation of columns due to the marked dependence of partition coefficients on the temperature.

$$k_d = e^{\Delta S_0/RT} - e^{\Delta H_0/RT} \simeq ae^{-\Delta H_0/RT}$$

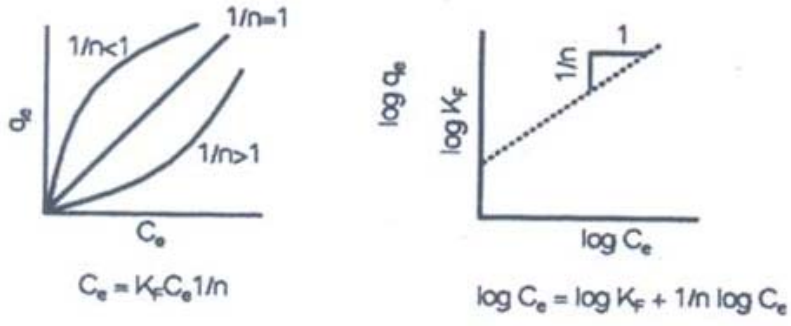
where ΔS^0 and ΔH^0 are standard entropy and enthalpy of sorption.

Usually ΔS^0 = 0-12 kcal and at 20^0C, K_d will decrease by 50%. This leads to a large value of R or large decrease in retention volume.

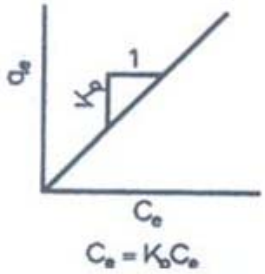
ADSORPTION ISOTHERMS



(a) Langmuir



(b) Freundilish



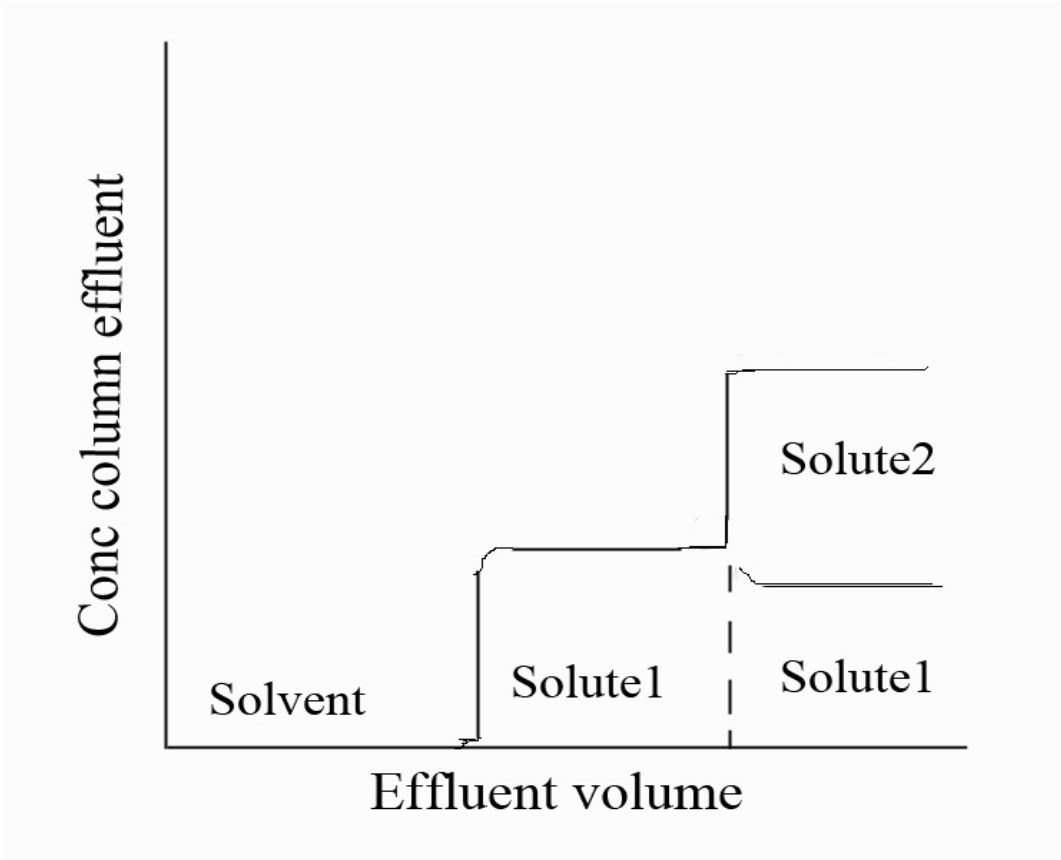
(c) Linear

DEVELOPMENT OF THE CHROMATOGRAM

The sample is generally introduced at the top of a column or near one edge of a sheet or thin layer of sorbent. When mobile phase is allowed to percolate through a stationary phase, sample components develop into separate zones (bands, peaks). This is known as chromatographic development. Development of chromatograms may be conducted either by frontal analysis or elution analysis or by displacement development.

1. Frontal Analysis

In this we pass the sample solution continuously through an adsorbant. The active centres of the adsorbant are occupied by the more strongly adsorbed components and the least strongly adsorbed components accumulate in the traveling front. The solvent comes out first followed by the least strongly adsorbed components followed by additional solutes which are more strongly adsorbed.



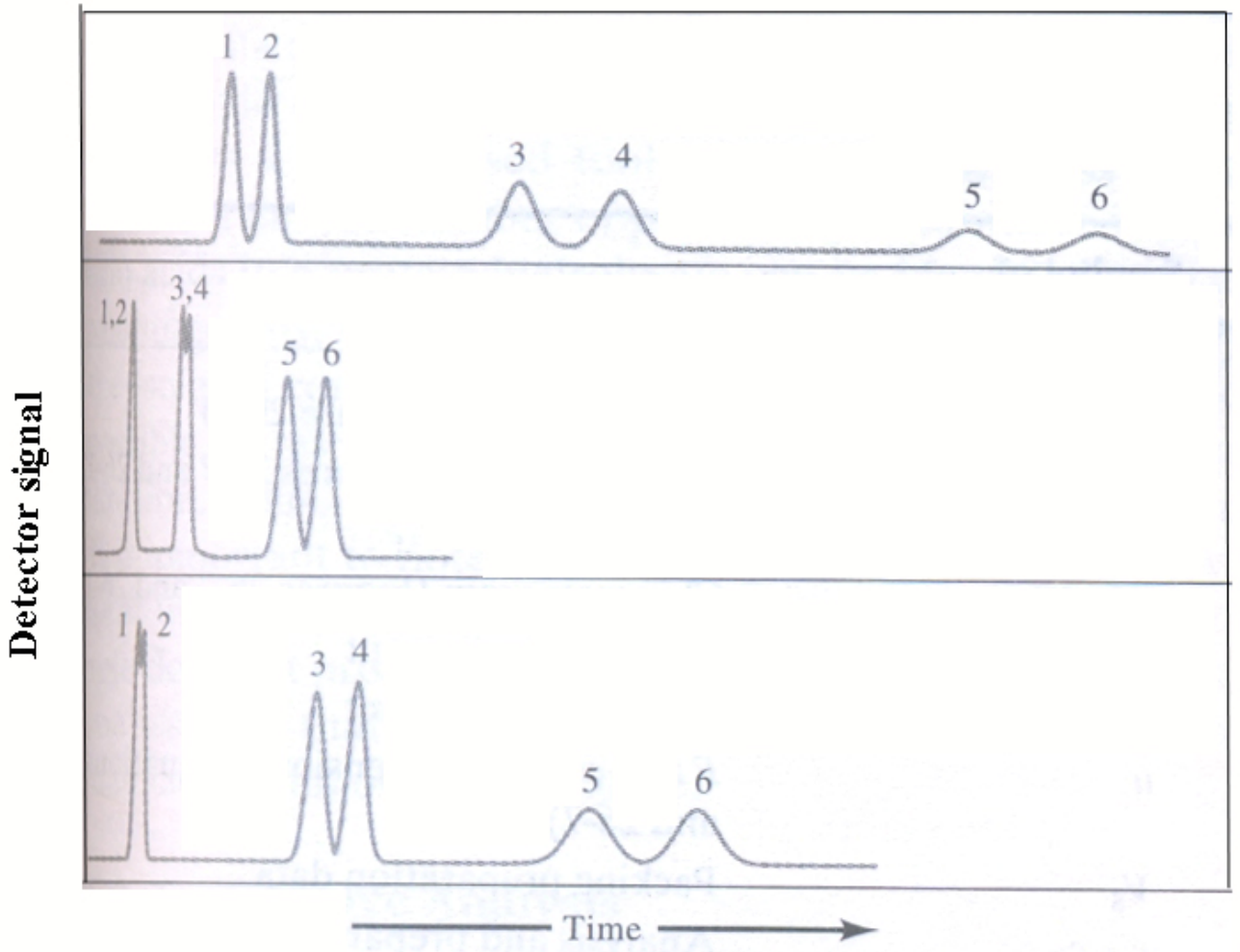
Frontal analysis leads to resolution only of the least strongly absorbed solute. Processes of this type are used for the removal of relatively small components of undesirable components when then are strongly absorbed.

2. Elution development

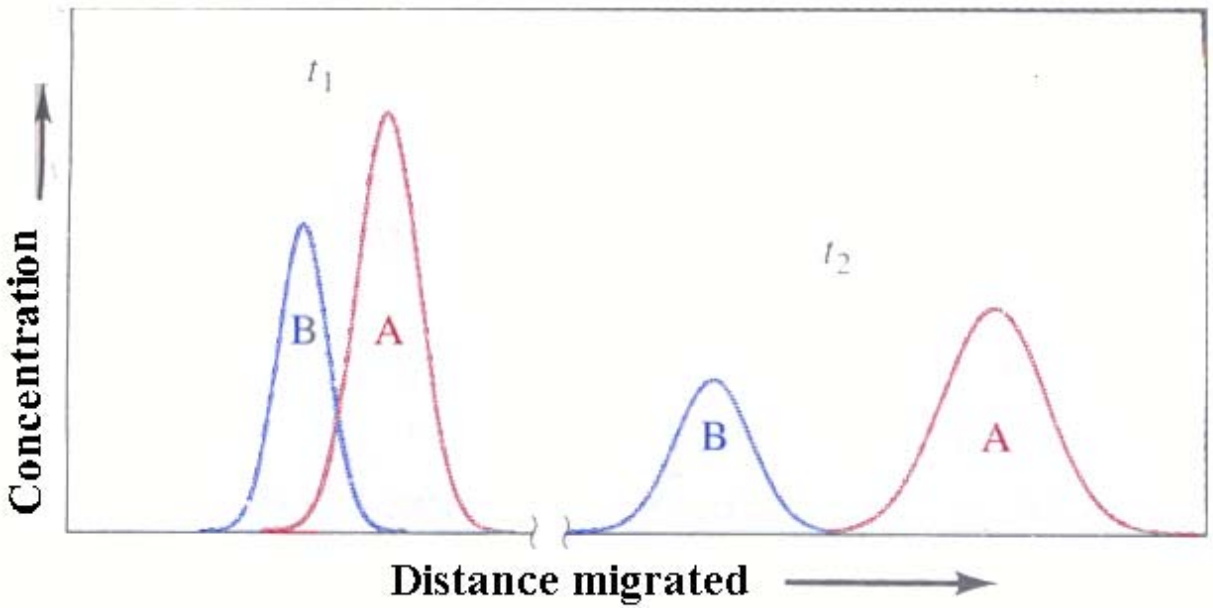
A small sample is introduced at the upstream end of the stationary phase. Pure solvent, called as the eluent is passed through the system which leads to a differential migration of the solutes in the mobile phase.

Subsequently the solutes will emerge out of the column depending upon the individual partition coefficients. If these are sufficiently different for different components the mixture will split into separate bands that migrate at different rates and emerge out.

ELUTION DEVELOPMENT



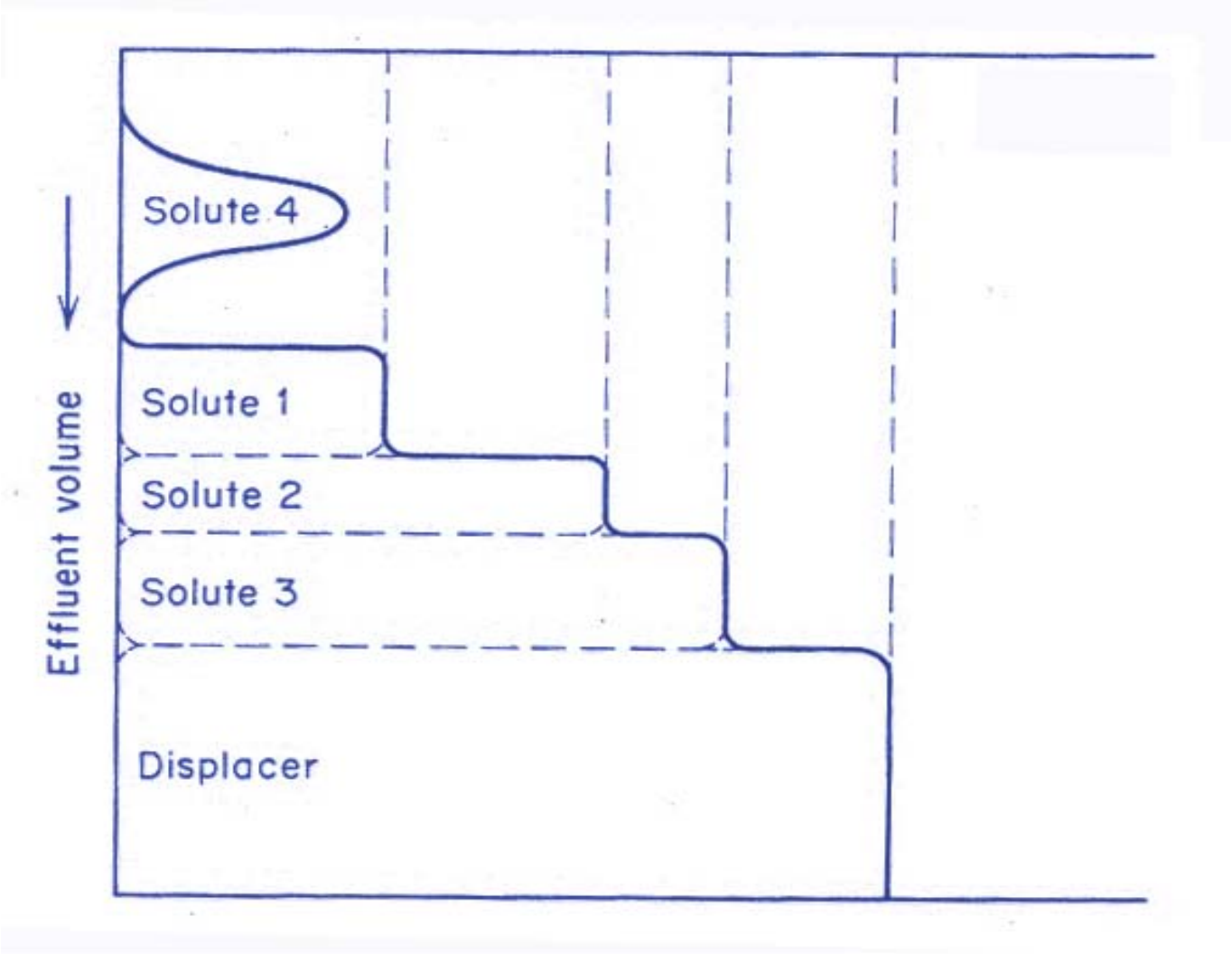
ELUTION DEVELOPMENT



3. Displacement Development

In this type of development separation of the sample ingredients is achieved by running a more strongly adsorbed displacing agent through the column. All the sample components are forced out of their sorption sites and compelled to move ahead of the front produced by the displacer. In their turn, the sample components displace each other. The first to leave the column will be the least adsorbed followed by the next least etc.

DISPLACEMENT DEVELOPMENT



It is common practice to choose as a displacer a substance that is closely related to the substances (a higher homologue) undergoing separation.
In this system a rather heavy loading of the column is permissible. This is useful for the preparative work.

GRADIENT ELUTION

Gradient elution development is characterized by the intentional variation of the eluting conditions during the course of separation. These include composition of the mobile phase, column pressure and column temperature. In gas chromatography this is easily achieved by varying the column temperature and/or gas glow.

In liquid chromatography, composition of the eluent is easily varied. Thus mobile phase with stronger displacing properties is continuously run through the column. Thus the zones are forced more closely together and they become narrower. Several solvent mixing devices are available today for efficient separation of the solutes.

DYNAMICS OF CHROMATOGRAPHY

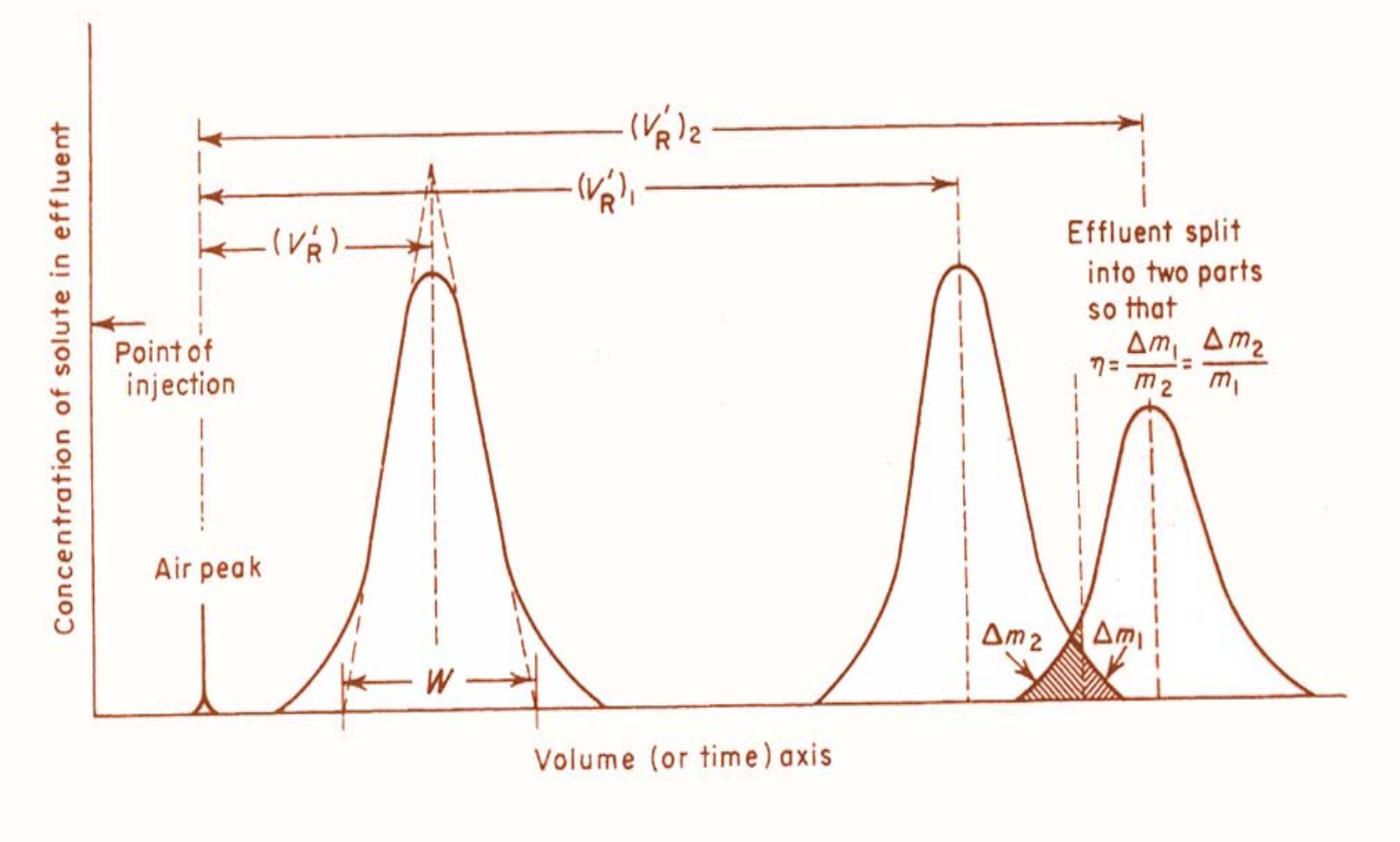
(i) Efficiency

The measure of the column efficiency is expressed by the plate number N given by,

$$N = 16(V_R/W)^2 \text{ or } 8\,(V_R/W_e)^2$$

where W is the width of the elution peak measured in volume units, W_e is the width at C_{max} or 0.368 C_{max}. C_{max} is the concentration of the material in the effluent at peak maximum.

COMMON ELUTION CURVES AND TERMINOLOGY



The retention volume may be replaced by retention time and the width by time.

Under ideal conditions, the chromatogram approaches a Gaussian curve and the width could be 4σ. Basically plate number compares the narrowness of a peak to the length of time ($t_R = V_R/F_c$) the component has been in a column .

Plate height H is the length of the column/number of plates (σ^2/L).

(ii) Zone spreading

When a mobile phase boundary passes the solute zone the rate of sorption and desorption can not keep pace with that of the solvent. Hence the progress down the column by individual solutes resembles a random stop and go process. The net forward travel of each component is actually an average value and there is a normal dispersion of values around the mean.

Further the solutes initially occupy a number of plates. The effect will be that a number of chromatograms are started successively which lead to zone width. Zone spreading is considered to be due to a series of molecular diffusion and local non equilibrium patterns originating in the velocity in equalities of the flow pattern but the extent of spreading is governed by diffusion between fast and slow stream paths.

(iii) Resolution

The degree of separation of two components is a problem common to all chromatographic methods. In truth complete separation can never be achieved because a chromatographic peak approaches in shape a gaussian distribution. In actual practice minimization of the degree of overlap or cross contamination between the adjacent zones to a desired experimental level is aimed at. Resolution is thus essentially a measure of the degree of separation of zones.

Mathematically,

$$R_S = \Delta Z / 4\sigma$$

where ΔZ is the separation of the zone centres.

For column elution methods,

$$R_S = (V_{R,2} - V_{R,1}) / 0.5 (W_2 + W_1)$$

Resolution is influenced by relative retention ratio, partition coefficients ratio, plate number and column length.

QUANTITATIVE EVALUATION AND SEPARATION

Elution is generally continued until all the components have left the column. This permits reuse of the column. Analysis of column effluent can be done by any method suitable for continuous analysis e.g spectrophotometry, refractometry, polarography, radiochemical etc.

Frequently automatic equipment is employed in conjunction with fraction collector and recording. Ideally the property chosen for monitoring the effluent should be linear function of the solute concentration and the detector response should also be linearly related to the operative property of the mobile phase.

Quantitatively the total amount of a solute eluted is measured by the area under a peak or peak height or gravimetric methods.

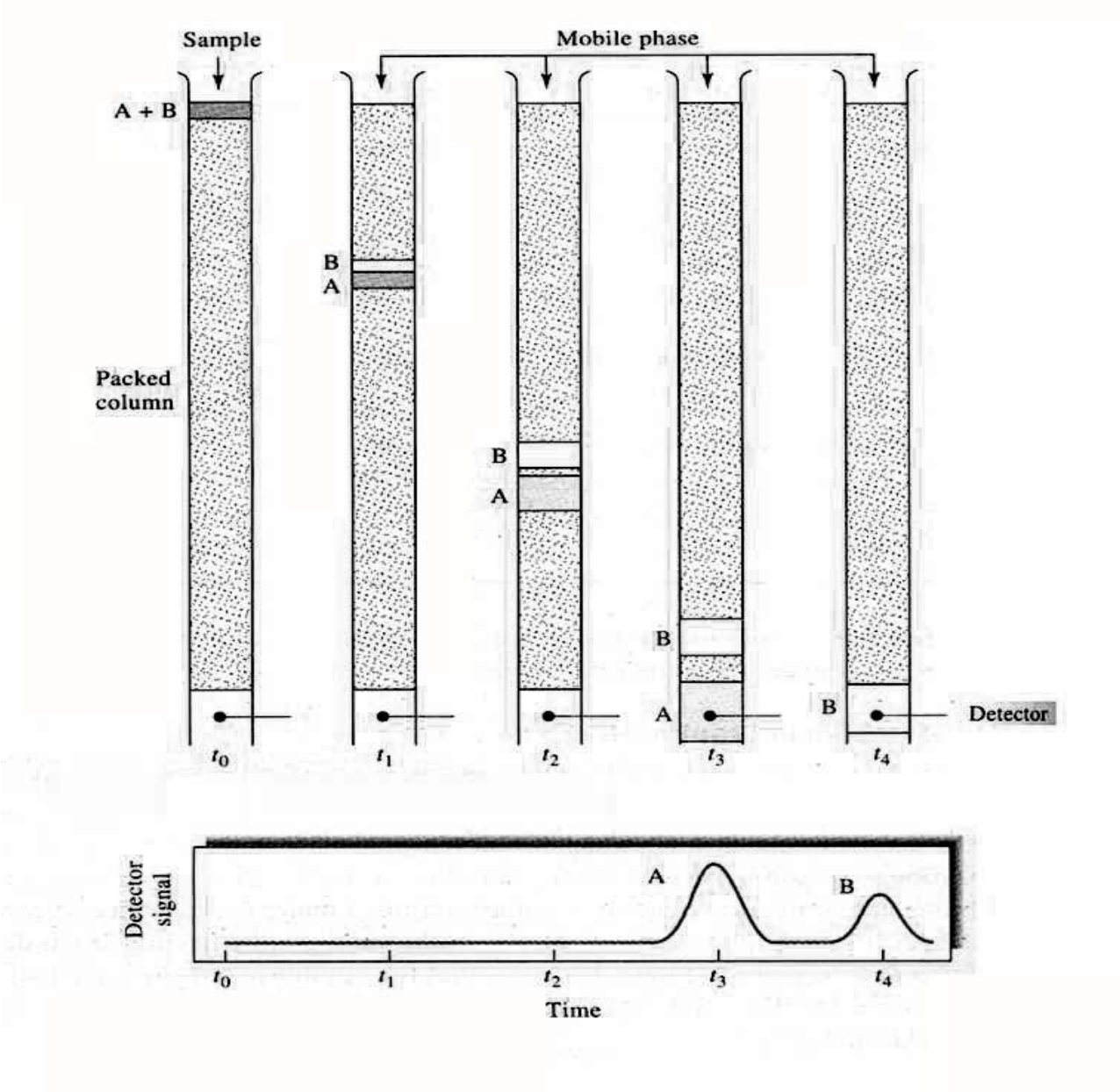
FRACTION COLLECTION

Fraction collectors enable the elute to be collected in portions as desired and permits one to characterize various species present in each fraction by independent methods. These are based on lapse of equal interval of time, constant fraction size or detector out put or drop counting. A high degree of automation is possible in all these cases.

GAS CHROMATROGRAPHY

Gas chromatography differs from other forms of chromatography in that the mobile phase is a gas and the components are separated as vapours. The separation is accomplished by partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support. A sample containing the solutes is injected onto a heated block where it is immediately vaporized and swept as a plug of vapour by the carrier gas stream into the column inlet. The solutes are adsorbed by the stationary phase and then desorbed by fresh carrier gas.

ELUTION METHOD OF GAS CHROMATOGRAPHY



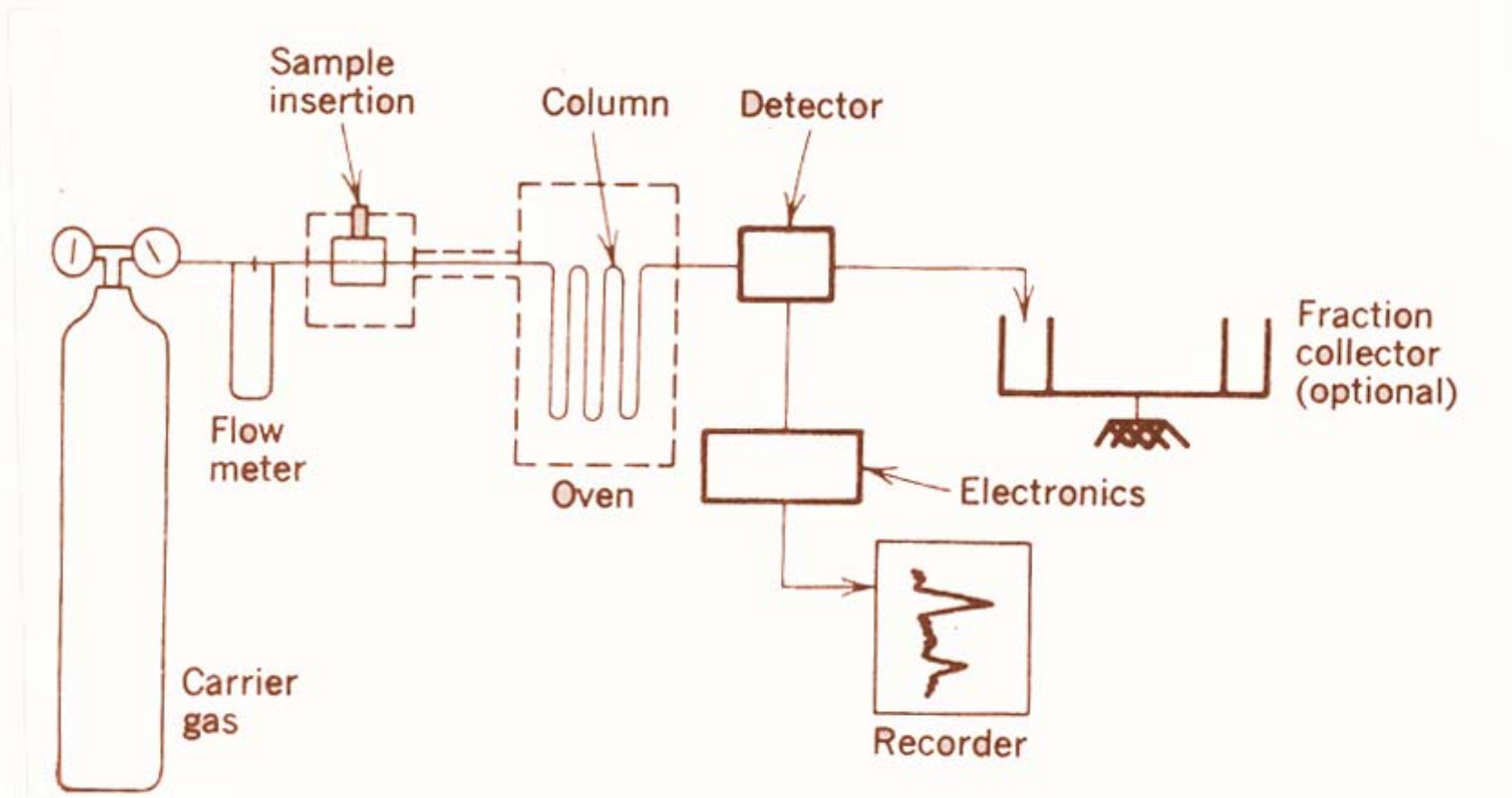
The process is repeated in each plate as the sample is moved toward the outlet. Each solute will travel at its own rate through the column. Their bands will separate into distinct zones depending on the partition coefficients, and band spreading. The solutes are eluted one after another in the increasing order of their k_d, and enter into a detector attached to the exit end of the column. Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.

The appearance time, height, width and area of these peaks can be measured to yield quantitative data. Use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of few minutes. Higher working temperatures up to 500^0 C and possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.

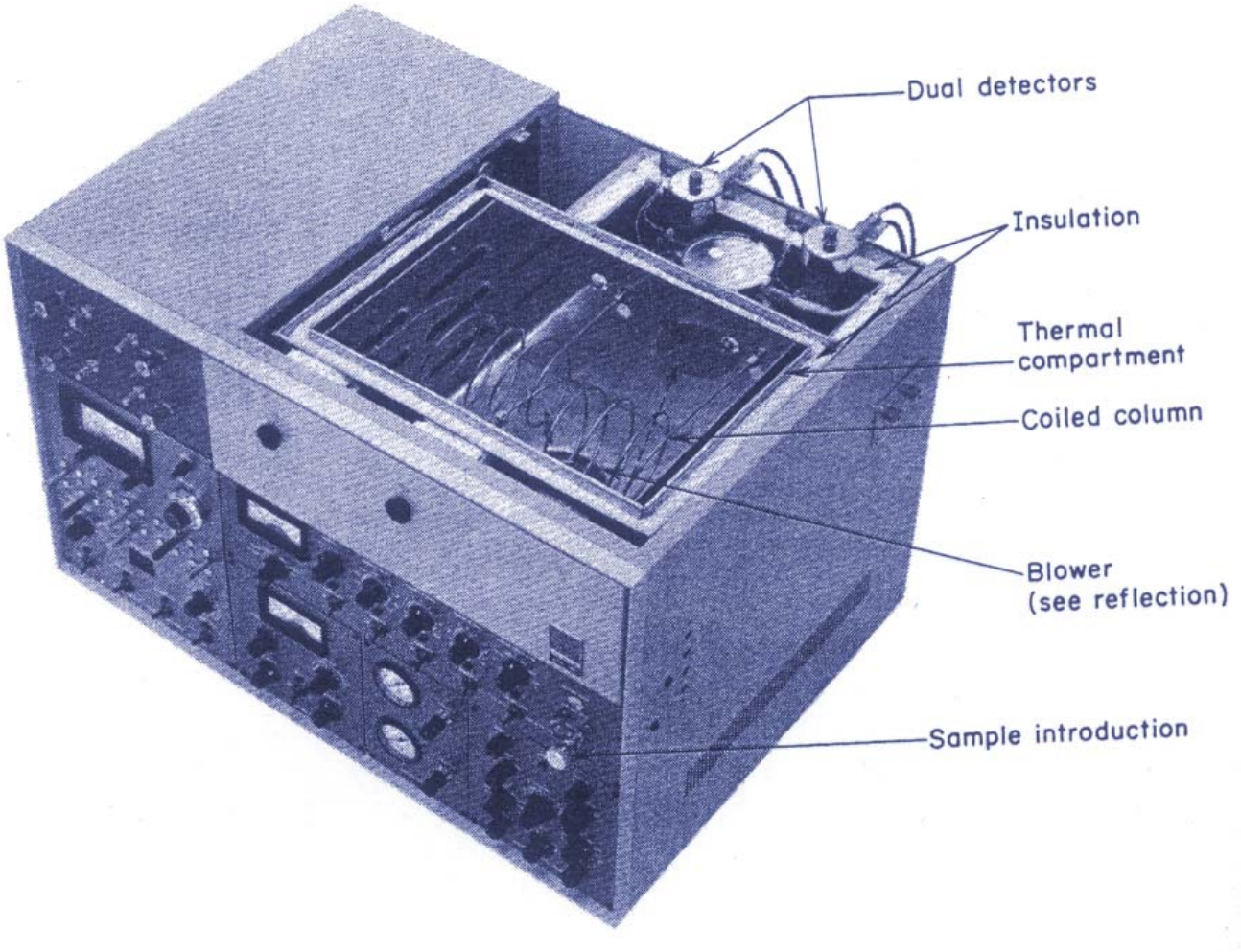
Gas chromatography is composed of six parts:

- Supply of carrier gas in a high pressure cylinder with attendant pressure regulators and flow meters
- Sample injection system
- The separation column
- Detector
- Recorder
- Separate thermostated compartments for columns and detectors

SCHEMATIC OF GAS CHROMATOGRAPH



GAS CHROMATOGRAPH



1. Pressure regulator

Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml / min), capillary restrictors, and a pressure gauge (1-4 atm). Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.

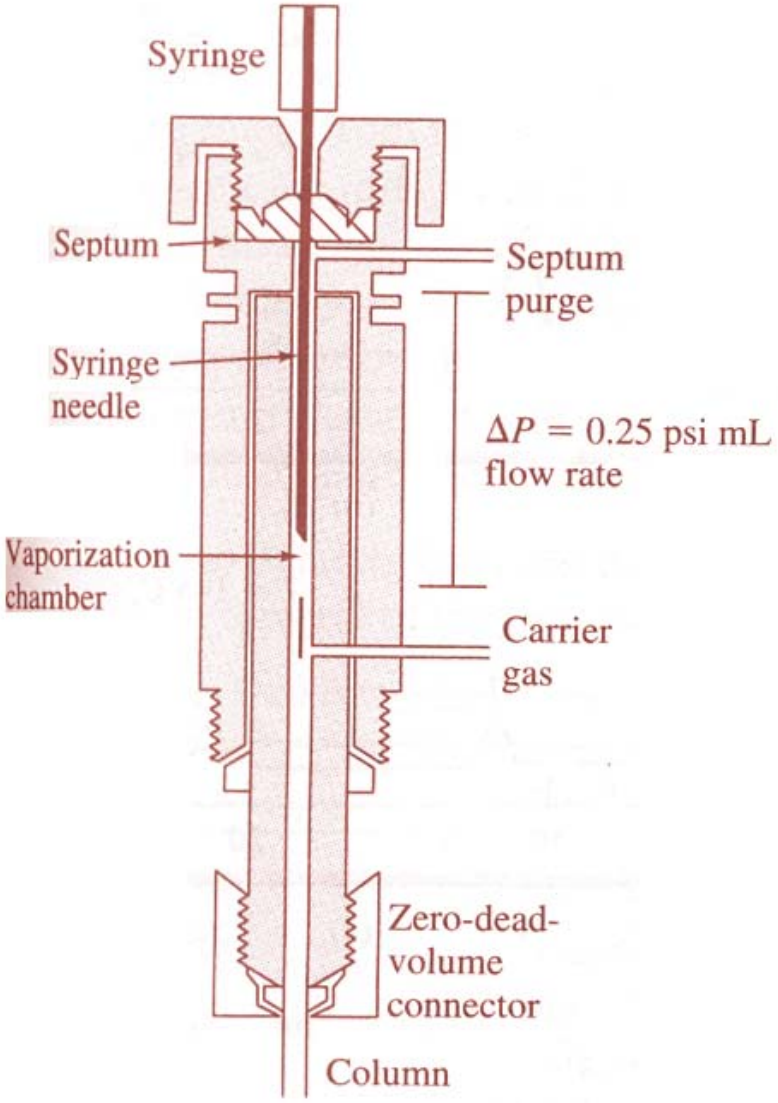
The operating efficiency of the gas chromatograph is directly dependant on the maintenance of a constant gas flow, but the contaminants in the carrier gas may affect the column performance and detector response. When ionization detectors are used. Hence a molecular sieve of $5A^0$ is used as a trap for the removal of hydrocarbons and water vapour.

Helium, N_2, H, Ar are used as carrier gases. He is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapours. N_2 is preferable when large consumption of carrier gas is employed.

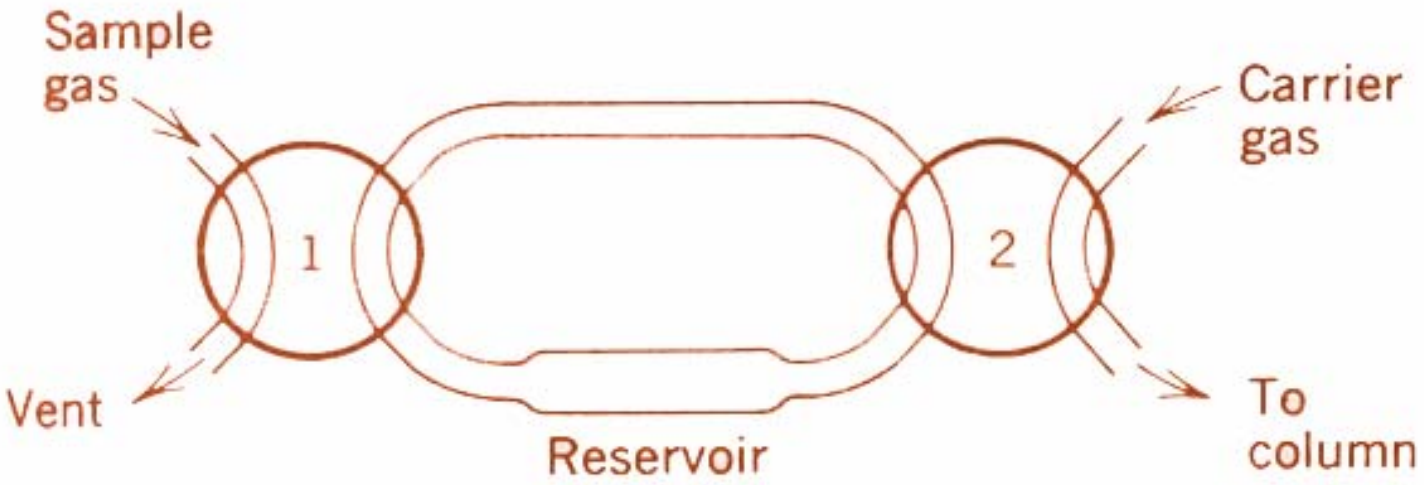
2. Sample Injection

Liquid samples are injected by a micro syringe with needle inserted through a self scaling, silicon-rubber septum into a heated metal block by a resistance heater. The insertion of sample is the most exacting problem in gas chromatography which needs lot of practice like an art. Insertion, injection and withdrawal of the needle should be performed quickly, smoothly and with proper reproducibility. Gaseous samples are injected by a gas tight syringe or through a by-pass loop and valves. Typical sample volumes range from 0.1 to 0.2 ml.

SAMPLE INLET SYSTEM



GAS SAMPLE INJECTION



3. Chromatographic column

The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape. Copper is useful up to 250^0C. Swege lock fittings make column insertion easy. Several sizes of columns are used depending upon the requirements.

COLUMN SPECIFICATIONS

Parameters	Open tubular	1/16 *	1/8 *	1/4 *	3/8 *
ID (mm)	0.25 0.50	1.2	1.65	3.94	8
Length (m)	100	20	20	20	30
Plates	3,00,000	60,000	48,000	30,000	15,000
Plates / m	3000	3000	2400	1500	750
Liq.phase	……..	3	5	10	20
Film thickness	1	5	5	10	20
Sample size (µ1)	0.01	1.0	2.0	20	1000

* All dimensions in inches

4. Supports

Plays a key role. Structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.

The support should be inert but capable of immobilizing large volume of liquid phase as a thin film over its surface. But surface area should be large to ensure rapid attainment of equilibrium between stationary and mobile phases. Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.

Diatomeceous earth, kiesulguhr treated with Na_2CO_3 for 900^0 C causes the particle fusion into coarser aggregates. Micro amorphous silica is converted into crystobalite which is marketed as chromosorb w, celite, diatoport w, gas-chrom, ana-chrom etc.

Diatomite is crushed, blended and calcined above 900^0 C which forms a pink powder known as chromosorb P. This is less fragile, high density, free flowing and capable of holding a large volume of liquid phase. This has 80% void space with 9μ pore size but the white one has 90% void space and 2 μ pore size. These pink columns are more efficient.

Both these have active sites on their surfaces which cause tailing with more polar solutes. These are due to metallic impurities, silanol (SiOH) and siloxane groups which give rise to H bonding effects. Acid washing removes mineral impurities and reduces the surface activity caused by –OH groups associated with Fe and Al but not silanol and siloxane groups. Silinization by dimethyl dichlorosilane or hexamethyl disilazane reduces surface activity and tailing. Since this reduces surface area of the support also more than 10% loading can not be used.

Coating of the support with stearic acid incorporated in silicone oil eliminates tailing in the separation of fatty acids. For amines KOH may be used for coating.

Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases. 0.05 – 0.2% loadings permit the analysis of high boiling substances at fairly low temperatures.

Porous polymer beads differing in the degree of cross linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250^0 C. These are marketed as Porapak P,Q,R,S etc.

The mesh size of the support determines the average particle diameter which in turn determines the HETP. Thus theoretically smallest particles should be used but it causes the permeability {α(particle dia)^2} and pressure drop {1/(dia)^2} to drop in longer columns. Coarser particles can hence be used. Best columns are 80/100 mesh for 1/8 inch with diatomaceous earth type supports. For effective packing of any column the internal diameter should be at least 8 times the diameter of the supports.

5. Liquid phases

An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support. No single phase will serve for all separation problems at all temperatures.

(i) Non Polar – Parafin, squalane, silicone greases, apiezon L, silicone gum rubber (SE-30). These materials separate the components in order of their boiling points.

(ii) Intermediate Polarity – These materials contain a polar or polarizable group on a long non polar skeleton which can dissolve both polar and non polar solutes. For example diethyl hexylphthalate is used for the separation of high boiling alcohols.

(iii) Polar – Carbowaxes – Liquid phases with large proportion of polar groups. Separation of polar and non polar substances.

(iv) Hydrogen bonding - Polar liquid phases with high hydrogen bonding bonding e.g. Glycol.

(v) Specific purpose phases – Relying on a chemical reaction with solute to achieve separations.
e.g $AgNO_3$ in glycol separates unsaturated hydrocarbons.

The maximum temperature at which a liquid phase may be used is determined by its volatility. Excessive volatility shortens the column life, contaminates the gas stream and affects the baseline stability. The operating temperature is determined by its viscosity or solidification point.

6. Column loading

15% loading means 15% liquid phase and 85% support. The lower the amount of the solid phase, the smaller is the sample that can be handled.

7. Open tubular columns (capillary)

An open glass capillary tube coated with liquid phase on the interior of the tubing. This permits use of very long columns (30-100 meters). Because of the low pressure drop, such columns give a very high overall column efficiency. Theoretical plates of 100,000 are possible. The diameter could be 0.07 – 0.25 mm. But the sample capacity is only about 0.1 to 0.3% of normal samples.

8. Support coated open tubular columns

These are fuzzy-wuzzy columns. A thin layer of porous material is deposited on the inner wall of an open tube and then coated with a liquid phase. This causes the internal surface area to increase. Thus the β value and film thickness are reduced by an order of magnitude. Thus fewer theoretical plates are necessary (shorter columns), resulting in shorter analysis time, and better resolution.

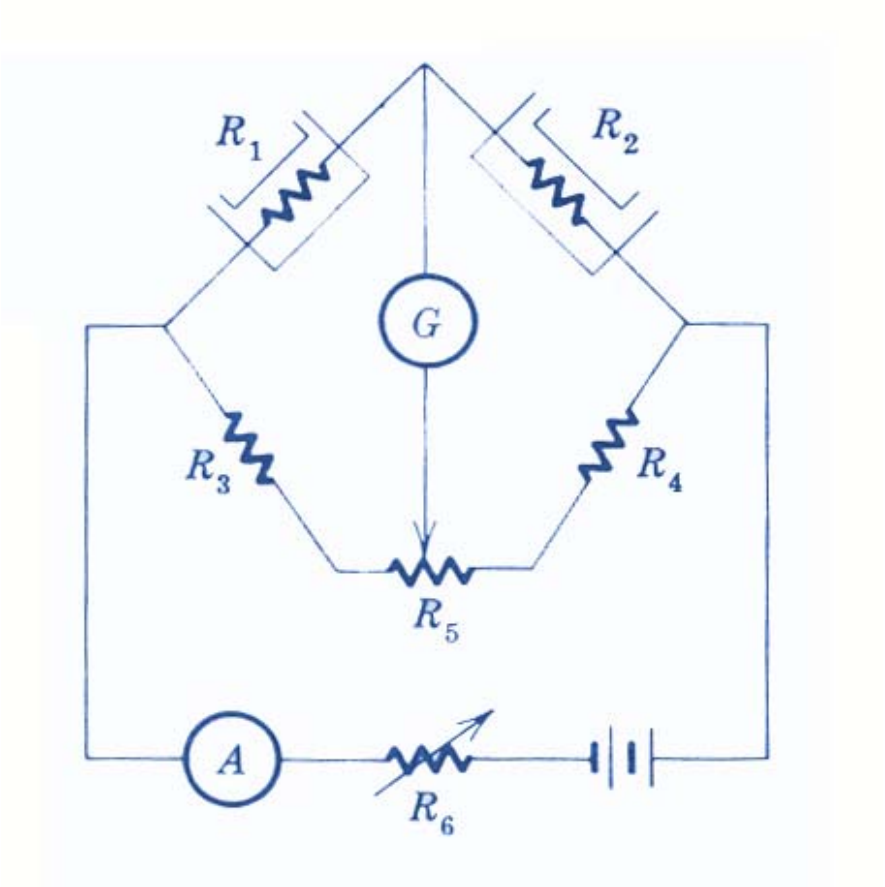
9. Detectors

Detectors sense the arrival of the separated components and provide a signal. These are either concentration dependant or mass dependant. All detectors are characterized by linear dynamic range (LDR - largest signal / smallest signal). Signal to noise ratio is 2 for the smallest signal. The detector should be close to the column exit and correct temperature to prevent decomposition.

(i) THERMAL CONDUCTIVITY DETECTOR

It consists of 4 heating elements located in a heating cavity of brass or steel block which serves as a heat sink . Thermistors or resistance wires are used with Pt-Ir springs. Filaments are gold sheathed tungsten or teflon coated tungsten with high temperature coefficient of resistance. The cell cavity has 2.5 ml capacity for large TCDs or 0.025 ml for micro TCDs (5 ml).

THERMAL CONDUCTIVITY DETECTOR



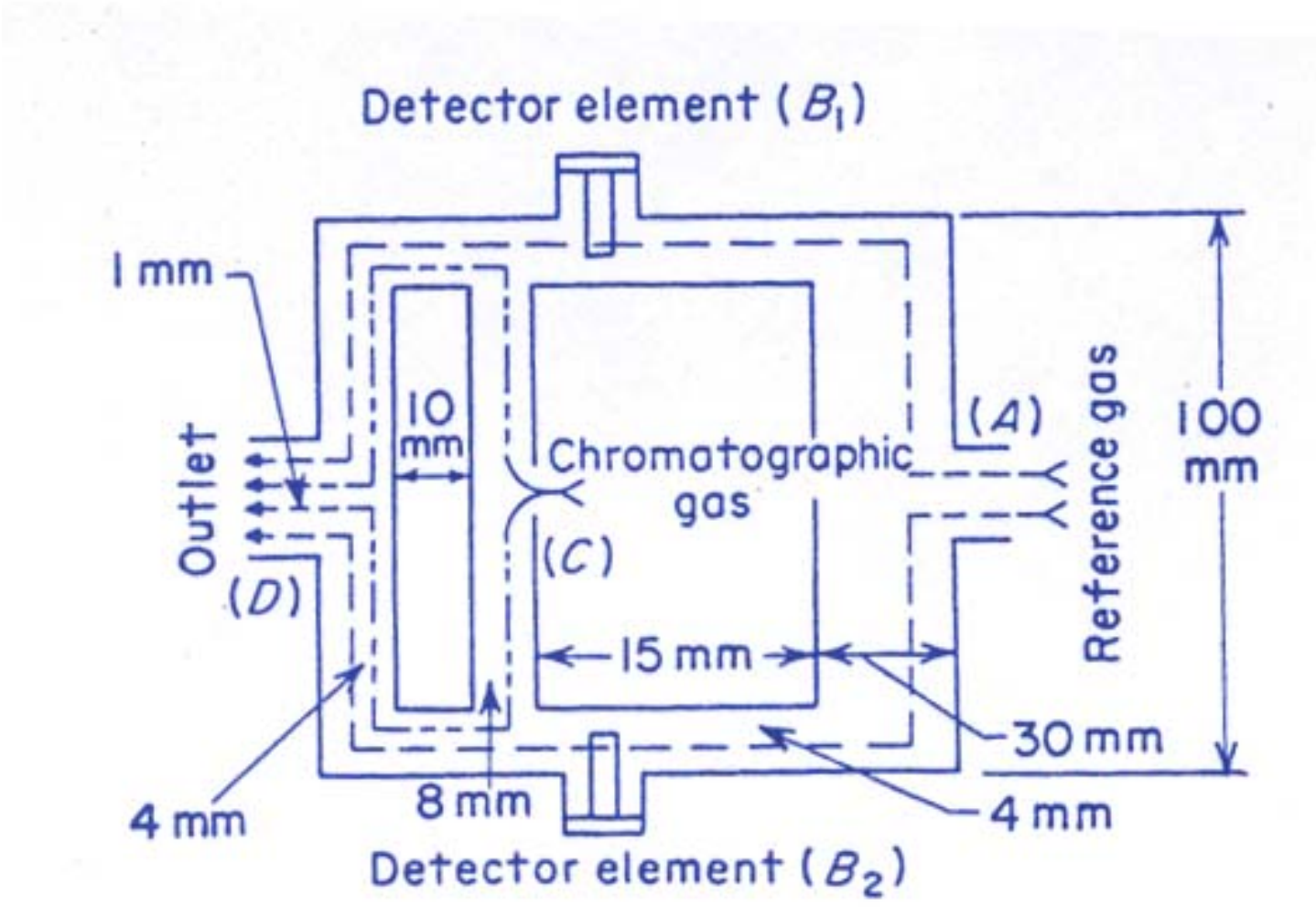
Thermal conductivity of the carrier gas and sample + carrier gas is measured. The TCD cells are connected to form the arms of a Wheatstone bridge. When pure gas passes both reference and sample wires are cooled to the same extent. When the solute emerges, the rate of cooling in the sample changes and the Wheatstone bridge is out of balance. This is recorded as a peak. Both He and H are useful as carrier gases since their thermal conductivities are different from the sample components.

(ii) GAS DENSITY DETECTOR

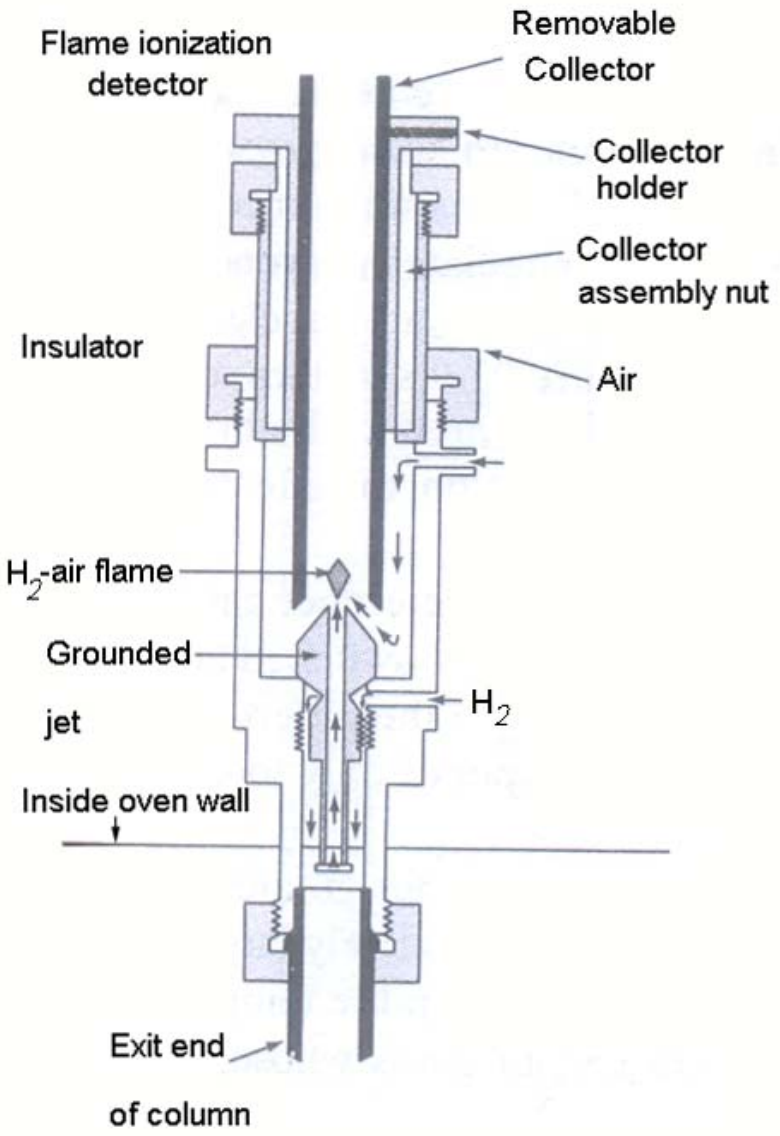
The sample and carrier gas are split into two streams, where cooling is monitored. Two flow meters B_1 and B_2 are installed into the stream and are wired in a Wheatstone bridge. The reference gas enters at A, splits into two and exits at D. The effluent enters at C, splits into two mixes with the carrier gas and exits at D. The effluent does not come into contact with detector elements and hence no contamination and carbonization. But it takes the path AB_2D with a temperature rise in B_2 and decreases in temperature of B_1 in AB_1D. The bridge imbalance is recorded as a signal.

The detector accommodates a sample volume 5 ml sample volume of 5 ml, operating temperatures of 100-300^0 C.

GAS DENSITY DETECTOR



FLAME IONIZATION DETECTOR



(iii) FLAME IONIZATION DETECTOR

The column effluent enters the FID after a particulate filter. Hydrogen and air mixture is burnt to obtain a plasma of around 2100^0 C which has sufficient energy to ionize any organic solute passing through it. The ions are collected at the anode and electrons are collected at the cathode. The resulting ionic current is monitored by measuring the voltage drop across a series resistor (300V). Up to 10^7 - 10^{10} Ω resistors are used. Currents as low as 10^{-14} A can be measured.

When the solute is burnt, a large increase in the electrical conductivity is seen due to the number of carbon atoms. The detector is insensitive to water, permanent gases, inorganic components, CO, CO_2 and etc. It is useful for aqueous extracts and for air pollution studies. But current decreases for substituted amines, halogens, OH groups etc. Linear dynamic range (LDR) is 10^6. A sample splitter is necessary. Precise temperature control is not a rigid requirement.

FID is insensitive to water and permanent gases such as CO, CO_2, CS_2, SO_2, H_2S, NH_3, N_2O, NO, NO_2, SiF_4, $SiCl_4$ etc. Therefore it is advantageous in moist air samples for the analysis of organic components. CO and CH_2 get converted to CH_4 over nickel catalyst and the sensitivity is 5 ng / sec^{-1}. For higher organic gases the sensitivity is 10 pg / sec^{-1} . LDR is 7.

(iv) PHOSPHOROUS DETECTOR

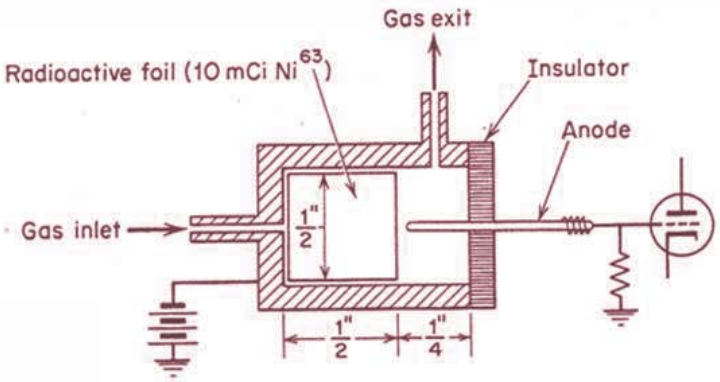
This is a modified flame ionization detector with the tip being made of CsBr. When a compound containing phosphorous is burned, alkali metals are released which ionizes and increases the current flow. Up to 10^{-12} g phosphorous compounds can be analysed in this way. The LDR is 1000. This detector is useful for the determination of phosphate additives in gasoline and pesticides.

(v) ELECTRON CAPTURE DETECTOR

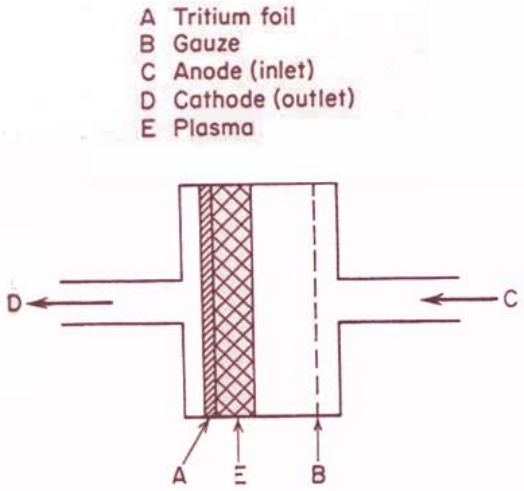
This is based on the electron capture by compounds having affinity for free electrons. This causes the loss of signal due to recombination phenomena.

As the nitrogen carrier gas flows through the detector, β particles from a tritium source (or Ni-63) ionize the nitrogen molecules and form slow electrons. These migrate to the anode under a fixed potential which can be varied from 2-100 V and generate 10^{-8} – 10^{-9} A base current.

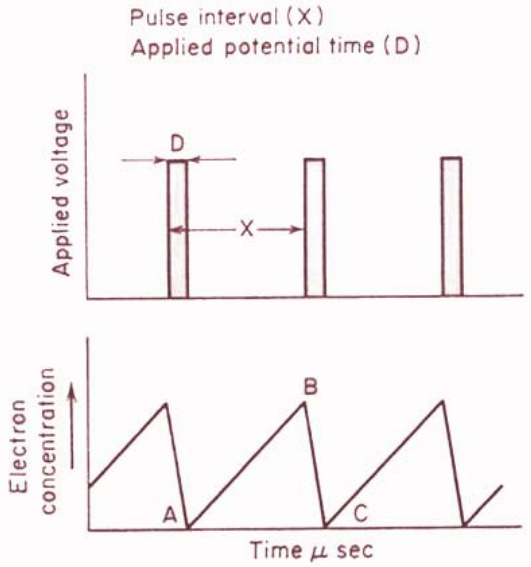
ELECTRON CAPTURE DETECTOR



(a) Pin cup design



(b) schematic diagram



(c) pulse sampling mode.

When an electron capturing solute emerges from the column, it passes through a low energy free electron moving area. The solute reacts with an electron to form a negative molecular ion or a neutral radical or a negative ion. Since these ions move more slowly than the free electrons, a reduction in net current occurs which is proportional to its concentration.

$$i = i_0 e^{-kxc}$$

where k is constant depending on the field strength, c is the absorption cross section of the vapour, x is a geometric factor, i_0 is the initial current and i is the final current.

The ECD can be operated either in pulsed mode or under a constant voltage. Thus either a season signal results or a pulsed current.

ECD is extremely sensitive to organic and inorganic halogen containing compounds, hydroxides, peroxides conjugated carbonyls, nitrites, nitrates, ozone, oxygen, organo metallic and sulphur containing compounds.

But it is insensitive to hydrocarbons, amines, ketones etc. Pesticides and organo metallics in gasoline are the most preferred applications.

(vi) OTHER DETECTORS

Flame emission, conductivity detectors, rf discharge detectors, infrared and mass spectrometer are other detectors.

RECORDING

The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals. An integrator is a good addition too.

THERMAL COMPARTMENT

Precise control of column, injection block, and column oven and detector units up to 0.1^0 C is desirable. Maximum operating temperature should be 500^0 C. Separate heaters for each with a rapid heating is a must. Also rapid cooling is essential for multiple operations.

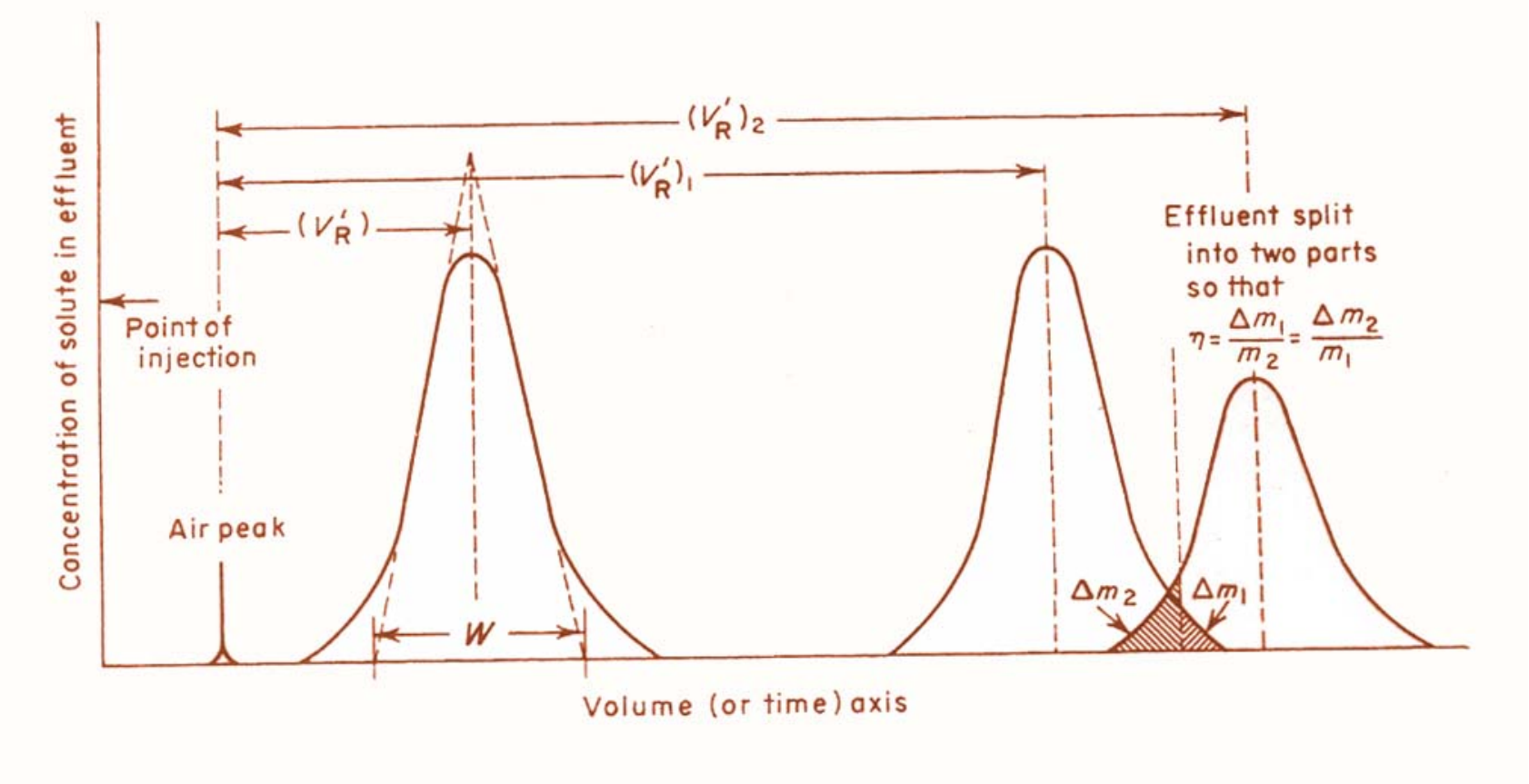
Gas chromatographic theory covers complex interactions of all the variables. But a brief treatment of basic parameters is considered here.

RETENTION BEHAVIOUR

For a given column operating at temperature T_c and carrier gas flow rate F_c, the retention time for any component in the column is a constant. On a chromatogram, the distance on the time axis from sample injection to the peak of the eluted component is called uncorrected retention time t_r.

$$V_R = t_r F_c$$

IDEALIZED ELUTION PEAKS



Gas flow rate must be corrected to the column temperature and outlet temperature P. The air spike measures the transit time for a non retained substance. Converted to volume V_m, it represents the interstitial volume of the gaseous phase in the column plus the effective volume contribution of the injector port and detection. Thus V^l_R has to be corrected for dead space.

$$V^l_R = t_r F_c - t_{air} F_c = V_R - V_M$$

Since gas moves more slowly near the inlet than at the exit of the column, a pressure gradient correction or a compressibility factor j to V^l_R must be applied to get the net retention volume.

$$V_N = j\, V^{l}_{R} \text{ where } J = \frac{3}{2}\,\frac{[\,(Pi/Po)^2 - 1\,]}{[\,(Pi/Po)^3 - 1\,]}$$

Since $V_R C_M = V_M C_M + V_S C_S$

$$V_R = V_M + K_d V_S$$

$$\text{or } K_d V_S = V_R - V_M = K_d\,(w_L/\rho_L)$$

where w_L = weight of liquid phase,
ρ_L = density of liquid phase

$V_g = (273/\rho_L)\,(K_d/T_C)$ = specific retention volume

where or $K_d = V_g.\,\rho_L\,(T_C/273)$ or $V_g = (273/\rho_L)\,(K_d/T_C)$

TEMPERATURE DEPENDENCE

$$\log V_g = \frac{\Delta H}{2.3\, RT_C} + \text{const}$$

ΔH = partial molar heat of the solute in the liquid phase

By plotting, $\log V_g \rho_L$ Vs $1/T_C$, $\Delta H /2.3\, R$ may be obtained which is a linear function.

Lower operating temperature leads to increased retention. Every 30^0 C reduction will double the retention volume.

A knowledge of ΔH and V_g helps in calculating the order in which peaks will emerge.

e.g > 67^0 C methyl cyclopentane and 2,4 dimethyl pentane

< 67^0 C 2,4 dimethyl cyclopentanone and methyl cyclopentane

RELATIVE RETENTION

Expressed as ratio of the retention time of a standard.

$$\alpha = t'_R / t'_R \text{ std} = (K_d)_2 / (K_d)_1$$

α is independent of column length, carrier flow rate, compressibility factor , liquid solid support ratio etc.

Retentions for various solute classes are tabulated for typical stationary phases which helps in estimating whether a mixture can be separated or not. Such tabulations will be found in the J.chrom, science, journal of gas chromatography, chromatography reviews, texts etc.

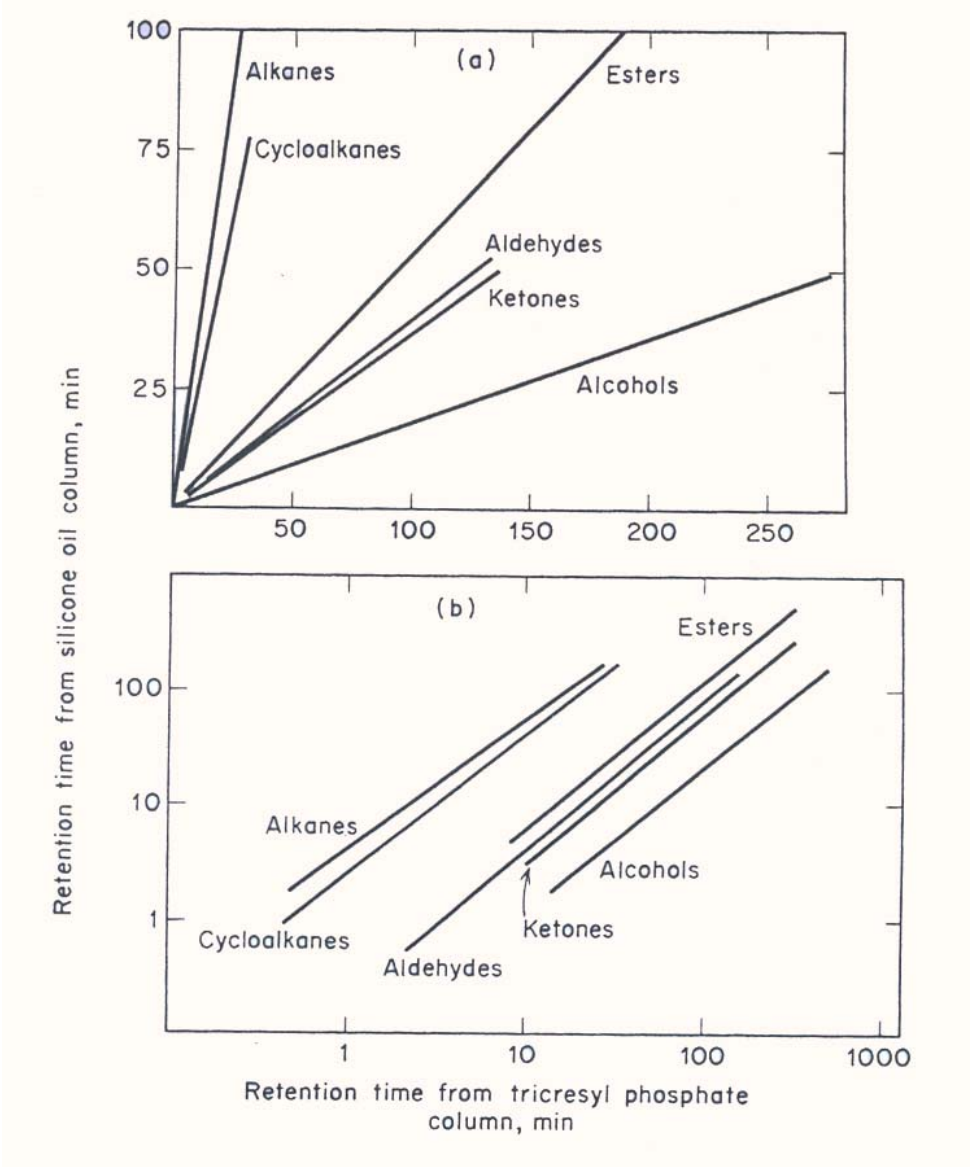
Kovat's Retention Index relates isothermal data i.e retention volume to those of n paraffins eluting directly before the sample.

$$I = 100\,n \text{ where n is the number of carbon atoms.}$$

$$= 100\,i\left(\frac{\log R_x - \log R_N}{\log R_{(N+i)} - \log R_N}\right) + 100\,n$$

where R_x, R_N, $R_{(N+i)}$ are retentions of unknown, paraffins of carbon n and (n+i). Hence it is carbon n-retention relation.

LINEAR AND LOG PLOTS



APPLICATIONS OF GAS CHROMATOGRAPHY

1. Reaction gas chromatography

The injected substances pass through a reaction zone ahead or within the injection port or in a column or post column.

(a) Subtractive processes

1. St.chain alkanes + cyclic alkanes and branched alkanes $\xrightarrow[\text{Mol.size}]{5A^0}$

Hexane , heptane, octane $\xrightarrow{268^0\ C}$ **hexane is not absorbed from a mixture of hexane , heptane, octane**

2. Olefins + aromatics $\xrightarrow[\text{in 20\% } H_2SO_4]{\text{20\% } HgSO_4}$ **only aromatics**

$\xrightarrow[\text{in 95\% } H_2SO_4]{\text{4\% } Ag_2SO_4}$ **Retains both olefins and aromatics**

3. **Water**

- $\xrightarrow{(MgClO_4)_2}$ **Removed**
- **Mol.siev 4 A^0**
- $\xrightarrow{P_2O_5/CaSO_4}$
- $\xrightarrow{CaC_2}$ C_2H_2

4. **CO** $\xrightarrow{I_2O_5}$ CO_2

2. Pyrolysis

Useful for nonvolatile compounds. Sample is cracked in a flash pyrolysis unit containing a heated filament for 15-20 seconds at high temperatures or passed through a high voltage corona discharge in a ceramic cylinder. Smaller fragments with lower boiling compounds give chromatograms of the pyrolysis products which are characteristic of specific compounds under specific conditions.

(a) Elemental analysis – high temperature - C, CO_2 , water and small molecules.

(b) Class reactions

Fatty acids $\xrightarrow[\text{Methanol}]{BF_3}$ Methyl esters

Sterols, sugars, OH groups $\xrightarrow[\text{Trimethyl chlorosilane}]{\text{Hexamethyl di silazane}}$ Silane derivatives

Fatty alcohols, OH $\xrightarrow[(AC_2)O + \text{pyridine}]{\text{acetylation}}$

Amino acids $\longrightarrow$ Methyl ester hydrochlorides

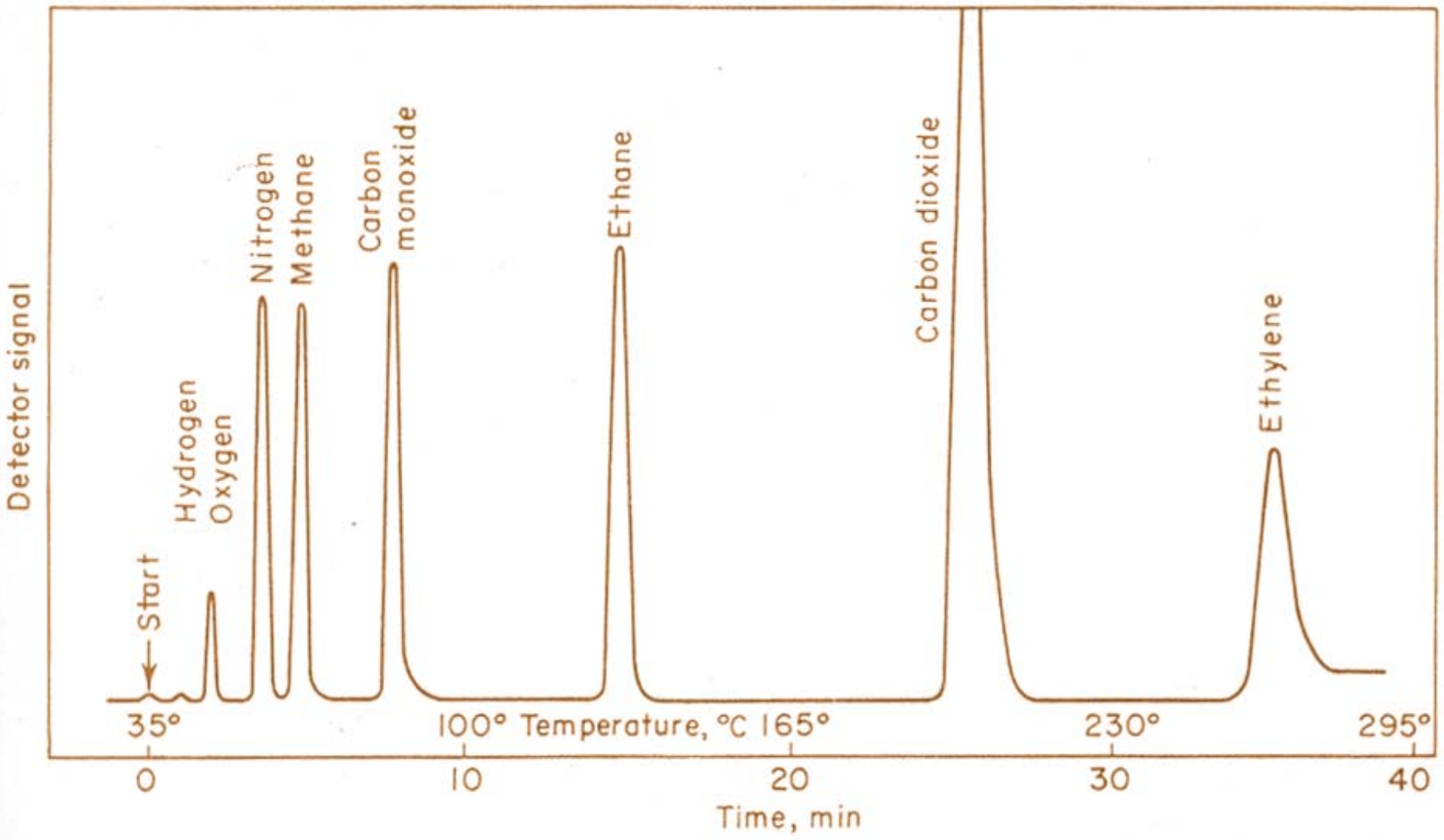
Kits for these reactions are available.

PROGRAMMED TEMPERATURE GAS CHROMATOGRAPHY

Separation of compounds having a wide range of boiling points can be improved or accelerated by heating the entire column at a fixed rate during the run. As the temperature is raised, solubilities will decrease and vapour pressure will increase and the compounds will start migrating.

Temperature programming results in time saving, sharper peaks and uniform peaks. Heating from ambient to 400^0 C within 8 minutes is possible while cooling can be effected from 400 – 100^0 C within 3 minutes.

ANALYSIS OF GAS MIXTURES BY TEMPERATURE PROGRAMMING



The number of theoretical plates for a programmed temperature GC is given by,

$$N = 16 [(V_T)_R / W]^2 \text{ (equivalent to isothermal GC)}$$

Essentially same degree of resolution is obtained provided heating rate is 1.5^0 ΔT above the isothermal temperature.

FLOW PROGRAMMING

Here carrier gas flow rate is progressively increased during analysis thus sweeping the components more rapidly through the column. Since peak height is proportional to the retention time and hence the flow rate, the height of the emerging peaks is raised as the analysis proceeds. A major advantage of flow programming is that, thermally unstable compounds can be analysed without the loss of the material and so also volatile liquid phases.

The flow programmer is a numatically controlled system which permits the pressure along the column to rise logarithmically during a predetermined time interval. It is a differential flow valve installed in the carrier gas supply line.

Higher gas flow rates generally cause lower column efficiency and the resolution may be poorer than optimum conditions. Higher flow rate also improves base line drift problems. Both FID, TCD are useful with flow programming.

GAS SOLID CHROMATOGRAPHY

Permanent gases are usually analysed using silica columns. In these columns for CO_2 can be separated from acetylene but other gases emerge as a single peak.

H_2, O_2, N_2, CO can be separated on a 4ft column packed with $5A^0$ molecular sieve

Isomers + St. chain alkanes $\xrightarrow{5A^0}$ st. chain alkanes are absorbed

QUANTITATIVE EVALUATION

1. Output of the recorder and the detector must be linear.

2. Carrier gas flow rate must be constant. Then the peak area can be measured by triangulation, integration or planimetry or gravimetry.

	Std. Deviation
Planimetry	4.0
Triangulation	4.0
Height X1/2 width	2.6
Gravimetry	1.7
Electronic	0.44

PREPARATIVE-SCALE GAS CHROMATOGRAPHY

1) Useful for IR, NMR, MS etc.

2) Columns of 1.0 to 1.5 cm in diameters and sample size can vary from 1-1000 mg

   1-2.00 g.

Danger of overloading is there if vapour phase of the sample is larger than ($0.5\ V_R/\sqrt{N}$).

Column diameter may be increased up to 3.2 cm dia (10 times more sample than 1 cm column).

Another approach is repetitive (automatic) injections of small samples on narrow dia columns of 3/8 - 3/4" dia. Sample size ranges from 5-30 ml. A high % of liquid phase is essential.

SHORT FAT COLUMNS – High volume preparative work etc.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Earlier liquid chromatographic work was usually carried out in 1.5 cm Diameter and 50-500 cm length columns with stationary phase particles diameters of 150-200 µm. These separations took long time, lasted for several hours and output flow rates were a few tenths of milliliters per minute. Attempts to increase the speed by applying vacuum or pumping were not effective because increase in flow rates increased the plate heights according to Van Deemter equation:

$$H = A + B/u + Cu$$

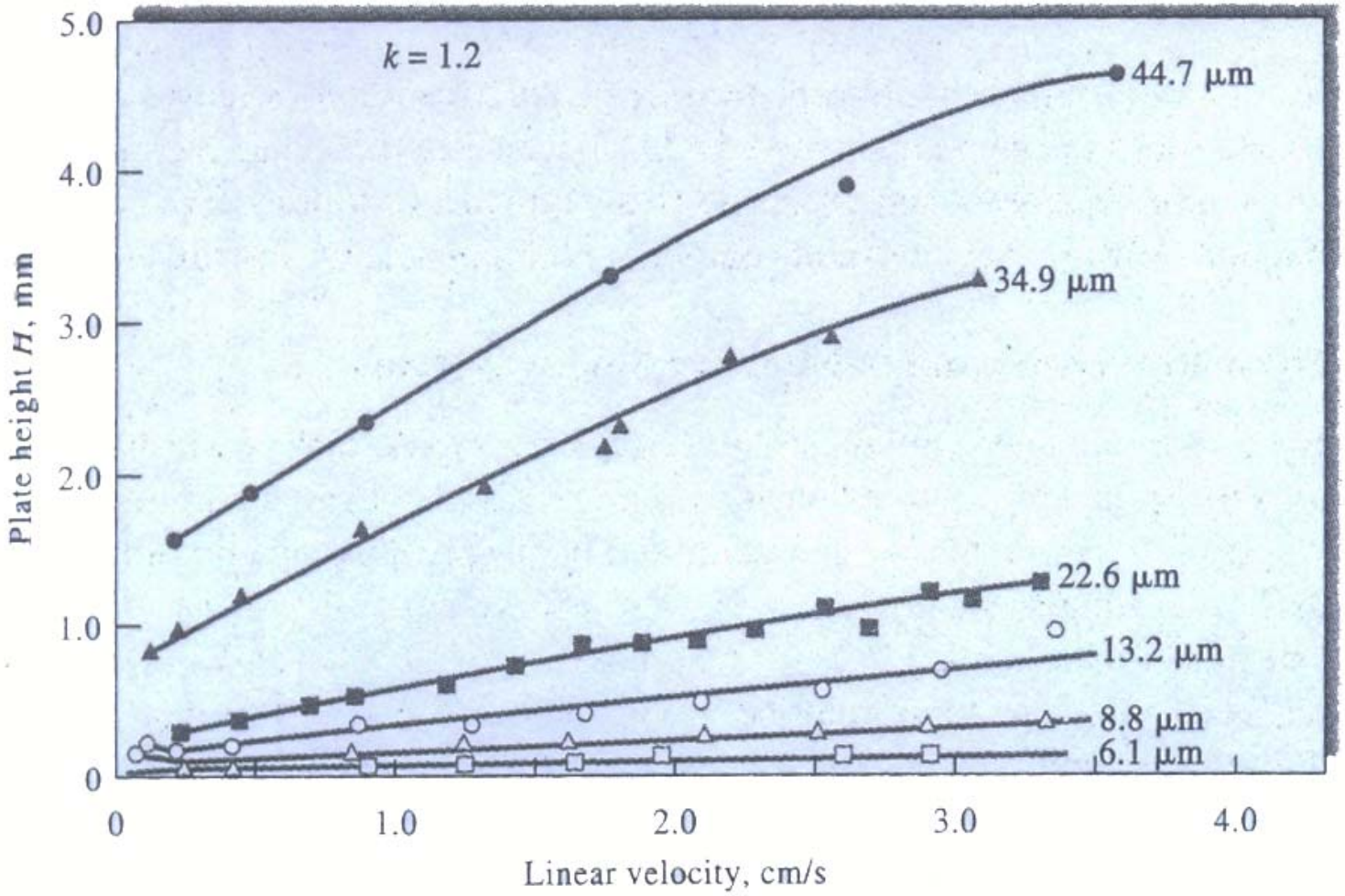
$$= A + B/u + (Cs + Cm)\,u$$

where H is the plate height (cm), u is the linear velocity of the mobile phase (in cm/sec), A,B and C are coefficients related to the phenomena of multiple flow paths, longitudinal diffusion and mass transfer between the mobile and solid phases.

Therefore decreased efficiencies were obtained. Soon scientists realized that major increases in column efficiency could be brought about by decreasing the particle size of the stationary phases to 3-10 µm. This technology required sophisticated instruments operating at high pressures.

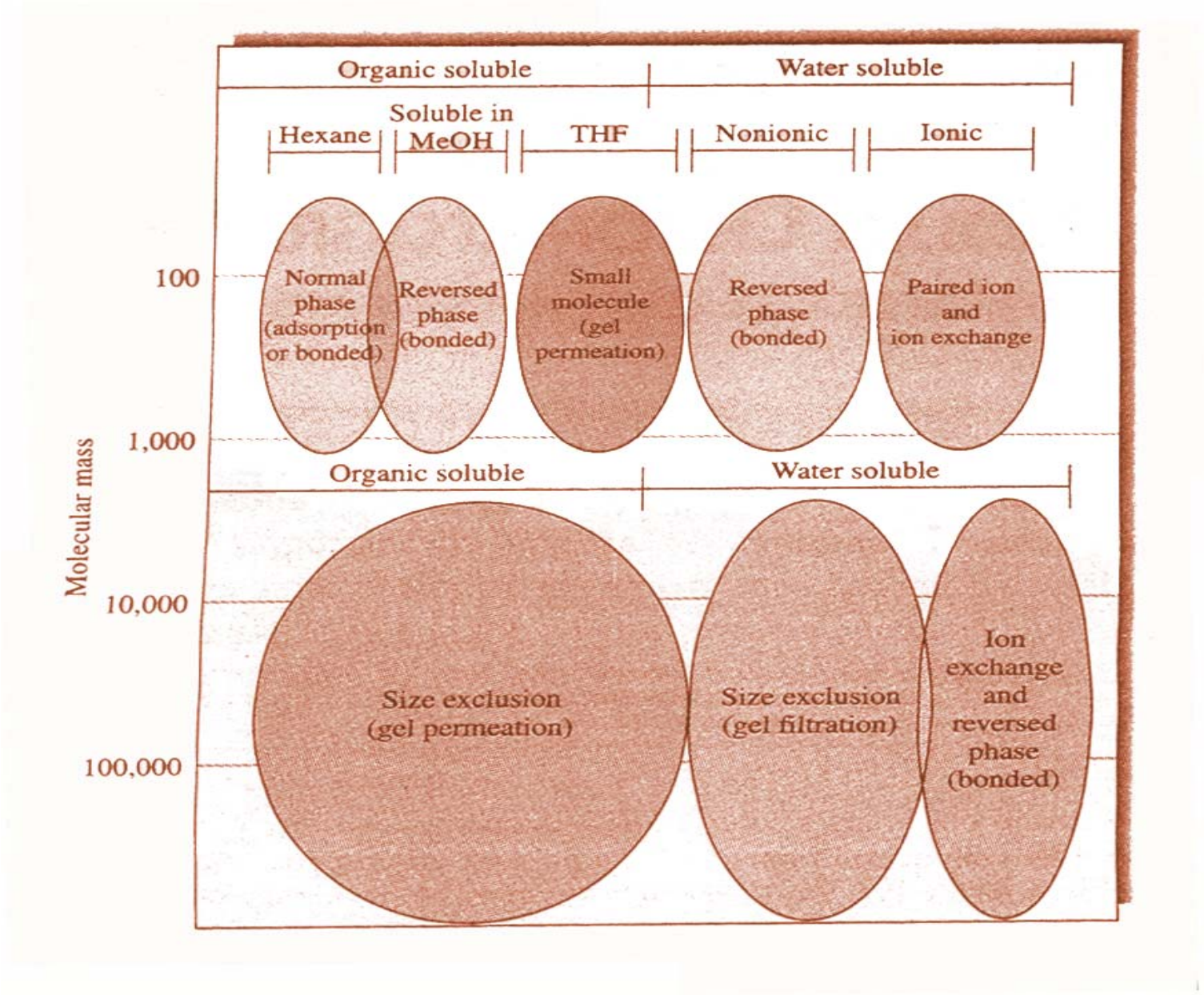
Once these techniques were developed it was realized that HPLC provided best analytical separations technology with high sensitivity, adaptability for quantitative separations. It was suitable for separating nonvolatile or thermally fragile molecules such as amino acids, proteins nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides antibiotics, steroids, metal organic species and metal compounds polymers and several other classes of compounds.

EFFECT OF PARTICLE SIZE ON PLATE HEIGHT



Molecular weights up to 10,000	Exclusion chromatography reverse phase partition
Lower molecular weight ionic species	Ion exchange chromatography Reverse phase partition
Smaller polar nonionic	Partition chromatography Reverse phase partition
Homologous series structural isomers	Adsorption chromatography Reverse phase partition

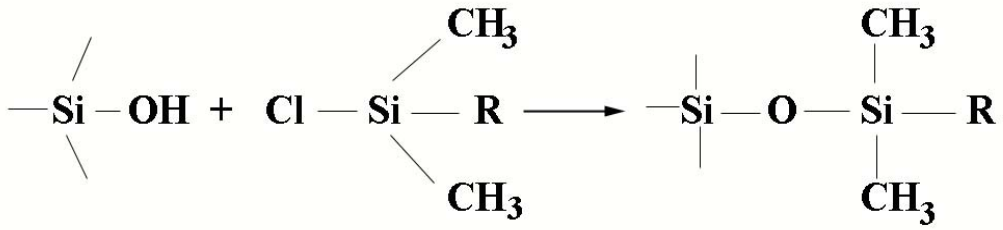
CLASSIFICATION OF HPLC TECHNIQUES



Early chromatographers used highly polar stationary phases such as water or triethylene glycol supported on silica or alumina and hexane or isopropyl ether as mobile phase. This is called as normal phase chromatography in which least polar compound eluted first.

In reverse phase chromatography, stationary phase is non polar (usually a hydrocarbon) and mobile phase is polar such as water, methanol, acetonitrile etc. Here most polar component appears first and increasing polarity increases the elution time.

Bonded phase coatings are siloxanes formed by the reaction of hydrolysed surface with an organochloro silane.



The coating is of the order of 4μ mol. m^{-2} in the bonded phase. Packings are classified as reverse phase when the bonded coating is nonpolar. R is n-octyl or n-octyl decyl groups. The particles have a brush or bristle like appearance.

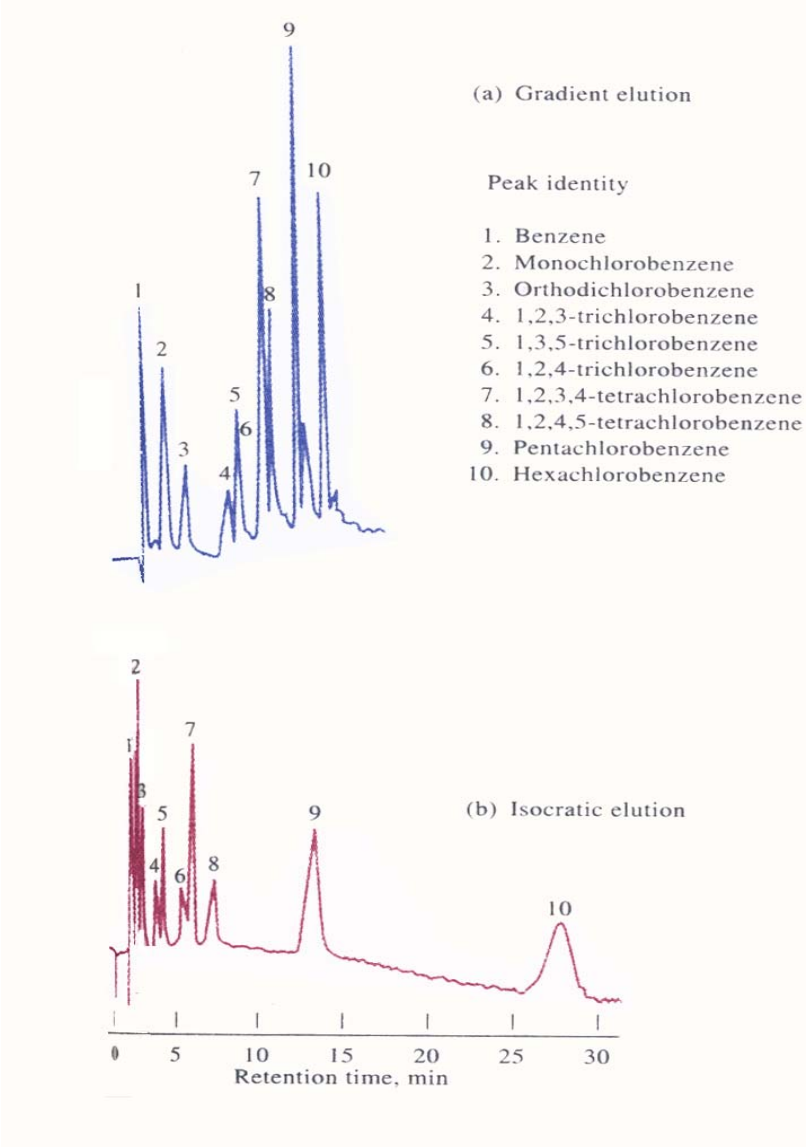
Nearly 75% HPLC work now is composed of reverse phase partition chromatography and world wide HPLC market is of the order of 1 billion dollars.

Pumping pressures of several thousand pounds per square inch are required. Hence equipment tends to be more elaborate and expensive.

Reservoirs of 200 - 1000 ml, equipped with sprayers, vacuum pumping , distillation system, heating and stirring, dust filters, Millipore filters under vacuum constitute other HPLC components.

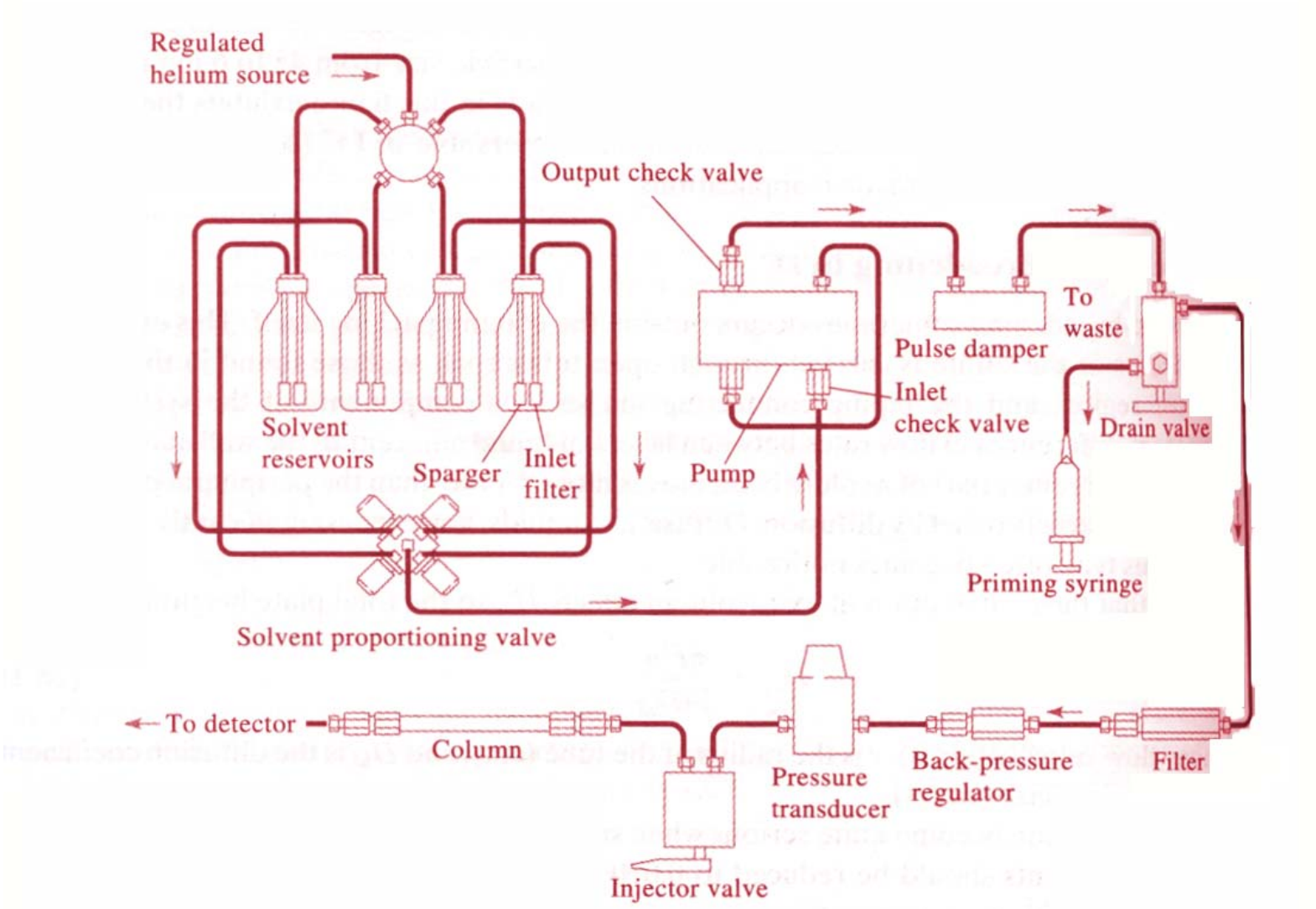
A single solvent used as mobile phase is called isocratic elution. A mixture of 2-3 solvents programmed to increase polarity in a series of steps or continuously is called gradient elution.

COMPARISION OF GRADIENT AND ISOCRATIC ELUTION



Benzene, monochlorobenzene, o- dichlorobenzene , 1,2,3 trichlorobenzene, 1,3,5 trichlorobenzene, 1,2,3,4 tetra chlorobenzene, penta chlorobenzene and hexa chlorobenzene.

INSTRUMENTATION FOR HPLC



Pumping systems must have the following capabilities:

(1) Generation of 6000 psi(lb/in)
(2) Pulse free input
(3) 0.1-10 ml/min flow rate with 0.5% reproducibility
(4) Corrosion resistance
(5) No explosion hazard but no leaking

A variety of pumps are being employed in HPLC work as shown below.

1) Reciprocating pumps
2) Displacement pumps
3) Pneumatic pumps

Computer controlled servo type delivery type systems are ideal.

Sample injection:

(i) Syringe which can with stand up to 1500 psi.

(ii) Stop – flow injections.

(iii) Sampling loops must be capable of with standing 7000 psi.

(iv) Micro sample injection valves can introduce 5-500 µl sample size.

Columns:

Stainless steel, or heavily walled glass tubing of 10-30 cm, capable of withstanding 6000 psi, 4 to 10 mm inside dia meter, 0.5 µm particles provide 40-60000 plates/meter.

1 - 4.6 mm dia, 3.5 mm particles, 3-5 cm length columns offer 1, 00,000 Plates/meter. Such columns have low solvent consumption and speed up the separations.

Guard column:

Filter and separate irreversibly bonding compounds. The particles have similar composition to that of the analytical column but slightly larger particle size to minimize pressure drop.

Column thermostats must be capable of controlling $\pm 0.1^0$ C. This is accomplished by using column heaters or water jackets.

(I) Pellicular nonporous, spherical glass or polymer beads of 30- 40 µm coated with a thin layer of silica, alumina, polystyrene, divinyl benzene or ion exchange resins are used for guard columns preferably.

(II) Porous particle packing, 3-10 µm, of Si, Al_2O_3, DVB, ion exchange resin are also useful materials.

Detectors are of two types. These are based on the bulk property of the eluents or solute properties such as absorption, fluorescence etc.

1. **Bulk property – RI, dielectric constant, or density.**

2. **Solute property – UV absorbance, fluorescence diffusion current etc. Most of the HPLC work is accounted by 71% UV, 15% fluorescence and 14 % by other measurements.**

Detector *	Mass LOD	State of the art
UV-vis	100pg - 1ng	1 pg
Fluorescence	1 – 10 pg	10 fg
Electrochemical	10 pg - 1ng	100 fg
RI	100 ng – 1µg	10 ng
Conductivity	500 pg - 1 ng	500 pg
M.S	100 pg – 1ng	1 ng
FT – IR	1 µg	100ng

* Quantitation is accomplished by peak height or peak area

ASSIGNMENT

- LC Pumps
- HPLC Columns & Pickings
- HPLC Detectors
- Sample injection systems
- Solvent handling in HPLC
- Typical applications of HPLC to Separations of Pesticides
- Typical applications of HPLC to separations of Insecticides
- Typical applications of HPLC to separations of Polymers
- Ion chromatography
- Biochemical applications
- High pressure thin layer chromatography

ALL THE BEST