i. **<u>Automatic integration</u>**: Electronic integrators are the most rapid and precise means of determining peak areas. They have a digital output derived by feeding the detector signal into a voltage-to-frequency converter which produces a pulse rate proportional to the input signal. The total number of pulse is a measure of the peak, and this can be printed out directly or stored until required.

EFFICIENCY AND RESOLUTION

The ideal chromatographic process is one in which the component of a mixture form narrow bands which are completely resolved from one another. The narrowness of a band or peak is a measure of the efficiency of the process while resolution is assessed to resolve peak of components with similar retention time (t_R) or retardation factor (R_f) values.

Efficiency, N for column separation is related to t_R as follows:

$$N = \frac{(tR}{\sigma} \qquad \sigma = standard\ deviation$$

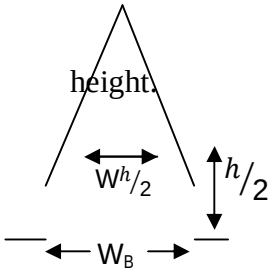
It is also easier to measure in terms of base line width

$$N = 16\ (\frac{tR}{WB})^2$$

$$N = 5.5\ (\frac{tR}{Wh/2})^2$$

WB = baseline width.

$W^{h}/_{2}$= peak width measured at half of peak height.



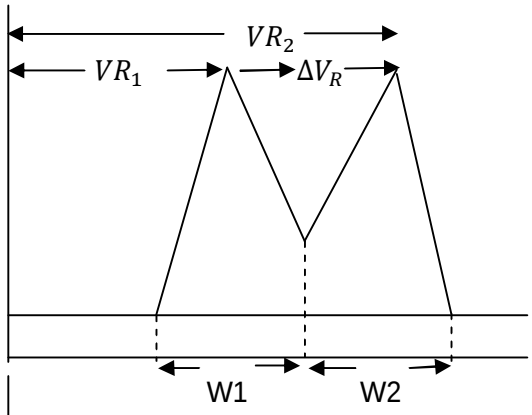
The parameter N is usually referred to as the "Plate Number" but an alternative mean of quoting efficiency is in terms of plate height, H or HETP (Height Equivalent to Theoretical Plate). N is related to H as:

$N = {}^{L}/_{H}$ L = total length of the column

In practice, Rs (resolution) is measured from a chromatogram by relating the peak-to-peak separation to the average peak width. This is expressed by the equation:

$$Rs = \frac{2\Delta VR}{(W1+W2)}$$

where V_R = retention volume.



ΔV_R is the separation of peak maxima, W1 and W2 are the respective peak widths. R_f value of 1.5 or more indicates cross contamination of 0.1% or less.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

It has its origin in classical column chromatography, although in both theory and practice, it is similar to gas chromatography. In column chromatography, the sample is introduced into a liquid mobile phase which flows through a column usually containing silica or alumina under the influence of gravity. Flow rate are in the order of $0.1cm^3\ min^{-1}$, which result in extremely lengthy separation time, and quite inadequate efficiency for separation of multi-component mixture. The poor performance is largely due to very slow mass transfer between stationary and mobile phases and poor packing characteristics leading to a large multiple path effect.

It was later recognized that the problems could be overcome through the use of smaller particles of stationary phase, and that rapid separations would require higher flow rate, necessitating the pumping of mobile phase through the column under pressure.

The means of meeting this basic requirement were developed in HPLC. The mobile phase is typically pumped at pressure up to about 3000psi, and flow rate of $1\text{-}5cm^3min^{-1}$ can be achieved through 10-25cm stainless steel column packed with particles as small as 3µm in diameter.

HPLC can be used largely for the separation of non-volatile substances including ionic and polymeric sample. This is complementary to gas chromatography. The use of HPLC in all its

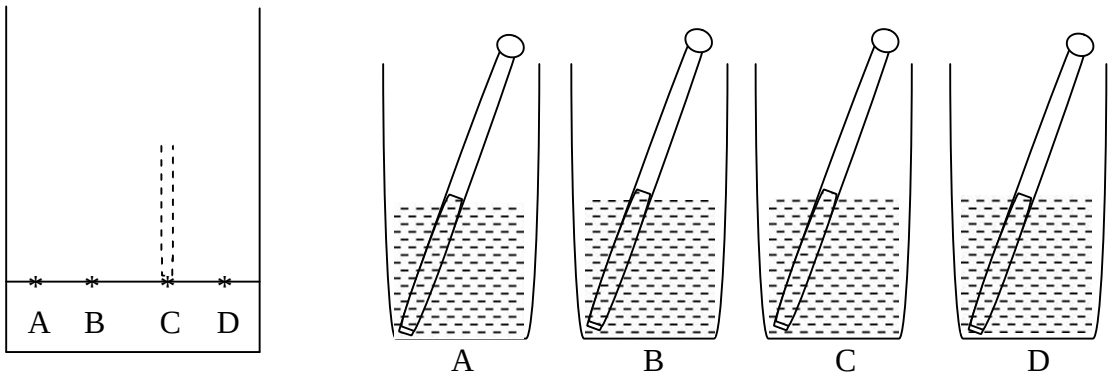
form is growing steadily and may eventually exceed that of GLC. This is because all four sorption mechanisms can be exploited and the technique is well suited to a very wide range of compound types including ionic, polymeric and labile (volatile) materials.

PAPER CHROMATOGRAPHY

Partition chromatography on sheet or strips of filter paper is one of the simplest and the most widely used of chromatographic technique. "Whatman 3mm" is used for preparative work.

METHOD

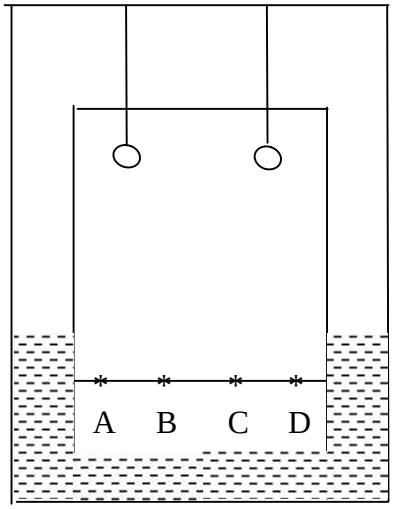
Samples are applied on the paper from the solution. Extracts from biological tissue often require preliminary purification before they can be applied. The reason for this is that large amount of protein or salt can interfere with the partition process by extracting water from the solvent. Proteins can be precipitated with alcohol and salts removed by electrolytic method (ion-exchange). Having selected the size and grade of paper to be used, a pencil line is drawn parallel to one edge and at a suitable distance from it. A number of small crosses are mount on the line corresponding to the number of samples to be applied and the nature of each sample written on in pencil. A drop of each sample is spotted on the appropriate position with a short length of capillary tubing or a platinum loop.



The spot should be about 5mm in diameter. Large spots lead to poorer separation. If more test substance is required than is present in 1 drop (especially with dil. solution), the spot is

allowed to dry by hair dryer and a similar application made. When the spots are dry, the paper is ready for development, in which solvent flows through the paper to produce separation. The solvent for development depends upon the nature of substances to be separated. It is essential to use one – phase solvent which corresponds in composition to the organic layer. Development can be carried out either by allowing the solvent to travel up the paper (the ascending technique) or down the paper (the descending technique).

Some of the solvent is poured into the bottom of the tank so as to saturate the atmosphere with its vapour.



Both ascending & descending techniques are employed. The ascending method usually gives better result with very volatile solvent. The disadvantage of this technique is that compounds with low R_f values are often incompletely separated. With the descending technique, the developing solvent can be allowed to run off the edge of the paper under the influence of gravity so that one is able to increase considerably the effective length of the run and this improves the separation. When the solvent has travelled the required distance, the position of the solvent formed is noted by tearing the paper slightly (or cut it) at the edges. Drying is carried out in the fume cupboard by electric hair dryer. The next is to locate the separated compounds. If they are coloured, there is no problem, but many compounds, especially of biological interest are colourless and hence invisible. Several methods are used, both physical and chemical methods to spot the compounds present.

The physical methods utilize particular properties of the compound such as fluorescence or radioactivity, while chemical methods involve reacting the substance with reagents to get coloured products.

Physical Methods

1. ***Fluorescence***: A number of unsaturated organic compounds fluoresce i.e. they have the property of absorbing UV light of short (invisible) wavelength and emitting light of longer (visible) wavelength. These compounds, although invisible on chromatogram in ordinary light, can readily be detected under UV lamp. The wavelength of the light emitted and hence the colour when seen is a characteristic of the compound and is therefore useful for the purpose of identification.
2. ***Radioactivity***: The wide spread use of nuclear power labeled radioactive compounds for research. This can be detected on chromatograms by means of special counter (Geiger Muller counter and Scintillation counter).

Physical methods of location have the advantage over chemical methods in that the substances on the chromatogram are not converted into other compounds and so they can be removed for further studies.

Chemical Methods

Colourless compounds are converted to coloured compounds by treatment with "locating reagents". The locating reagent can be a gas (H_2S), for location of metallic ions which form metallic sulphide. Solutions of reagents can be applied by dipping the paper into a solution of the reagent or by spraying the solution on the paper e.g. the most widely used reagent for detecting amino acid is "ninhydrin".

Ninhydrin is a white solid which when applied to the paper at a 0.1 – 0.25% solution is a suitable solvent.

The reaction can be represented as:

Amino acid	+	ninhydrin	⟶	coloured product
Colourless		colourless (white)		blue - lilac

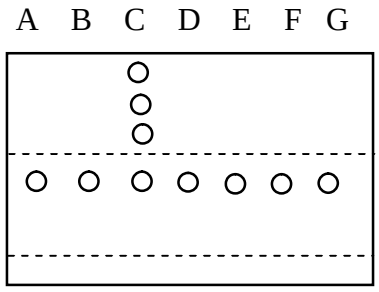
Also, sensitive methods of detecting reducing substances e.g. glucose and maltose (malt sugar) is by paper chromatography. This is dipped into appropriate volume of $AgNO_3$ and NaOH in alcohol. A grey spot is an indication of reducing sugar.

Both physical & chemical methods described are for qualitative analysis.

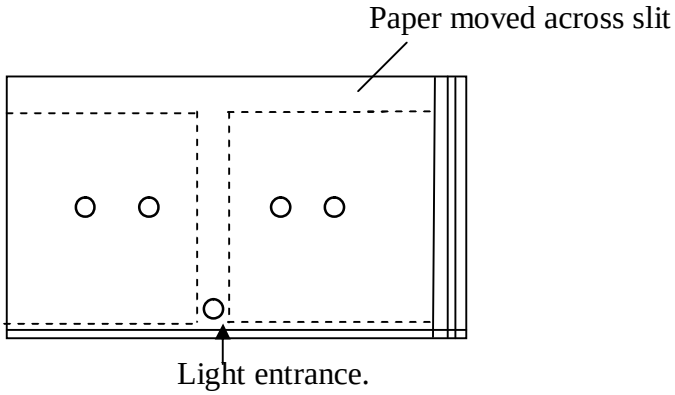
Quantitative Measurement

Sometime, it is required to make a quantitative measurement of a particular measurement in a mixture in addition to the usual qualitative ones.

The paper is developed dried and the marginal strips cut out and treated with suitable reagents. The compound so eluted can then be determined by one of the multitude of methods available for substances in solution (colour reactions, titration, microgravimetric procedures etc.) If a reagent is available, which react with the component to produce a colour, the intensity of which is proportional to the amount of that component present then quantitative measurement can be made directly on the paper. Such methods are always comparative in nature. For more accurate work, the intensity of each spot is measured with a photoelectric device known as **"photo densitometer"**. A thin strip is cut from the chromatogram (area enclosed by the dotted line), placed between two sheets of glass, and into the holder of the densitometer. The machine is set at zero on the section of the strip free from any compound and the strip is then moved in small stages passed the beam and there will be deflection. The deflection is proportional to the concentration of the material. Such deflection or dryer is recorded.



A, B, D, E, and F are spots containing known amounts of compound (standards).



The graph of that deflection is plotted against concentration (standards). It obeys Beer-Lambert's law.

Advantages of Paper Chromatography

One of the major advantages of is the sensitivity with which compound can be located after separation. Amount as little as 0.1µg can be detected with routine reagent. The lower limit for the detection of most compounds is between 1-50 µg (ppb).

Paper chromatography has played major role in elucidating the chemistry of compounds.

THIN – LAYER CHROMATOGRAPHY

Comparatively, paper chromatography is a versatile technique but its use is limited by the fact that separation can be conducted only with fibrous materials such as cellulose, since other valuable media such as silica gel, alumina and gel filtration beads cannot be made into sheets. This problem can be overcome by supporting thin – layer of these substances on an appropriate base. The usual method is to support them on glass base: in which case the product is referred to as **"chromatoplate"**.

A thin layer supported on solvent – resistant plastic sheet is called **"chromatosheet"**. Both are available commercially. Two types of layers are useful; solid layers which adhere to the support by the virtue of the adherent qualities of the material itself or because of a binding agent incorporated with it and also the loose layers.

Thin – layer separation resemble those of paper in some ways, but much wider choice of media means that separation by partition adsorption, gel exclusion filtration and ion – exchange can be performed by this technique.

The particular properties of thin layer also allow shorter development times to be achieved.

PREPARATION OF CHROMATOPLATE

Solid layers are prepared by applying 'slurry' of the chosen medium in a suitable liquid unto a clean glass plate. It is essential for optimum results to be achieved by making the layers to be uniform.

The particle size, surface structure and adhesiveness of medium must be carefully controlled. If reproducible results are to be obtained must be carefully controlled, if reproducible results are to be obtained. If the material adheres badly, a binding agent such as calcium sulphate ($CaSO_4$) is often