STANDARD BIOANALYSIS METHODS
(4)

CHROMATOGRAPHY

Dr. Rokaia Elamary

Lecturer of Microbiology/ Dept. Botany, Fac. Science

EXAMPLES ON PLANT ANALYSIS TECHNIQUES

Chromatography

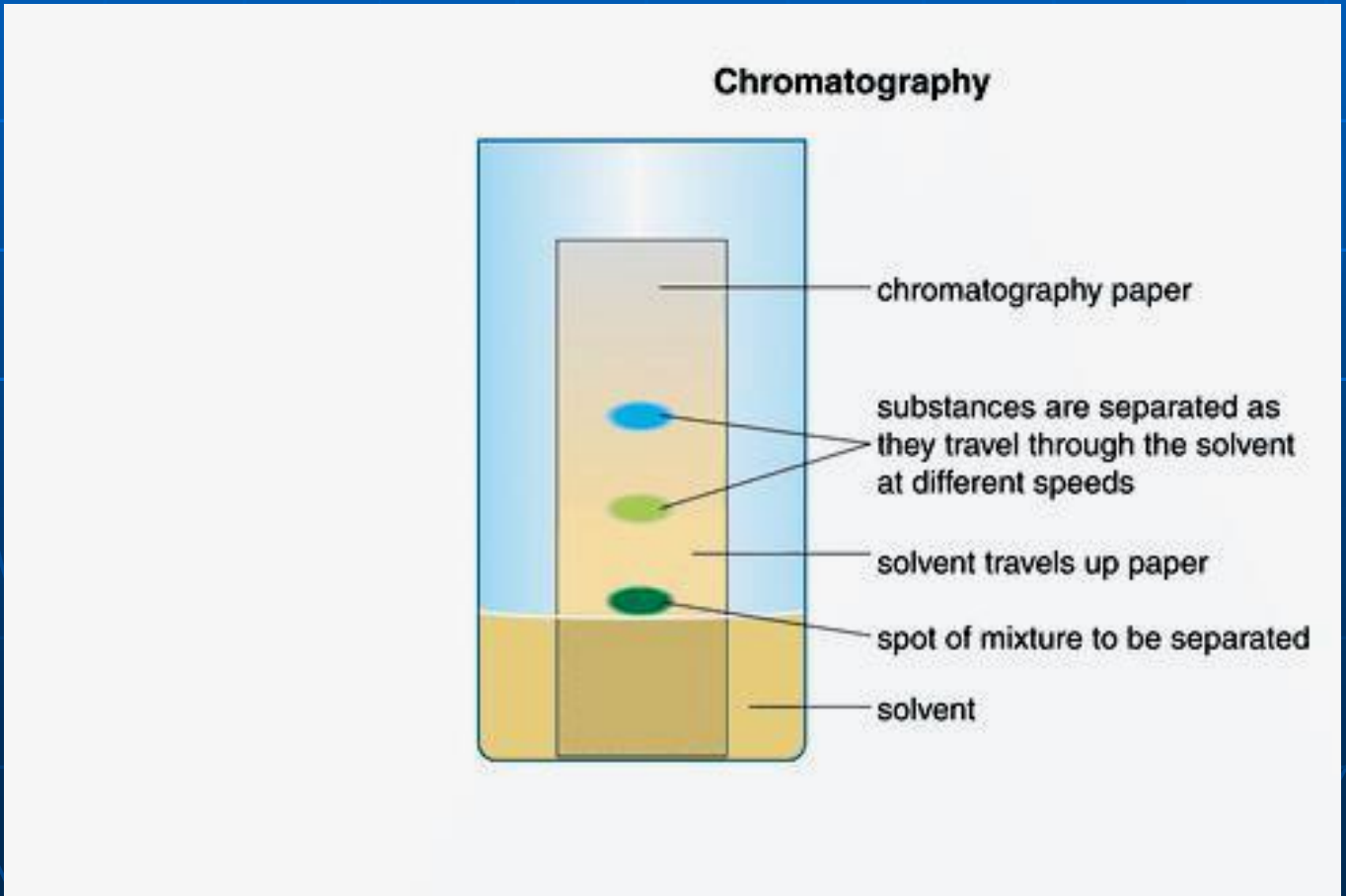
All chromatographic techniques flow the mixture, that is to be separated, through a material that **retains some components more than others**.

This causes different components to flow through the material at different speeds, so they separate.

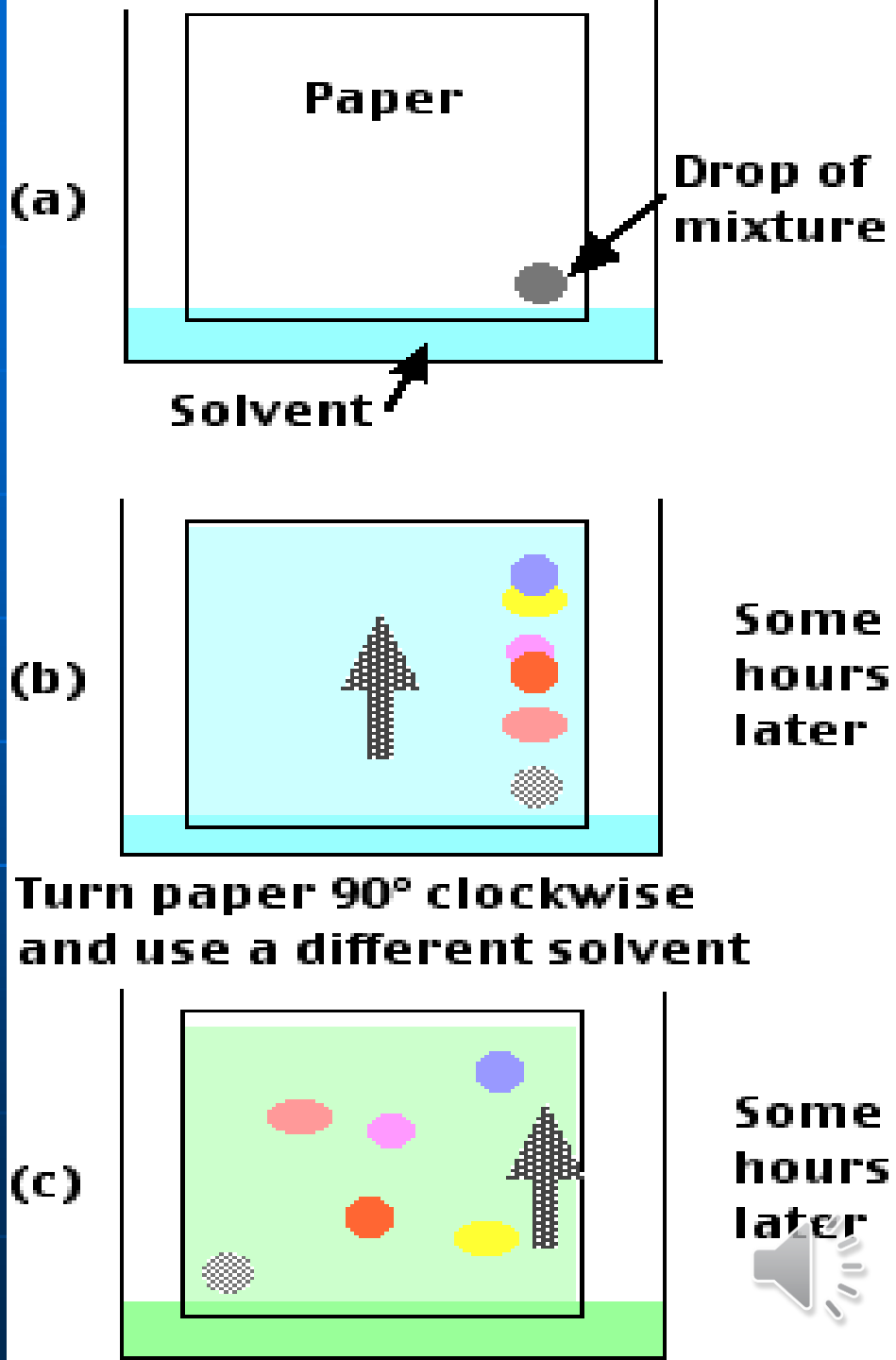
(1) Paper chromatography (PC)

- **A simple method that can be carried out on a sheet of filter paper serving as a medium for separation and support. Usually involves either partition or adsorption chromatography.**
- **In partition, the compounds are partitioned between a largely water- immiscible alcohols (i.e. solvents) such as *n*- butanol and water.**
- **The classic solvent mixture, *n*- butanol- acetic acid- water (4: 1: 5, top layer) abbreviated as BAW is still applicable for many plant constituents.**

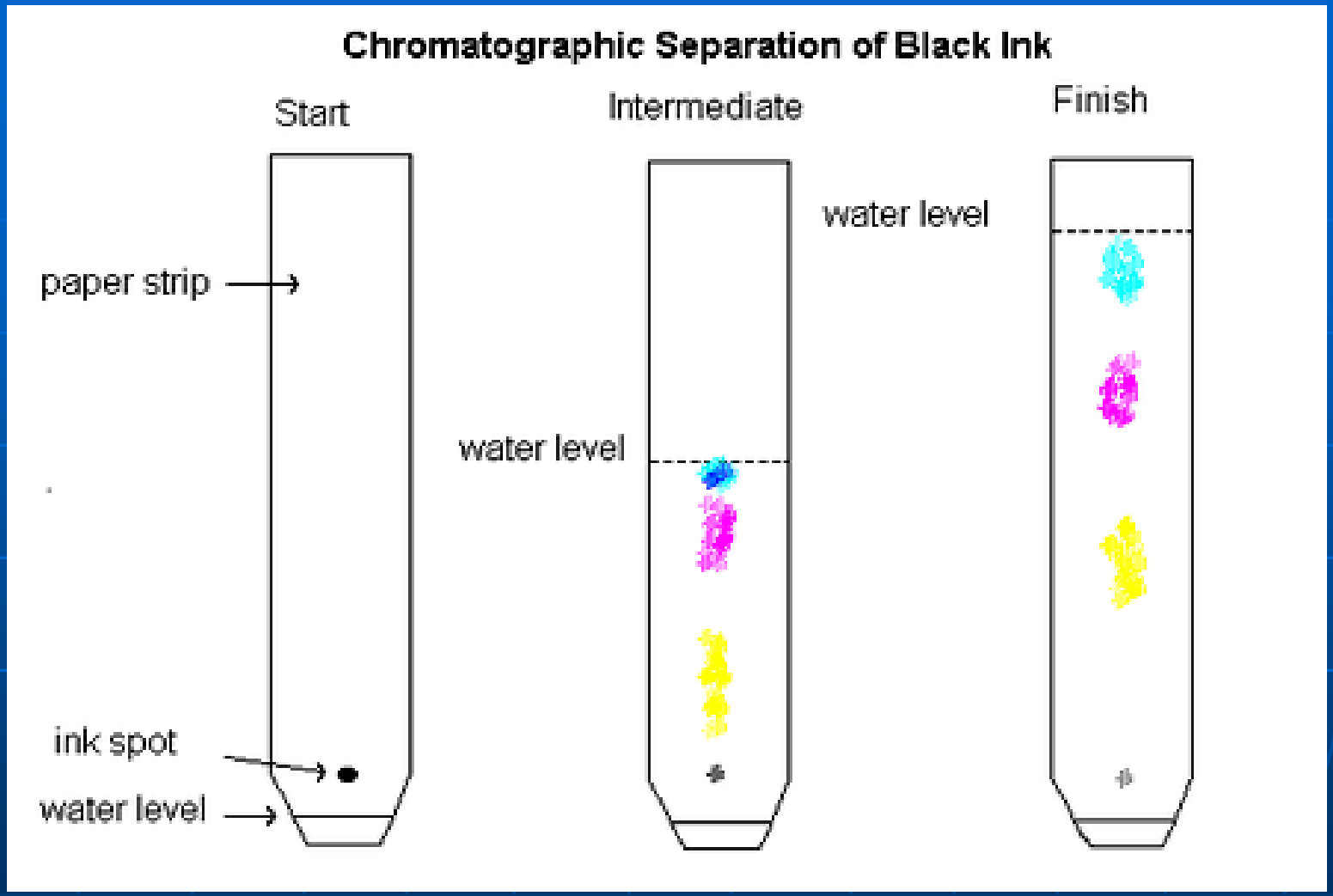
- **In adsorption**, adsorption forces are one of the main features of PC in aqueous solvents.
- **Pure water is a remarkable solvent and can be used for separating the common purines, pyrimidines, phenolic compounds and plant glycosides in general.**



- In most laboratories, descending PC is carried out in tanks that accommodate Whatman® papers of the size 46 by 57 cm. This method is more convenient for two-dimensional separations.



- A range of modified filter paper is available in the market for particular chromatographic separations. For example, the polar properties of cellulose can be reduced by incorporating silicic acid or alumina, making the paper more suitable for separating lipids.
- Special papers are also available for large scale separations.
- Papers can also be modified in the laboratory, for example, by soaking them in paraffin or silicon oil for carrying out reversed phase chromatography for lipids.
- In PC, compounds are usually detected as coloured or UV- fluorescent spots, after reaction with a chromogenic agent either by dipping or spraying.


Chromatographic Separation of Black Ink
Start
Intermediate
Finish
paper strip
ink spot
water level
water level
water level

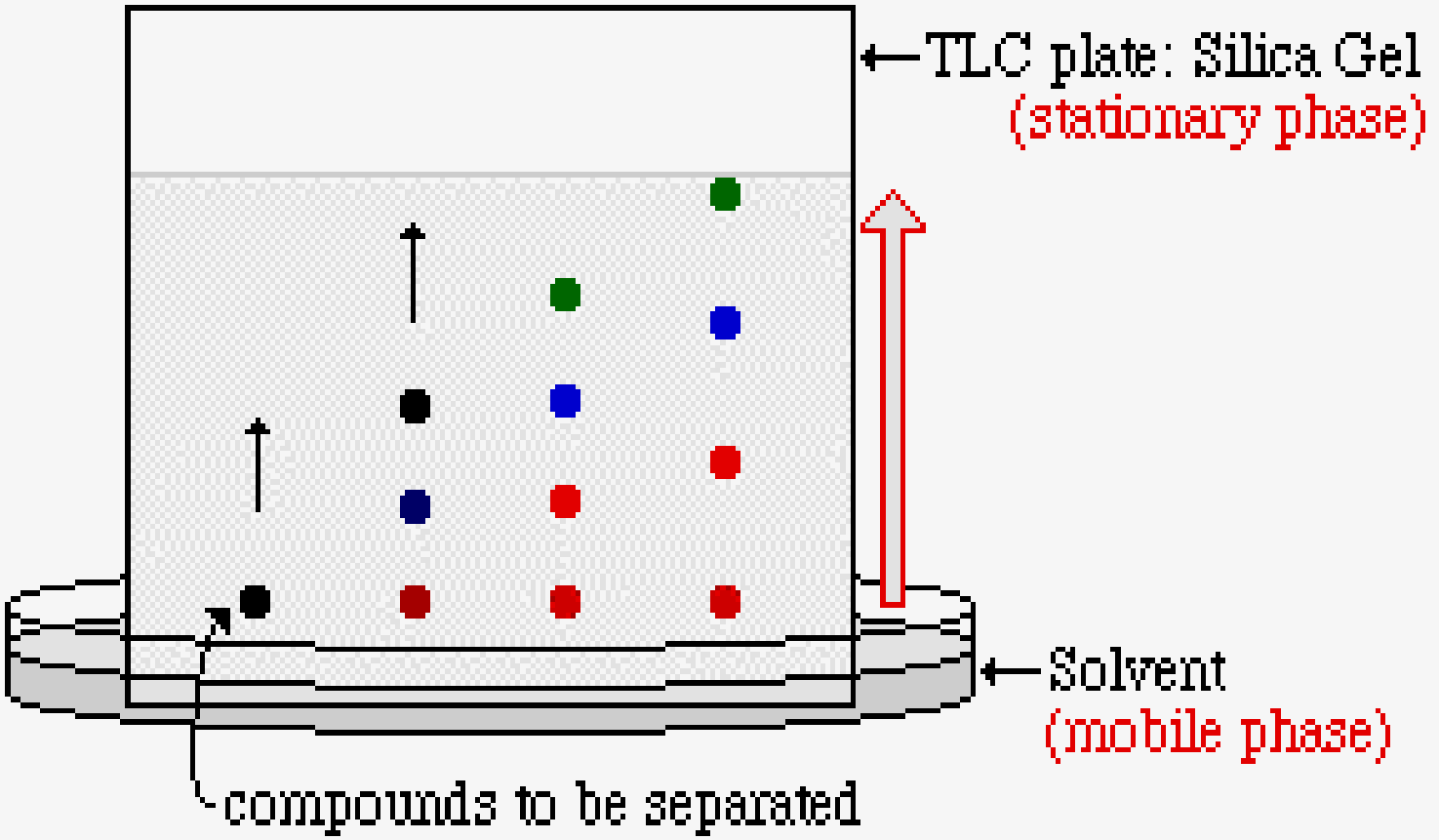
- The *Rf* value is "the distance a compound moves in chromatography relative to the solvent front".

- It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, and this is divided by the distance between the origin and the solvent front (i. e. the distance the solvent travels).

- This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this value by 100.

(2) Thin layer chromatography (TLC)

- The advantages of TLC compared to PC are versatility, speed and sensitivity.
- Versatility is due to the fact that a number of different adsorbents besides cellulose may be spread on to a glass plate or other support and employed for chromatography.
- Silica gel is the most widely used, but layers are made up from aluminum oxide, celite, Ca hydroxide, Mg phosphate, polyamide, sephadex®, polvenylpyrrolidone (PVP), cellulose or mixtures of two or more of these materials.


TLC plate: Silica Gel
(stationary phase)
Solvent
(mobile phase)
compounds to be separated

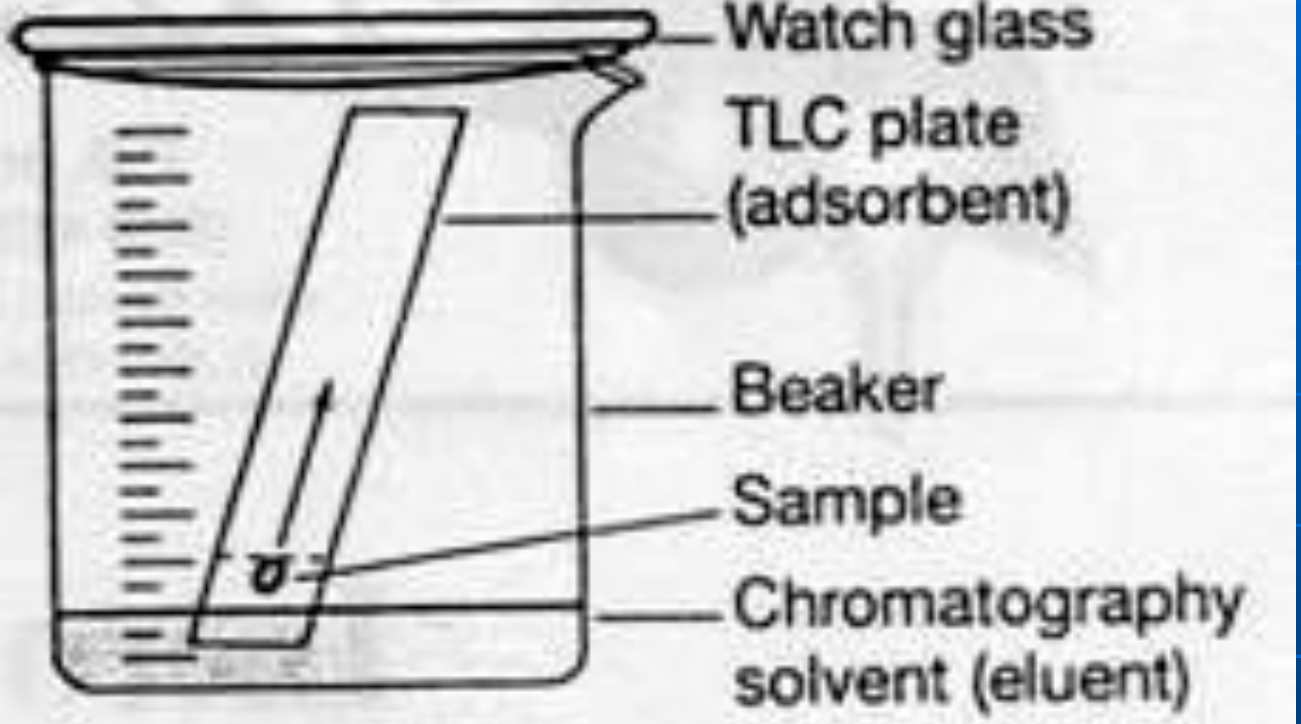


Figure 2. Chromatography Chamber

* The greater speed of TLC is due to the more compact nature of the adsorbent when spread on a plate.

*The sensitivity of TLC is that separations on less than µg amounts of material can be achieved if necessary.

*One of the original disadvantages of TLC was the labour of spreading glass plates with adsorbent, this was eased after the introduction of automatic spread devices.

*Precautions:

- The glass plates should be cleaned carefully with acetone to remove grease
- The slurry of silica gel (or other adsorbent) in water has to be vigorously shaken for a set time interval (i. e. 90 s) before spreading.

*Depending on the particle size of the adsorbent, calcium sulphate hemihydrate (15 %) may have to be added to help adhering the adsorbent to the glass.

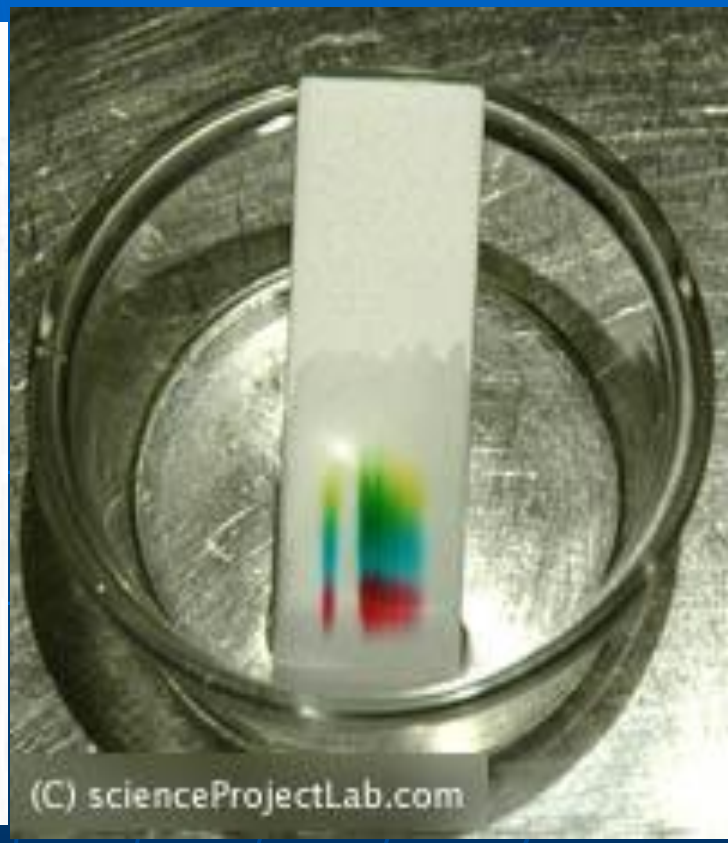
*After spreading, plates have to be air dried and then activated by heating in an oven at 100- 110°C for 30 min.

*Spreading plates made easier by using ready-coated plates with different adsorbents on glass, aluminum, or plastic sheets. Laboratory- coated plates are advantageous for moisture control of the adsorbent.

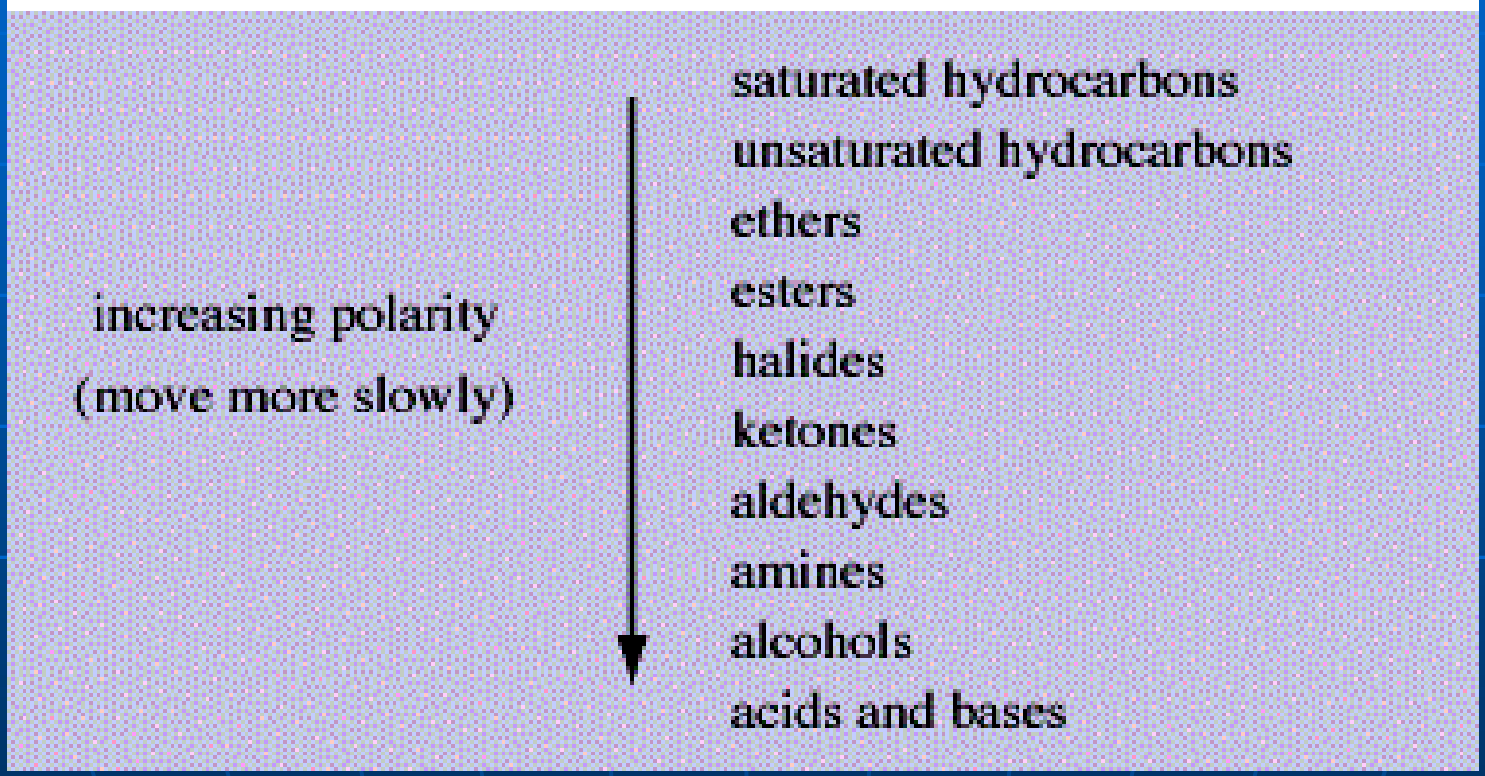
*A wider range of solvents have been applied to TLC than PC

TABLE 13–4 ADSORBENTS USED FOR THIN-LAYER CHROMATOGRAPHY

Adsorbent	Materials Separated
Silica gel	Amino acids, polypeptides, fatty acids, steroids, phospholipids, glycolipids, plasma lipids
Alumina	Amino acids, steroids, vitamins
Kieselguhr	Oligosaccharides, amino acids, fatty acids, triglycerides, steroids
Celite	Steroids
Cellulose powder	Amino acids, nucleotides
Hydroxylapatite	Polypeptides, proteins
Polyethylenimine	Nucleotides, oligonucleotides



The expected elution order of organic classes.

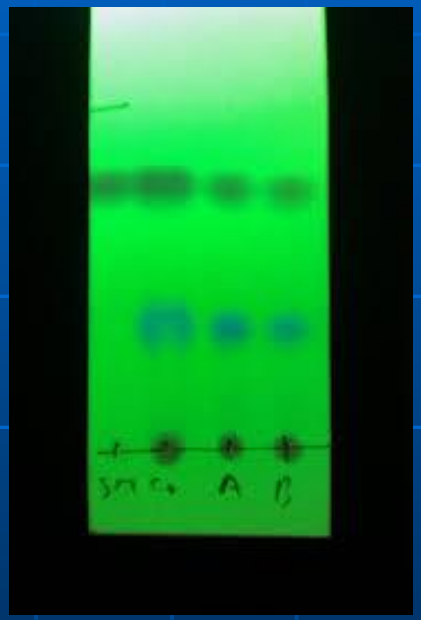


- Detection of compounds on TLC is carried out by spraying (normally). The small area of the plate makes it a relatively simple procedure.

- One advantage over PC is that plates can be sprayed with conc. H_2SO_4 that is a useful detection reagent for steroids and lipids.

- Separated constituents are recovered by scraping off the adsorbent at the appropriate places on the developed plate, eluting the powder with a solvent such as ether and finally centrifuging to remove the adsorbent.

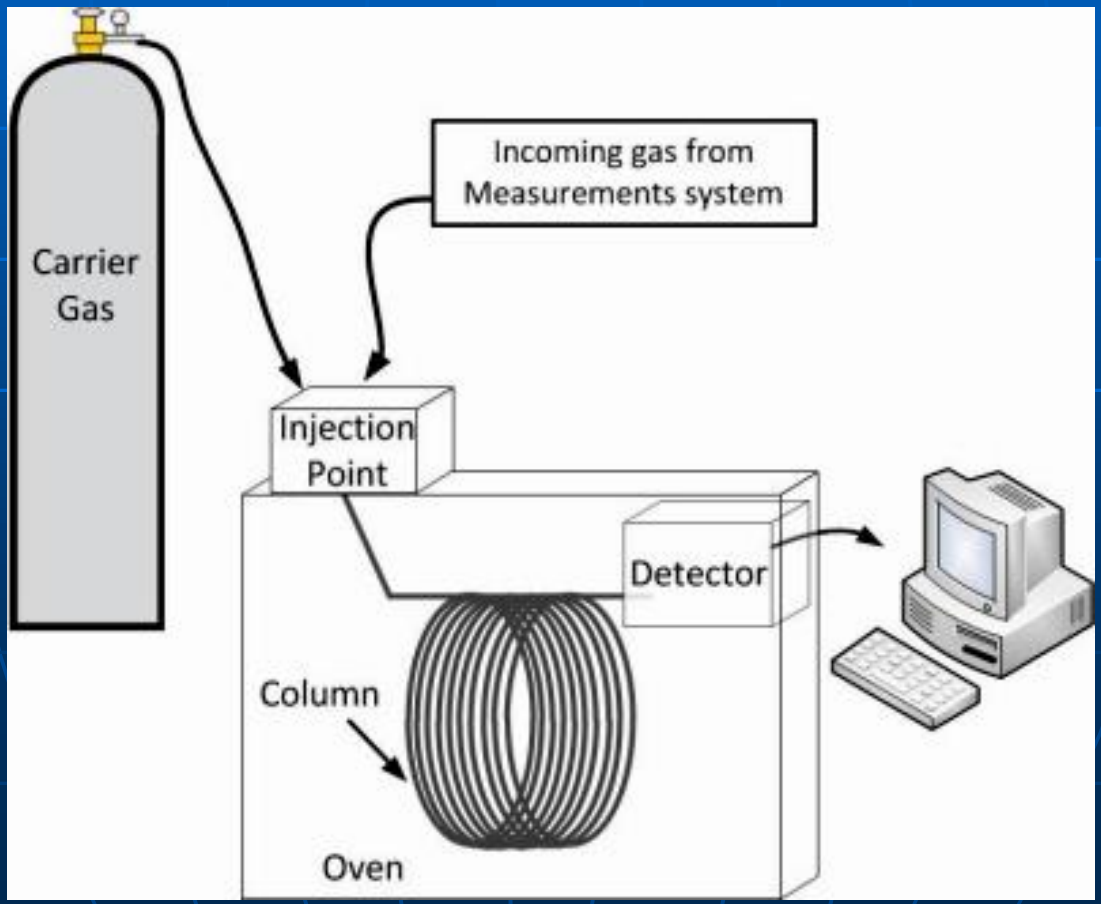
- Compounds that absorb in the near UV (i. e. 254 nm) can be detected either by adding a fluorescent dye to the slurry when the plate is being prepared or by spraying the developed plate with a similar dye.



- Preparative TLC is carried out using thick (up to 1 mm) instead of thin (0.1- 0.25 mm) layers of adsorbent

(3) Gas liquid chromatography (GLC)

- **GLC apparatus is more expensive and sophisticated than TLC or PC. It consists of four main components:**



1- The **column** is a long narrow coiled tube (e. g. 3m x 1mm) usually made of metal. The column is packed with a stationary phase

(e. g. 5- 15% silicon oil)

on an inert powder.

2- The **heater (oven)** to heat the column from 50 to 350°C at a standard rate and to hold temperature at the higher limit if necessary.

- The temperature of the column inlet is separately controlled. The sample dissolved in ether or hexane is injected by a fine syringe into the inlet port through a rubber septum.

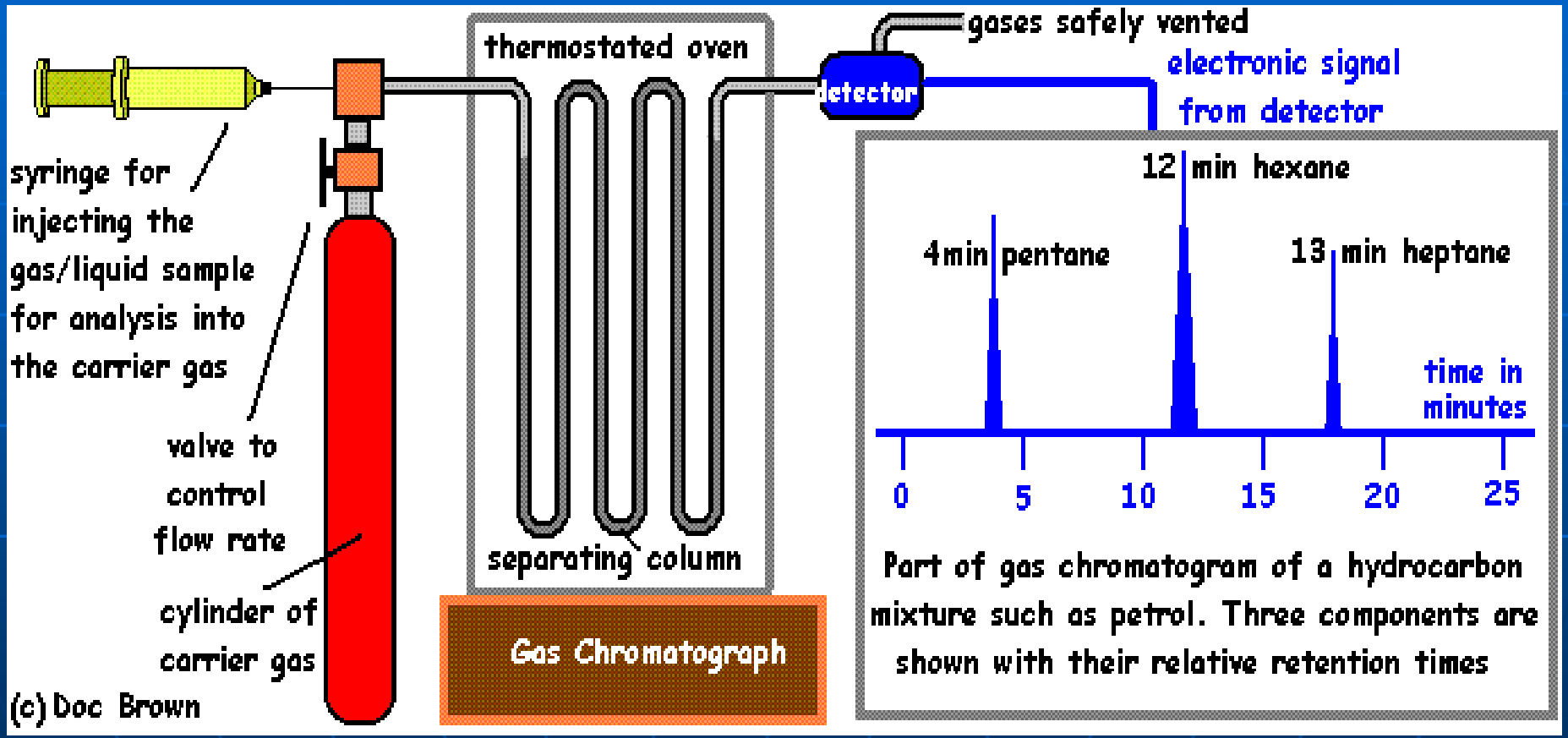
3- Gas flow consists of an inert **carrier gas** such as nitrogen.

- Separation of the compounds on the column depends on passing this gas at a controlled rate.

4- A **detection device** for measuring the compounds as they pass from the column.

- Detectors are flame ionization, thermal, electron capture, …etc. The first method requires hydrogen gas to be added to the gas mixture and burned in the detector.

- The detector is attached to a recorder or computer in which software can control the process as a whole and separate the compounds as peaks of varying intensities.


thermostated oven
gases safely vented
detector
electronic signal
from detector
syringe for injecting the gas/liquid sample for analysis into the carrier gas
valve to control flow rate
cylinder of carrier gas
separating column
Gas Chromatograph
(c) Doc Brown
12 min hexane
4min pentane
13 min heptane
time in minutes
0
5
10
15
20
25
Part of gas chromatogram of a hydrocarbon mixture such as petrol. Three components are shown with their relative retention times

- The results of GLC can be expressed in terms of **retention volume *Rv*** which is "the volume of carrier gas required to elute the component from the column". It can be expressed also in terms of **retention time *Rt*** which is the time required for elution of the sample.

- The main variables in GLC are the nature of stationary phase and operation temperature. Many classes of substances are routinely converted into derivatives before subjected to GLC.

- GLC provides both **quantitative** and **qualitative** data on plant substances, since the measurement of **peak area** is directly related to the concentrations of different components in the original mixture.

- **The separated components GLC can be subjected to further analysis such as mass spectrometry (MS). The combined GC- MS apparatus is now very common.**