Pharmacognosy

For

FIRST YEAR PHARMACY STUDENTS

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Contents

Part I

Unorganized drugs

UNORGANISED DRUGS

Definition: Unorganised drugs are materials derived from parts of plants or animals by certain processes and have non-cellular uniform structure i.e. they do not constitute definite organs in plants or animals, but mostly, are constituents in these organs.

General characters of unorganised drugs:

- They are either a natural secretion or a pathological product in plants or animals.
- They are prepared from its natural origin by many processes including **extraction** with certain solvent e.g. **Agar**, **incision** e.g. **Gums** and **opium** and **expression** e.g. **olive oil** or they may be **natural secretions** such as **Myrrh and Beeswax.**
- Unorganised drugs comprise solids such as **Colophony** and **Gum Arabic**, semisolids such as **Oleoresins** or fluids such as **oils** and **balsams**. So, for identification the physical characters viz, colour, odour, fracture and solubilities in different solvents, as well as the chemical tests are the possible means.

Classification of Unorganised Drugs:

Unorganised drugs are classified into the following groups regarding to their origin and chemical nature.

1. Resins e.g. Colophony.
2. Gums e.g. Gum Arabic.
3. Extracts e.g. Agar.
4. Dried latex e.g. Opium.
5. Dried Juice e.g. Aloes.
6. Waxes e.g. Beeswax.
7. Saccharine subatances e.g. Honey.
8. Oils and fats e.g. Castor oil and Lard.
9. Volatile oils e.g. Clove oil.

I- RESINS

Definition: Resins are natural or prepared, solids or semi-solid exudations from plants or insects feeding on plants.

Types of Resins:

1. Natural resins: These are naturally occurring exudates of plants e.g. Mastic.

2. Prepared resins: These comprise resins which are prepared by certain process from plants in distinction of synthetic resins and they include:

- Resins prepared by extraction of the drug with alcohol pouring the concentrated extract into an excess of acidified water, collecting, washing and drying of the precipitate e.g. Gluco-resins of Jalap and Podophyllum.
- Resins prepared by steam distillation of a natural oleoresin e.g. Colophony from crude Turpentine.

3. Synthetic resins: These are polymeric substances formed readily by condensation or by addition of certain chemical compounds e.g. phenol and fromaldehyde. These compounds interact initially to yield a long series of phenolformaldehyde which termed Bakelite resins.

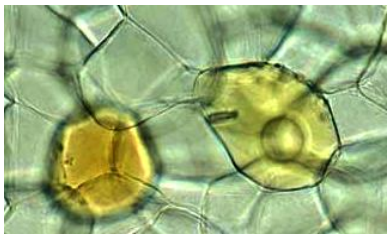
Formation of Resins:

Natural resins are commonly produced by secretory tissues which secrete a mixture of substances, one of which is the resin which is held in solution by terpenes or volatile oils. These secretory structures may be:

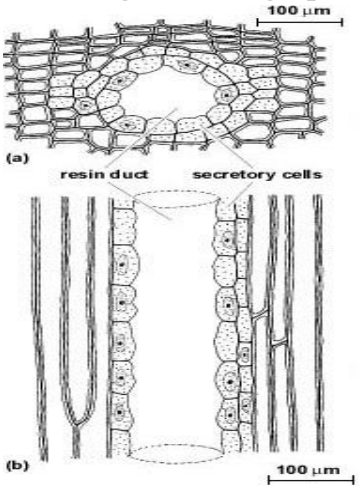
1. **External:** present on the surface of the plant as glandular trichomes of Cannabis in which the secretion is collected under the raised cuticle of the secreting head.

2. **Internal:** present in the mass of plant tissues. It may by:

- Unicellular, called resin-cell which is ovoid cell containing the resin mixture as in **Ginger**.



- Multicellular, which may be a gland with secretory epithelium e.g. **Clove** or a tube or duct with lining secreting epithelium e.g. **Umbelliferous fruits**.



In addition to the initial secretion ducts, normally present in the plant, others may be formed as a result of incision of certain trees. The number of the ducts thus formed may be very large and produce large quantities of oleo-resin. This oleoresin flows over wound, fanning a temporary protection for it.

Not only the secretory tissues contain resins, but also all the cell elements of a tissue, such as **Guaiacum wood**, may become filled with resins.

Characters of Resins:

1. They are insoluble in water, mostly soluble in alcohol or ether.
2. Resins are uncrystallisable and have little taste.
3. On heating at moderate temperature, they often forming sticky or adhesive fluids without volatilisation or decomposition.
4. Resins burn with smoky flame, owing to the high carbon content in the molecule.
5. They pre transparent when pure, but become opaque and brittle when they contain water.
6. Resins are non conductor of electricity, but when rubbed they become negatively charged.

7. They range in specific gravity from 0.9-1.25.
8. Chemically, resins are the oxidised terpenes of the volatile oils.

RESIN COMBINATION:

1. **Oleoresin:** Oleoresin are mixtures of volatile oils and resins. They are semisolids or liquids depending on the amount of volatile oil present, e.g. Turpentine, Canada balsam and Copaiba.
2. **Gum-resins:** This means resins occur in mixture with gums The gum is usually similar to Gum Arabic e.g. Gamboge.
3. **Oleo-Gum-Resin:** When resins occur in association with volatile oil and gum e.g. Asafoetida, Galbanum and Myrrh.
4. **Glycoresins:** In few cases, resins may be combined in a glycosidal manner with sugars as in the resins of Jalap, Ipomea and Podophyllum.
5. **Balsams:** They are resinous substances that contain varying amounts of aromatic balsamic acids, viz, benzoic acid or cinnamic acid or both or esters of these acids. They often contain small amounts of volatile oil.

Chemical Composition of Resins:

The main constituents of resins may be classified under the following:

1. **Resin acids:** Among resins consisting chiefly of resin-acids is Colophony.
2. **Resins esters:** These are present in addition to their decomposition products i.e. resin alcohols (resinols) and resin phenols (resino-tannols) e.g. Benzoin, Storax and balsam Peru and Tolu.
3. **Resenes:** These are chemically inert compounds which predominate in resins of Myrrh, Mastic and Olibanum.

OFFICIAL RESINS AND RESIN COMBINATIONS

A - RESINS

1- COLOPHONY

Colophony is the solid resin left after steam distillation of the volatile oil from the oleoresin obtained from ***Pinus palustris, P. toeda*** and other species of ***pinus***, Fam. Pinaceae.

COLLECTION AND PREPARATION:

The oleoresin is collected by Cup and Gutter method where blazes are made in pine trees, few feet apart and the groove is enlarged at intervals. As the grooves are lengthened the cups are moved higher and they are emptied at intervals and the oleo-resin sent to the distillery. Considerable increase in the amounts of oleo-resin occurs by stimulating the tissues of the groove by application of 50% sulphuric acid or plant growth substances. The oleoresin is warmed with water, the temperature is then raised to boiling and the distillate (containing water and oil) is collected for separation of the oil while the molten resin is run through wire strainers into barrels and allowed to cool.

CHARACTERS:

1. Colophony occurs in translucent glassy masses of a pale yellow or amber colour frequently covered with a yellow powder.
2. It is brittle with glassy and conchoidal fracture being easily pulverisable. It fuses gradually at about 100°C, and at a higher temperature burns with a smoky flame.
3. It is insoluble in water, but soluble in alcohol, chloroform, ether,

benzene, carbon disulphide, glacial acetic acid, fixed and volatile oils, dilute alkalies and partly soluble in light petroleum.

CONSTITUENTS:

1. 93% abietic acid, before distillation the resin contains d- and L-pimaric acid but during distillation d-pimaric acid is stable and L-pimaric undergoes isomeric changes into abietic acid.
2. Resenes and esters of fatty acids.

Tests for identity:

1. To about 10ml of 1% solution of colophony in acetic anhydride, add 1 drop of sulphuric acid, a bright purplish-red colour is produced, which rapidly changes to violet.
2. Shake about 0.05gm of freshly powdered colophony with 5ml light petroleum, for few minutes, and filter. Shake the filtrate with an equal volume of dilute copper acetate solution, the light petroleum layer assumes a bright bluish-green colour.

USES:

1. In pharmacy, for the preparation of zinc oxide and other adhesive plasters and ointments.
2. Large quantities of the darker grades are destructively distilled to yield resin spirit and oil and manufacture of dark varnishes and printing ink.
3. The medium grades are largely used for manufacture of soap and the lighter grades for sealing-wax.

2- CANNABIS (Indian hemp. Marihuana, or Pot)

Cannabis is the dried flowering tops of the pistillate plants of ***Cannabis sativa*** Linne var. Indica Fam. Cannabinaceae.

The plant is an annual, dioecious herb indigenous to central and western Asia and is cultivated in India and other tropical and temperate regions for the fiber and seed. Cannabis was used in China and India, spread slowly through Persia to Arabia where the resin was known as "**Hashish**", and probably was introduced into European and American Materia Medica about the time of Napoleon. Through many years of selective cultivation, two genetic types of cannabis have evolved. One, designated the drug type, is rich (up to 15 %) in the psychoactive constituent Tetrahydrocannabinol (THC). The other, referred to as the hemp type, contains little active principle (Cannabidiol is the predominant cannabinoid) but has the elongated bast fibers desired in the manufacture of rope.

The THC is concentrated into a resin which is secreted into trichomes found on the small leaves (bracts) and bracteoles (leaf-like structure that encloses the ovary) of the flowering tops of the female plant.

For drug purposes either the resin (hashish) is used or the flowering tops of the female plant (marihuana).

Hashish

N.B:

1. The male plant produces an equivalent amount of active constituents; however, it is not concentrated into a resin but found throughout the plant.
2. The amount of resin found in pistillate flowering tops of *C. sativa* markedly decreases when the plants are grown in the more

temperate climates. Thus Indian cannabis yields 20% or more of resin; Wisconsin hemp, 6 % or less.

The unusual sensations induced in humans by the uncontrolled use of cannabis are obtained more promptly and with less drug by inhaling the smoke of burning cannabis than by oral dosage. By smoking marihuana, the drug effects are noticeable almost immediately and reach a peak in 30 minutes. Users experience euphoria and relaxation. Intoxicated persons also develop an altered time sense with an enhanced awareness of their environment. Thought processes are slowed, short-term memory impaired, and one has difficulty concentrating.

Other constituents; 20% fixed oil that is expressed and used in the manufacture of paints and soaps, the cake meal is used as cattle food.

Chemical Tests (for THC):

Modified BEAM'S Test

Extract the powder with methanol, filter, and evaporate to dryness, Dissolve the residue in light petroleum ether, filter into a separating funnel and extract successively with 5% $Na_2CO_3$and 5% H_2SO_4, wash with water, decolorize with charcoal if necessary and evaporate the filtrate. Add to the residue a few drops of N/10 alcoholic KOH when a purple color is given.

USES:

For treatment of nausea and vomiting associated with cytotoxic drugs that are used in cancer chemotherapy. Its mechanism of action is possibly through binding to opiate receptors in the forebrain and indirectly inhibiting the emetic center in the medulla oblongata. In addition, because marihuana and THC stimulate the appetite, THC is used in the treatment of anorexia associated with weight loss in patients with AIDS.

3- RESINA JALAPAE (Jalap Resin)

Jalap resin is a mixture of resins obtained by precipitating with water the alcoholic extract of the dried tuber of ***Ipomoea Purga***, Fam. Convolvulaceae.

The plant is cultivated in India. Jamaica and South America but mostly is exported from Eastern Mexico.

CHARACTERS:

1. Jalap resin occurs in dark brown masses or fragments, frequently covered with a brownish powder being translucent at the edges.
2. It has brittle, resinous and glassy fracture.
3. It has characteristic odour and somewhat bitter and acrid taste.
4. Jalap resin is insoluble in water, readily soluble in alcohol and in solutions of caustic alkalies. It is partially soluble in ether, chloroform, light petroleum, benzene and in oil of turpentine.

CONSTITUENTS:

1. The main constituent of the resin is the ether-insoluble convolvulin, a substance of high molecular weight having 18 hydroxyl group esterified with valeric, tiglic and exogonic acids.
2. Ether soluble fraction, scammonin.

USES:

Jalap resin is a powerful hydragogue cathartic. Jalapin the decolourised-ether insoluble portion of jalap resin, is used for this purpose.

4- RESINA PODOPHYLLI (Podophyllum Resin)

Podophyllum resin is a mixture of resins obtained from the dried roots and rhizomes of ***Podophyllum peltatum***, known as American podophyllum Resin, and of ***Podophyllum hexandrum*** known as Indian Podophyllum Resin, Fam. Berberidaceae.

The American plant is collected in Virginia. Kentucky and Indian, while the Indian drug is found in Thibet and Afghanistan.

PREPARATION:

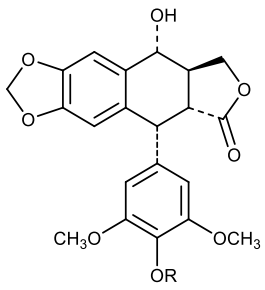
The resin (podophyllin) is prepared from podophyllum by percolation with alcohol and followed by precipitation from the concentrated percolate upon pouring into acidified water. The precipitated resin is washed with water, dried then powdered.

CHARACTERS:

1. Podophyllum resin occurs in brownish-grey masses, or frequently in light brown to greenish-yellow amorphous powder.
2. It has characteristic Liquorice like odour and bitter acrid taste.
3. Podophyllum resin darkens in colour on exposure to light or to temperature above 25°C.
4. It is very irritating to the eyes, and to the mucous membranes.
5. Podopyllum resin is practically insoluble in cold water, partially soluble in hot water, from which it is precipitated on cooling.
6. It is partially soluble in chloroform and in dilute solution of ammonium hydroxide, entirely or almost soluble in alcohol.

CONSTITUENTS:

The chief constituents of the resin belong to the group of lignans, which are C_{18} compounds. The most important ones present in podophyllum resin are:



Podophyllotoxin, R = CH_3
Demethylpodophyllotoxin, R = H

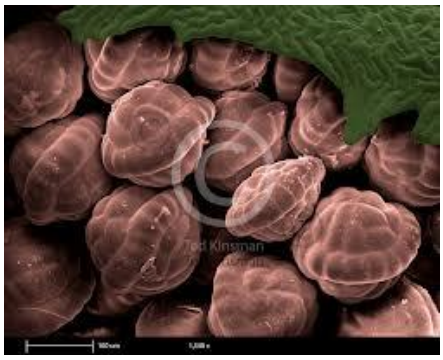
- Podophyllotoxin (about 20%)
- β-Peltatin (about 10%) free or as glucoside.
- O-Peltatin (about 5%) free and as glucoside.
- The closely related demethyl-podophyllotoxin and its glycoside.
- Dehydropodophyllotoxin.

USES:

1. As a purgative.
2. Externally in the treatment of certain types of warts and soft venereal.
3. It has cytotoxic action.
4. Podophyllotoxin possesses tumor-inhibiting properties.

B- OLEORESINS

OLEORESINA FILICIS (Filix mas oleoresin)



PREPARATION

Male fern oleoresin may be prepared by exhausting male fern, in moderately coarse powder, by percolation with ether. Remove the ether, and evaporate the remainder of the percolate on a water bath until an oleoresinous extract remains. Determine the proportion of filicin in the extract by the assay process and add, if necessary, a sufficient quantity of a suitable official fixed oil to produce oleoresina Filicis of the required strength.

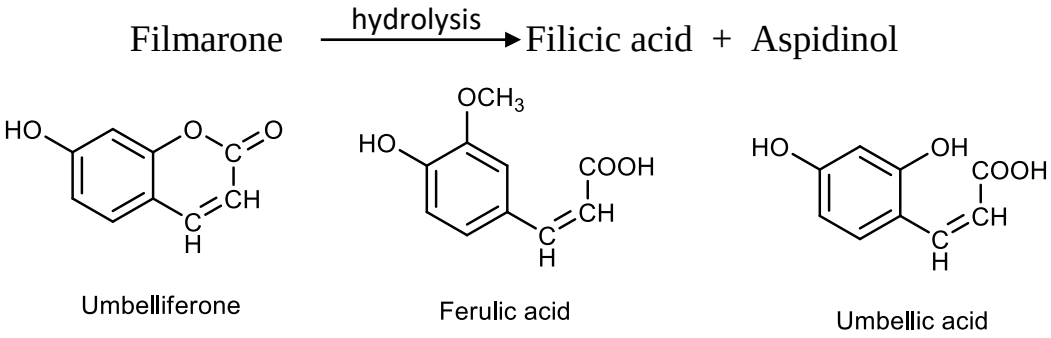
Male fern oleoresin contains not less than 24%, and not more than 26% w/w of crude filicin.

CHARACTERS:

1. Male fern oleoresin is a thick dark green liquid, frequently depositing a granular crystalline substance.
2. Male fern oleoresin is insoluble in water, soluble in ether and not less than 85% of it is soluble in light petroleum.
3. It has agreeable odour and bitter astringent taste.

CONST1TUENTS:

1. Phloroglucinol derivatives which occur as mono-, bi-, tri-, and tetracyclic compounds. The constituent is about 5% of a yellow amorphous substance of acid nature termed filmarone, to which the taenicide activity of the drug is due.
2. Filicic acid, aspidinol, flavaspidic acid, flavaspidinol and albaspidin.
3. Filmarone occurs as a bright yellowish-brown powder, insoluble in water, soluble in organic solvents, and in alkali hydroxides and carbonates. It is slowly hydrolysed in the drug or in solution.



Filicic acid is therapeutically inert. It forms water soluble barium salt, a property utilized in the assay of the extract. Upon heating the acid, its lactone, filicin is formed.

Test for Identity:

Mix about 0.1 gm of male fern oleoresin and 0.2gm of talc. Shake vigorously with 10 ml of hot alcohol and then filter. Add to 1 ml of the filtrate 9 ml of alcohol and 1 drop of ferric chloride, a light green solution which turns brownish is obtained.

USES: Male fern oleoresin is used as taenicide.

C- OLEO-GUM-RESINS

4- GUMMIRES1NA MYRRHA (Myrrh)

Myrrh is an oleo-gum-resin obtained from the stems and branches of ***Commiphora myrrha***. **C. *molmol*, C. *erythraea*** and possibly other species of Commiphora, Fam. Burseraceae.

It is collected chiefly in Somal and exported via Aden or Bombay some myrrh is collected in South of Arabia.

GENERAL DESCRIPTION:

Commiphora species yielding myrrh are shrubs to small trees up to about 10 m high. The whitish gray bark has schizogenous gum-oleo-resin cavities. Part used is the exudation from the natural fissures in the bark or from man-made incisions. The exudation is a pale yellow liquid, which soon hardens to form yellowish-red or reddish-brown tears or masses that are then collected.

CONSTITUENTS:

1. Myrrh contains 1.5-17% (usually ca. 8%) volatile oil composed of heerabolene, limonene, dipentene, pinene, eugenol, cinnamaldehyde, cuminaldehyde, cumic alcohol m-cresoL cadinene, curzerene (11-9%), curzerenone (11.7%), dihydropyrocurzerenone (1.1%), furanoeudesma-1,3-diene (12.5%), 1.10 (15)-furanodiene-6-one (1.2%), and lindestrene(3.5%).
2. Up to 40% (usually ca, 20%) resins consisting of α-, β-commiphoric acids; commiphorinic acid, α- and β-heerabomyrrhols, heeraboresene, commiferin, campesterol,

sitosterol, cholesterol, α-amyrone, 3-epi-α-amyrin, and others.

3. About 60% gum, which on hydrolysis yields arabinose, galactose, xylose, and 4-O-methylglucuronic acid.

Test for Identity:

Triturate about 0.5 gm of myrrh with 1 gm of sand and shake with 10 ml of ether. Filter, divide the filtrate into two portions, and evaporate in a porcelain dish. To the film left in one porcelain dish add a few drops of nitric acid, a purplish violet colour is produced. Over the film left in the other porcelain dish pass vapours of bromine, a violet colour is produced.

USES:

Myrrh has stimulant and antiseptic properties and is used :

1. As a mouth wash.
2. As a uterine stimulant and emmenagogue.
3. In parasitological cures especially schistosomicidal effect.

D- BALSAMS (BENZOIN)

Benzoin is a balsamic resin obtained from the incised stem of ***Styrax benzoin*** known in commerce as **Sumatra Benzoin** or form ***Styrax tonkinensis***. Known in commerce as **Siam Benzoin** Fam. Styraceae.

Sumatra benzoin is produced from trees growing in Malay peninsula, Sumatra, and Java; Siam benzoin is from trees growing in Laos, Vietnam, Cambodia, China and Thailand.

GENERAL DESCRIPTION:

Benzoin is the balsamic resin obtained from various Styrax spp. *Styrax benzoin* Dry, and yield Sumatra benzoin; *S. tonkinensis* (pierre) Craid ex Hartwich and other related Styrax species (Section Anthostyrax, Fam. Styraceae) yield Siam benzoin.

Benzoin-producing Styrax species are mostly small to medium trees (up to 20 m high) growing in tropical Asia.

Benzoin is a pathological product formed when the tree trunk is injured. It is produced by incising the bark; the exuded balsamic resin hardens on exposure to air

CONSTITUENTS:

Benzoin contains chiefly esters of cinnamic and benzoic acids together with free acids. Amounts and types of esters and acids vary widely with its source.

Sumatra benzoin contains about 90% resinous matter composed mainly of sumaresinolic acid and coniferyl cinnamate, with 10-20% benzoic acid and 10-30% cinnamic acid; other constituents present include 2-3% phenylpropyl cinnamate. 1% vanillin, and traces of cinnamyi cinnamate, styrene, and benzaldehyde.

Siam benzoin contains 70-80% resinous matter composed primarily of siaresinolic acid and coniferyl benzoate, with 11.7% benzoic acid. 2.3% cinnamyl benzoate, and 0.3% vanillin also present; cinnamic acid is reported absent.

Benzoins from three styrax species of Chinese origin are reported to be similar to Siam benzoin in balsamic acids content consisting only of benzoic acid.

Tests for Identity:

1. When about 0.5 gm of powdered benzoin is warmed with 10 ml of potassium permanganate solution, a faint odour of benzaldehyde is

developed only with Sumatra benzoin but not with Siam benzoin.

2. Digest about 0.2 gm of coarsely powdered benzoin with 5 ml of ether, for about 5 minutes, decant about 1 ml of the ethereal solution into a porcelain dish containing 2-3 drops of sulphuric acid and mix carefully, a deep purplish-red colour with Siam benzoin and deep reddish-brown colour is produced with Sumatra benzoin.

USES:

It used as antiseptic, astringent, and expectorant; in vaporizer fluids for inhalation to relieve respiratory discomforts; in Compound Benzoin Tincture, which is widely used as a skin protectant.

II- DRIED LATEX

Latex is an emulsion or suspension where the continuous phase is an aqueous solution of mineral salts, proteins, sugars, tannis, alkaloids etc.. and the suspended particles are oil-droplets, resin, gum, proteins, starch, caoutchouc. Latex is often white in colour, as in opium (***Papaver somniferum***), but may be buff to light brown as in papain yellow, as in ***Argemone mexicana***, or red as in the rhizome of ***Sanguinaria Canadensis***. It occurs in the plants in special structures termed laticiferous cells, tubes or vessels, from which it is obtained by incision into the plant.

Opium

Opium (Raw opium) is the, latex obtained by incision from the unripe capsules of ***Papaver somniferum*** Fam. Papaveraceae and dried partly by spontaneous evaporation and partly by artificial leat.

Collection and Preparation:

1. Incision: While the capsules are still green or just showing a tint of yellow, incisions are made into the walls, so as not to penetrate into the loculus for the following reasons.

- It results in loss of opium.
- Prevent the seed from ripening.

The incision cuts equatorially round the capsule across the laticiferous vessels, which are anastomosing throughout the phloem tissues of the capsule wall, the latex exudes in small drops and partially dries in the air. Incisions are usually made in the afternoon.

2. Time of Collection: Time of collection must be chosen so that neither rain nor dew is likely to spoil the exudation. It is preferably in the morning following the day of incision.

The type of instrument used and the manner of making the incisions and the final form differ according to the country of origin. In Yugoslavia, Bulgaria, Macedonia and Persia a single horizontal incision is made equatorially round the capsule, using a small sharply pointed knife, about 2 to 3 cm long, mounted in a wooden handle about 8 cm long.

In Yugoslavia and Macedonia the exudates is collected in conical tins lined with poppy leaf and holding about 750 gm. of moist opium. The masses turned out from the cans are soft, conical and covered with poppy leaf. Sometimes the latex is put into tins, and is carried in a semi-fluid condition to the factory, where it is dried in warm air, mixed by passing it through a mill and is made into flat oblong cakes about 1.5 to 2.5 cm thick, 18 to 20 cm long and 6-7 cm wide. The cakes are sometimes dried as they are or they may be first rolled in coarsely powdered poppy leaves and then slowly dried, for which purpose they are placed on trays of wire netting stretched on wooden frames and exposed to a warm atmosphere for a long period.

In Persia (Iran) the exudation is scraped off with a knife and collected in a small bowl or on poppy leaf, then it is mixed and made up into rectangular prick-shaped cakes about $10 \times 5 \times 7$ cm.

In Turkey a special cutter is used, this consists of a wooden handle with a flat end in which are embedded seven small knives, the tips of which project about 1.5 mm so that incisions cannot be made, too deeply. The cutter is drawn around the equator of the capsule thus making seven parallel incisions from which the latex exudes. The latex is removed with a special copper instrument, shaped like a small tray. The scrapings are massed into balls of various sizes, wrapped in poppy leaves and packed

with Rumex fruits then mixed in a mill and moulded into uniform sub-cylindrical cakes.

CHARACTERS:

1. Opium occurs in more or less rounded or cubical pieces or somewhat flattened or brick-shaped masses, usually about 8-10 cm diameter, varying in weight, but commonly weighing between 250 and 1000 gm, sometimes warped in tissue paper, cellophane or covered with Poppy leaves or Rumex fruits.
2. Externally, it is dark brown to chocolate brown, sometimes with fragments of Poppy leaves and with Rumex fruits adhering to the masses.
3. It is more or less plastic when fresh, becoming hard and tough, or occasionally brittle on keeping.
4. Internally it is dark brown, coarsely granular or nearly smooth.
5. Opium has a strong characteristic narcotic odour and characteristic very bitter taste.

CONSTITUENTS:

1. **Alkaloids:** Opium contains about twenty five different alkaloids, belonging to different chemical groups which occur in combination with meconic acid, of which about 5% is present, and with sulphuric acid. The most important alkaloids are morphine (10-20%), codeine (methyl morphine 0.3-0.4%), narcotine (2-8%), thebaine (0.2-0.5%). Narceine. papaverine and the remaining alkaloids are present in very small quantities constituting together rather more than 1% of the drug.
2. Small quantities of mucilage, sugar, wax, caoutchouc and salts of Ca and Mg.

Test for Identity: (Test for meconic acid)

Warm 20-30 mg of powdered opium in 2-3 ml of water for few minutes and then filtering. On adding a few drops of 5% ferric chloride solution to the filtrate, a purplish red colour is produced and the colour is not destroyed by addition of dilute hydrochloric acid or 5% mercuric chloride solution.

USES:

1. Opium is servers as a hypnotic, an analgesic and a sedative.
2. It is frequently administered to relieve pain and to calm excitement.
3. It is also used as an astringent in diarrhoea and dysentery and as a sedative in certain forms of cough.

All the important alkaloids have a narcotic action which decreases in the following order, morphine, papaverine, codeine, narcotine, thebaine.

III- DRIED JUICES

ALOE

Aloes is the solid residue obtained by evaporating the liquid which drains from the cut leaves of ***Aloe vera***, known in commerce as ***Curacao Aloes***, ***Aloe ferox*** and its hybrids, known in commerce as ***Cape Aloes***, or of ***Aloe Perryi***, known in commerce as ***Socotrine*** or ***Zanzibar Aloes***, Fam. Liliaceae.

Aloe vera is a species indigenous to North Africa, but cultivated in the West Indies.

Aloe ferox is a South African species. While Aloe Perri is a species found on the Island of Socotra, in Eastern Africa.

COLLECTION AND PREPARATION:

Cutting of leaves takes of Aloe vera (curacao Aloes) place in March in the noon, the leaves are placed quickly in V-shaped troughs set on a sharp incline to allow the juice to flow down into a vessel placed beneath. The juice is then evaporated in a copper vessel at a relatively low temperature and the thickened juice is poured into large gourds or boxes and the product is obaque or semi-translucent.

Comparative Characters of Different Types of Aloes

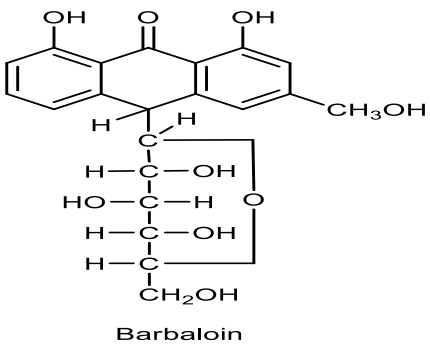
Itmes	Cape	Curacao	Socatrine	Zangiber
I. Physical properties **Colour**	Yellowish-brown	Chocolate-brown	Black	Livery-brown
Appearance	Transparent	Opaque	Opaque	Opaque
Fracture	Glassy	Waxy	Porous	Less porous
Odour	Disagreeable	Disagreeable	odourless	odourless
Taste	Sour, bitter taste	bitter	bitter	bitter
II. Chemical constitnents				
Barbaloin	+	+	+	+
β-Barbaloin	+	+	-	-
Isobarbaloin	±	+	-	-
Aloe-emodin	-	+	+	-
III. Chemical Tests				
Bromine test	+	+	+	+
Borax test	+	+	+	+
Cupraloin test	±	+	-	-
Nitric acid test	+	+	-	-

CHARACTERS:

Curacao Aloes: Occur in dark chocolate-brown, usually obaque masses. The fracture is dull waxy, uneven and frequently conchoidal. It has characteristic disagreeable odour and nauseous, very bitter taste.

CONSTITUENTS:

1. **Anthraquinons:** Aloin, barbaloin, isobarbaloin, β-barbaloin anthranol, aloetic acid, anthracene, aloe-emodin, emodin and chrysophanoic acid.



Barbaloin

2. **Saccharides:** Cellulose, glucose, mannose, L-rhamnose and aldopentose.
3. **Fatty acids:** γ-linolenic acid.
4. **Enzymes:** Oxidase, amylase, catalase, lipase and alkaline phosphatase.

5. **Amino acids:** Lysine, threonine, valine, methionine, leucine, isoleucine, phenylalanine.
6. **Vitamins:** Vitamins B_1, B_2, B_6, C, and E, folic acid, choline and β-carotene.
7. **Minerals:** Calcium, sodium, manganese, magnesium, zinc, copper and chromium.
8. **Miscellaneous:** Cholesterol, triglycerides, steroids, uric acid, lignins, β-sitosterol, gibberellin and salicylic acid.

Aloe vera terminology:

- **Aloe vera gel:** Naturally occurring, undiluted gel obtained by stripping away the outer layer of the Aloe vera leaf.
- **Aloe vera concentrate:** Aloe vera gel from which the water has been removed.
- **Aloe vera juice:** An ingestible product containing a minimum of 50 % Aloe vera gel.
- **Aloe vera latex:** The bitter yellow liquid derived from the pericyclic tubules of the rind of Aloe vera, the primary constituent of which is aloin.

Tests for identity:

Boil about 0.5 gm of powdered aloe with 50 ml of water for 2 to 3 minutes, clarify with kieselguhr and filter. The filtrate responds to the following tests:

1. **Borax test:** To 5 ml of the clear filtrate add 0.2 gm of borax and dissolve by heat, pour 2 to 3 drops of the dark fluid into water, a green fluorescence is produced. All the four kinds of aloes described to this test.
2. **Bromine test:** To 1 ml of the clear solution of aloes add an equal volume of saturated solution of bromine. A yellow precipitate of tetrabromaloin is formed. All the four kinds of aloes described to this test.

3. **Cupraloin test (for isobarbaloin):** Dilute 2 ml of the filtrate to 10 ml with water, add a drop of copper sulfate, warm and add about 0.5 ml of sodium chloride T.S. and then about 1 ml of alcohol and warm. A deep wine red colour is produced with Curacao Aloes and a quickly fading pale wine red with Cape Aloes. This test is not given with Socotrine and Zanzibar Aloes.
4. **Nitric acid test:** Mix 5 ml of the filtrate with 2 ml of nitric acid. A yellowish-brown colour, passing rapidly to vivid green is produced with Cape Aloes, a deep brownish red colour with Curacao Aloes and a yellowish-brown colour with Socotrine and Zanzibar Aloes.
5. **Free anthraquinone derivatives:** Shake 30 ml of the filtrate with 20 ml of benzene and allow separating. Shake 10 ml of the benzene layer with 10 ml of dilute ammonium hydroxide solution. A cherry red colour is produced in the ammoniacal layer with Curacao and Socotrine but not with Zanzibar and Cape Aloes.
6. **Modified Borntrager's test (barbaloin):** Mix 0.1 gm of Aloe with 5 ml of 5°/o ferric chloride solution, and 5 ml of dilute hydrochloric acid. Heat in a boiling water bath for 5 minutes and cool. Shake the solution with benzene and allow separating. To the benzene layer add an equal volume of dilute solution of ammonium hydroxide and shake, where a pinkish-red colour is formed in the ammoniacal layer- All varieties of aloes give this test.

Uses of Aloe vera:

- **Topical:** wound healing, sunburn, hair stonic and minor skin irritations.
- **Oral:** constipation, peptic ulcers, immune system enhancement, diabetes, and asthma.

IV- EXTRACTS

1. AGAR-AGAR

Agar is the dried mucilaginous substance prepared from ***Gelidium corneum, G. cartilagineum***, Fam. Gelidiaceae, and other closely allied red algae.

Japan is the main source of supply and prepare agar from Gelidium species while Ceylon, Southern California, Australia and United kingdom prepare agar from Gracilaria species.

PREPARATION:

In Japan, the seaweeds are collected by means of rakes or by removing them from the ocean floor by diving. The algae are taken ashore and dried, beaten and shaken to remove sand and shells. The weed is bleached by watering and exposure to sunlight. Decoction is made by boiling the algae with slightly acidulated water (1 to 60) for several hours, then filtered while hot. On cooling, a jelly is produced which is cut into bars then forced through wire netting to form strips. Drying is accomplished by successively freezing, thawing and drying at about 35ºC.

CHARACTERS:

1. It occurs as grayish-white granulated powder or in bundles of membranous strips, 4 to 10 mm wide, translucent and almost colourless.
2. The surface of strips is wrinkled and somewhat micaceous and various species of diatoms are embedded in it.
3. It is yellowish, tough and difficult to break when damp and brittle when dry.

4. It has slight odour of marine algae and a faintly salty mucilaginous taste.
5. It is insoluble in cold water but swells and is slowly soluble in boiling water to form a colloidal solution which forms a jelly on cooling.

CONSTITUENTS:

Agar is s heterogenous polysaccharide contains two principles which are agarose and agaropectin.

1. Agarose is a galactose polymer (free of SO_4) and is responsible for the gel strength of agar.
2. Agaropectin is possibly a sulphonated polysaccharide in which galactose and uronic acid units are partly esterified with sulphuric acid.

Tests for Identity:

1. Boil 1 gm of Agar for 10 minutes with 100 ml of water, replacing the water lost by evaporation, the solution yields a stiff jelly on cooling.
2. Boil 0.5 gm of agar with 10 ml of dil. Hydrochloric acid for 10 minutes, set aside for 10 minutes and decant, examine the residue microscopically, where sand particles, spong spicules and frustules of diatoms of Arachnoidiscus are found.
    - To about 4 ml of the decanted solution add 1 ml of barium chloride, where a white precipitate is given (for SO_4 radicle).
    - To another portion, add dilute sodium hydroxide solution for neutralisation then 4 boiling water bath, where a red precipitate is given indicating reduction by the reducing sugars produced by hydrolysis of the sugar moiety.
3. To a nearly boiling 0.2% solution of agar, add tannic acid T.S., no precipitate is formed (c.f. gelatin).
4. Mix a little of powdered agar with a drop of rhuthenium red T.S., where the particles are stained deep red.
5. Mix a little of powdered agar with a drop of N/50 iodine, where some particles are coloured deep crimson.

USES:

1. Medicinal, Pharmaceutical and Cosmetic. As a bulk laxative, particularly in chronic constipation; in the manufacture of emulsions, suspensions, gels, and hydrophilic suppositories.
2. Food: Used in canned meat and fish products as gel filler or gel binder, in confectionery, dairy products and sweet sauces.
3. Others: A major use of agar is in culture media for microorganisms. It is one of the most widely used media for biotechnology purposes.

2-GELATIN (Gelatine, Gelatinum)

Gelatin is the protein obtained by boiling the collagous tissue of animals such as skin, tendons ligaments, and bones with water, evaporating the aqueous extract and drying the residue in air.

The animals used for this purpose are Ox, ***Bose taurus***, and the sheep, ***Ovis aries***, Fam. Bovidae.

PREPARATION:

1. Raw material consisting of the skin and tendon is first subjected to a preliminary treatment, known as (liming) in which the material being soaked for about 20 days in dilute milk of lime. This process dissolves the fleshy matter, removes chondroproteins of the connective tissues and saponifies fats. The hides are then thoroughly washed in running water.
2. Bones are usually ground and defatted, by treatment with benzene, and then the mineral matter is removed by treatment with hydrochloric acid.

The treated materials from skins, tendons or bones are now heated with water in open pans with perforated false bottoms or sometimes under reduced pressure. The clear fluid is run off and is evaporated under reduced pressure until the gelatin content is about 45%. It is then run into shallow metal trays and allowed to set to a jelly. The jelly is removed and

placed in trays with a wire netting bottom, these trays are passed through a series of drying rooms at temperatures, increasing by about 10°C each time, from 30°C to 60°C, and sometimes bleaching with SO_2 is done.

CHARACTERS:

1. Gelatin occurs in thin sheets, or shreds, or powder which may be nearly colourless or pale yellow.
2. It is hard and brittle, when broken it at first bends and then breaks suddenly with a short fracture.
3. In cold water it swells and when heated dissolves.
4. It is soluble in acetic acid and glycerol, but not in alcohol and ether.
5. A 2% hot aqueous solution should gelatinise on cooling.

CONSTITUENTS:

Gelatin consists chiefly of the protein glutin and, when it is heated with soda-lime, ammonia is evolved, showing the presence of nitrogen of which it contains about 18%.

Tests for Identity:

1. An aqueous solution of gelatin gives a precipitate with solutions of chromium trioxide, tannic acid, trinitrophenol and lead subacetate.
2. With Millon's reagent it gives a white precipitate which becomes red on boiling.

USES:

Gelatin has been used as a nutrient. It is also used as a basis for glycerin suppositories, for the preparation of pastilles and for the preparation of nutrient media for the growth of bacteria.

V- GUMS

Gums are abnormal products, resulting from pathological conditions brought about either by injury or by unfavourable conditions of growth and are usually formed by changes in the existing cell-walls.

CHARACTERS:

1. Gums are amorphous, translucent solids.
2. They are insoluble in alcohol and organic solvents, but soluble in water to yield viscous, adhesive solutions, or are swollen by the absorption of water into a jelly-like mass.
3. They consist of calcium, potassium and magnesium salts of complex substances known as polyuronides.
4. They can be hydrolysed by prolonged boiling with dilute acids when they yield mixtures of sugars and organic acids.
5. The sugar that formed by hydrolysis are monosaccharides, usually pentoses such as arabinose, xylose or hexoses such as galactose.
6. The acids liberated by hydrolysis are uronic acids i.e. acids derived from monosaccharides by the oxidation of the primary alcoholic group which they contain as glucuronic and galacturonic acid. In certain gums e.g. acacia gum, part of the polyuronide acids, which are glycosidal compounds of one molecule of uronic and one molecule of a sugar.
7. Gums are produced by the conversion of the cell-walls of the tissue into gums, probably by means of enzyme.

N.B.:

1. Pectine and hemicelluloses also yield, on hydrolysis, uronic acid and sugars, thus showing a relationship with gums.
2. Mucillages are similar in constitution to gums, but are normal products of cell activity, being secreted in the cell and laid down like hemicelluloses.
3. Artificial gum (dextrin) is produced from starch, differs originally from gums in being entirely converted into dextrose (glucose) by dilute mineral acids, it is strongly dextrorotatory while natural gums being slightly laevorotatory.

1. GUM ARABIC (Gum Acacia)

Gum Arabic is the dried gummy exudation from the stem and branches of ***Acacia Senegal***, or of some other African species of Acacia, Fam. Leguminosae-Minosoideae.

The plant is a tree abundant is Sudan, in Central Africa and in West Africa but the best gum is produced in kordopan.

Collection and preparation:

Some gum exudes from trees as a result of the cracking of the bark, but the most esteemed, kordofan, variety is obtained from trees, about six years old, tapped in February and March, or in early in September. The tapper, with a blow from a small axe, makes a transverse incision just under the bark of the stem and large branches without injuring the cambium. By twisting the axe the bark is loosened, strips of it being then pulled off up and then down the cut and so removes a strip of bark 0.5 to 7 meter long. The cambium produces new phloem and in about twenty to thirty days the tears of gum which have formed on the surface may be collected.

Freshly collected gum is translucent but on exposure to the sun, it develops cracks and become friable.

CHARACTERS:

1. It occurs in rounded or ovoid tears of variable size, usually about 0.5 to 2 cm in diameter.
2. Externally, it is whitish or yellowish-white, opaque from the presence of numerous small fissures in the outer part of the tears.

3. Tears are easily broken up into a number of small transparent angular fragments with glistening vitreous surface.
4. It is almost odourless and has a bland and mucilaginous taste.
5. It is insoluble in alcohol but dissolves freely in water forming a translucent viscid liquid.

CONSTITUENTS:

1. It consists mainly of arabian (Ca, Mg and K salts of arabic acid). Hydrolysis of Arabic acid yields a sugar moieties consisting of 2 molecules of D-galactose, 3 molecules of L-arabinose and 1 molecule of L-rhammose, in addition to an aldobinoic acid which is 6-β-D glucuronoside-D-galactose.
2. It also contains enzymes such as diastase; and oxidase.

Tests for Identity:

1. Tests for oxidase enzyme:
    - Dissolve about 0.25 gm of the coarsely powdered drug in 5 ml, of distilled water by shaking in the cold. Add 0.5 ml hydrogen peroxide and 0.5 ml of benzidine solution, shake and allow to stand for few minutes, a deep blue colour or greenish-blue colour is formed due to the presence of oxidase enzyme.
    - Repeat the above test replacing the benzidine solution by tincture of guaiacum, a blue colour is produced due to the presence of the oxidase enzyme. If the gum is heated or bleached, it fails to give the above tests.
2. To 2 gm of gum Arabic add 15 ml of water and shake frequently until solution is effected and carry out the following tests on the obtained mucilage:
    - To 5 ml of the mucilage add 2 ml of saturated aqueous solution of borax. The mixture agglutinates to stiff translucent masses.
    - Dilute 1 ml of the mucilage with 4 ml of water and add few drops of lead subacetate solution, a heavy white precipitate forms.
    - Dilute 1 ml of the mucilage with 2 ml of water and add few drops of ferric chloride solution, a stiff brown jelly is formed, due to the tannin which is present in inferior gum.

- Dilute 1 ml, of the prepared solution with 4 ml of water, boil, cool, and add 2 drops of N/10 iodine solution. No blue colour is produced with official gum. A blue colour indicated starch, violet or red indicates dextrin which should not be present in the official gum.
- Dilute 1 ml with 4 ml of water and add a few drops of lead acetate solution, no precipitate is produced.

USES:

3. Mainly in the manufacture of emulsions and in making pills and troches (as an excipient).
4. As a demulcent for inflammations of the throat or stomach.
5. As a masking agent for acrid-tasting substances such as capsicum.

2- GUM TRAGACANTH (Tragacanth)

Tragacanth gum is the dried gummy exudation obtained by incision from the stem of ***Astragalus gummafer*** Fam. Leguminosae, and some other Asiatic species of Astragalus.

The plant grows in Asia minor, Syria. Iraq, Iran and USSR.

The mode of formation of tragacanth is entirely different from that of acacia, the gum exuding immediately after injury and therefore being preformed in the plant while acacia is slowly produced after injury.

The cell walls of the pith and medullary rays of the stem are gradually transformed into gum, a change which is termed (gummosis).

COLLECTION:

Gum can be obtained from the plants in their first year, but it is said to be of poor quality and unfit for commercial use. The plants are therefore tapped in the second year. When the stem is incised a gum exudes and dries, the form that it assumes being dependent on the form of the incision, vertical slits yielding flat, ribbon-shaped pieces and punctures

vermiform tears. When the stem is wounded the gum is forcibly pressed out, a piece about 2 cm being exuded in half an hour, it carries with it the starch grains present in the cells of the phloem and cortex in a more or less unaltered condition. The exuded gum is collected two days after incision. Some of the plants are burned at the top after having had incision made- The plant then sicken and gives off a greater quantity of gum (inferior quality).

CHARATERS:

1. Persian tragacanth occurs in thin, flattened, curved, ribbon-shaped flakes of a translucent, horny appearance and nearly colourless or faintly yellowish.
2. The flakes are often 3 cm long 1 cm wide and about 2 mm thick, and are marked with numerous concentric longitudinal and transverse ridges, giving the impression that the gum has been exuded in successive portion.
3. The flakes break with a short fracture, are odourless and almost tasteless.
4. Soaked in cold water they swell considerably, forming a tenacious gelatinous mass, but only 8 to 10% dissolves.

CONSTITUENTS:

1. Tragacanth can be separated into two parts on the basis of its behaviour when added to water:
   - Portion soluble in water is named tragacanthin.
   - The insoluble portion is named basorin.
2. Chemical investigation has shown the presence of three fractions in the gum:
   - An acidic portion named tragacamhic acid, which on hydrolysis yields galacturonic acid, galactose, xylose and fucose.
   - A neutral polysaccharide which on hydrolysis gives arabinose and galactose.
   - A small amount of a substance believed to be a glycoside of a sterol derivative.

3. Tragacanth contains also, traces of starch, cellulose, and nitrogenous substances- No oxidase enzyme is present.

Tests for Identity:

1. Place a little powdered gum tragacanth on a porcelain tile, add few drops of N/50 iodine solution and rub to form a smooth paste. It acquires an olive green colour.
2. Boil about 0.25 gm of gum tragacanth with 50 ml of HCl to 4 ml of the mucilage and heat for 30 minutes in a water bath. Divide the liquid into two parts.
    - To the first part add 1.5 ml of sodium hydroxide solution and 3 ml of Fehling's solution and warm in a water bath. A red precipitate forms due to the presence of reducing sugars from the hydrolysis of the gum.
    - To the second part add barium chloride solution, no precipitate is produced (distinction form agar).
3. Dilute 10 ml of mucilage prepared above with equal volume of distilled water and add 10 m of lead acetate a flocculent precipitate is formed (distinction of gum Arabic).

USES:

1. Tragacanth has been in use since ancient times. Most of its uses are based on its emulsifying, thickening, and suspending abilities as well as its stability to acid and heat.
2. Extensively used in vaginal jellies and creams, in emulsions (e.g. cod liver oil), low-calorie syrups and elixirs, and as binding agent or demulcent in tablets and lozenges.
3. It is also used in toothpastes and hand lotions.

Part II

Chromatography

Chromatography and its application in the field of natural products

Introduction:

One of the most important scopes of pharmaceutical sciences is the development of new drugs. Actually, this can be achieved via several methods like isolation from natural sources, biotechnological techniques and/or chemical synthesis. In this course we will focus on drug preparation from natural sources using what is known as chromatographic technique. This process starts with identification and collection of natural source followed by extraction of the constituents -from fresh or dried material- and isolation and identification of each constituent.

Extraction:

In the field of natural products, extraction can be defined as the process of solubilizing and separating the constituents from their source using a suitable solvent.

Based on the nature of the constituents, extraction can be either hot extraction or cold extraction.

Hot extraction is used in case of thermostable constituents as it is rapid while cold one is used for heat sensitive (thermolabile) constituents to maintain its integrity.

Types of extraction:

Maceration: Soaking the natural source in a suitable solvent for several days at room temperature. Shaking can facilitate releasing the constituents.

Digestion: It is the same like maceration but with heating at moderate temperature.

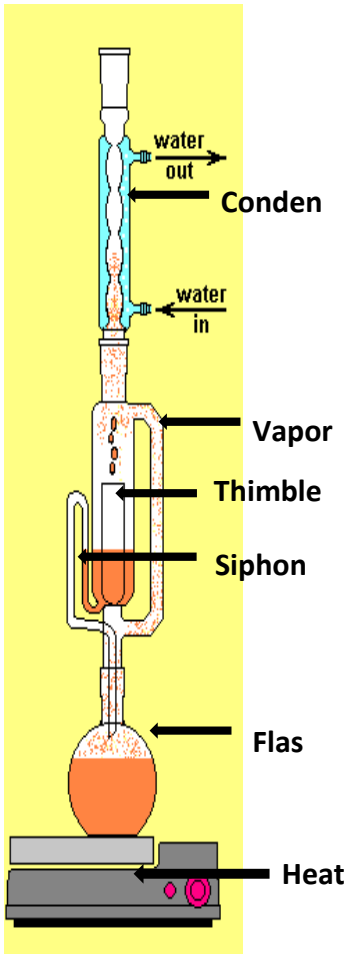
Infusion: Soaking the natural source in cold or boiled water for a short time.

Decoction: Soaking the natural source in a defined volume of boiled water and for a defined time.

Percolation: Soaking the natural source in boiled solvent for certain time but with continuous addition and drainage of solvent. (i.e. solvent in continuous movement).

- **Soxhlet:**

Represents a type of hot continuous extraction (percolation) in which the ground material (natural source) is placed in a thimble (E) made and the extracting solvent is placed in flask (A) and heated. The solvent's vapors is evaporated through vapor tube (B) and condensed in condenser (D). The condensate drips into the thimble containing the natural source to make extraction. As the liquid in the thimble reaches a certain point in siphon tube (C), it will drained into flask (A). This process is repeated continuously till complete extraction.



Advantages:

- Almost achieves complete extraction.
- Small volume of solvent is utilized.

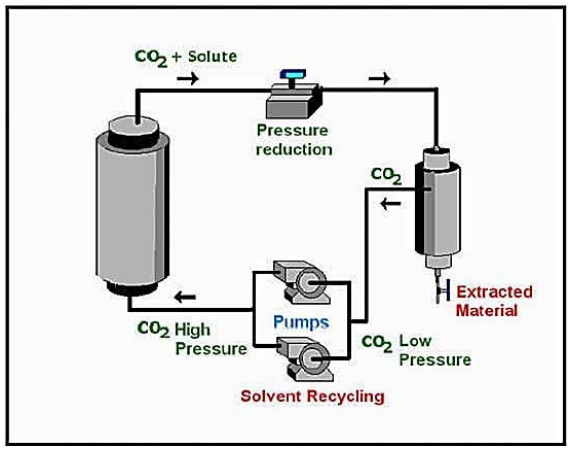
Disadvantage:

- Not suitable for thermolabile chemicals extraction.

Supercritical fluid extraction:

This method depends on using gases at their critical point to make use of penetrating power of gases and solvating power of liquids.

Critical point is the temperature at which the gas retains its gaseous properties irrespective of the applied pressure.



Example: Carbon dioxide, critical point is 31.1˚C; it is suitable for extraction of lipophilic constituents.

Advantages:

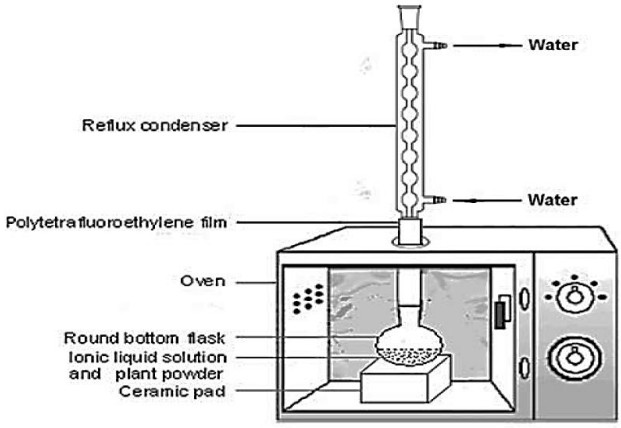
- No solvent residues.
- No environmental contamination and safe.

Disadvantages:

- High cost.

Microwave assisted extraction:

This method depends on release of molecules from tissues as a result of microwave energy. Resulted heat enhance the process.



Advantage:

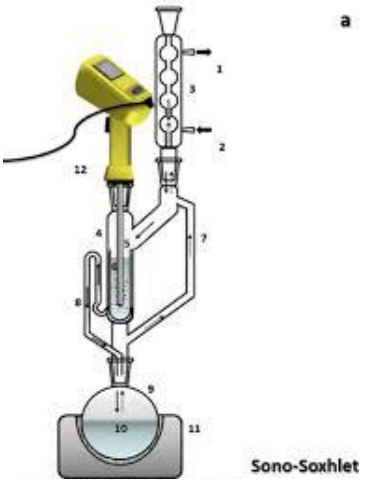
- It is suitable for polar small molecules.

Disadvantages:

- Possibility of chemicals destruction due to overheating so it must be avoided.

Ultrasound assisted extraction (Sonication extraction):

Ultrasound disrupts cell wall and increase contact between constituents and solvents resulting in high yield of the components.



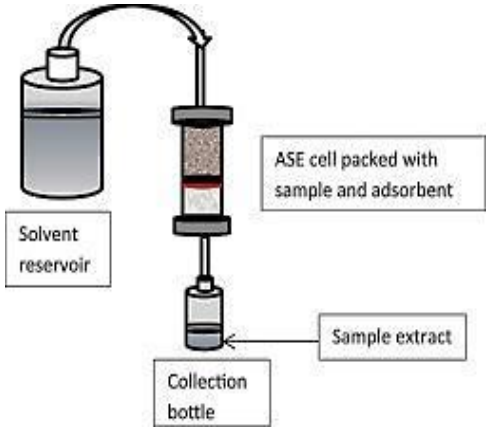
Advantages:

- It reduces time and solvent use.

Disadvantages:

- Free radicals can be formed.

Accelerated solvent extraction:



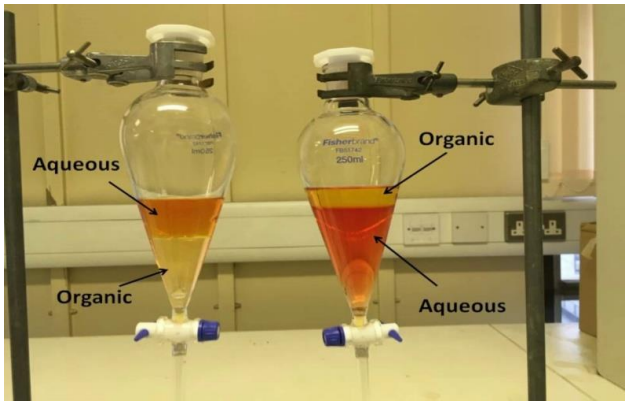
Automated method depends on packing the samples with inert material like sand and putting this mixture in layers separated by filter paper. It has controlled temperature and pressure and depends on solvent type.

Advantages:

- Short time, reduced solvent consumption and highly efficient.

Fractionation:

After the extraction process; the extraction solvent is evaporated using rotavapour, a stream of nitrogen or any other suitable method, the extract is suspended in water in a separating funnel and subjected to what is called fractionation process using a series of different organic solvents starting with the less polar one. In other words, the total extract is divided into several fractions based on the polarity of the constituents.



Soxhlet can be used to perform extraction and fractionation in the same step in a so called successive extraction. In such case, the extraction process starts with the less polar solvent till complete extraction of non-polar compounds then the next less polar solvent is used and so on.

Anyhow; after this process, the fractionation solvent is evaporated and the remaining residue is subjected to a suitable chromatographic process to isolate its components in a pure form.

Chromatography:

Generally, chromatography is a method for separation of components of a mixture based on the affinity (differential migration) of those constituents to either a stationary phase or a mobile phase.

Types of chromatography according to mechanism of separation:

- Adsorption chromatography: Based on the differential migration between a **solid** stationary phase and a **liquid** or **gaseous** mobile phase.
- Partition chromatography: Based on the differential migration between a **liquid** stationary phase and a **liquid** or **gaseous** mobile phase.
- Gel permeation chromatography (Molecular sieving): Based on molecular weight of compounds to be isolated.
- Ion-exchange chromatography: Based on net charge of compounds to be isolated.
- Electrophoresis: Based on differential migration to opposite electrodes.

Types of chromatography according to used technique:

- Column chromatography (Closed bed chromatography).
- Planar chromatography (Open bed chromatography).
  - Thin layer chromatography (TLC).
  - Thick layer chromatography (Preparative TLC).
  - Paper chromatography (PC).
- High performance liquid chromatography (HPLC).
- Gas chromatography (GC).

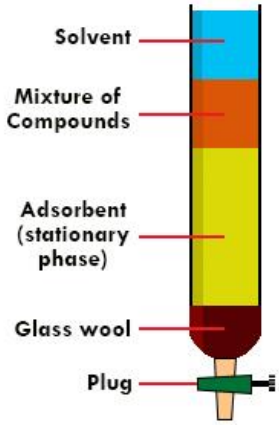
Terminology

1. **Adsorbent:** Finely divided homogenous solid having uniform particle size and large surface area which is capable of attracting molecules to its surface.
2. **Analyte:** Components of sample mixture
3. **Chromatogram:** The final record at the end of chromatographic separation process.
4. **Development:** Description of the process of chromatography. (running of the mobile phase through the stationary phase)
5. **Eluent:** Solvent used for separation in chromatographic techniques
6. **Effluent:** Any liquid out of the column
7. **Elution:** Separating out components of the mixture in pure or partially mixed form
8. **Resolution:** The ability of any chromatographic process to separate pure compounds.
9. **Retention time:** Time taken to elute a particular solute (compound) out of the column
10. **Rate of flow(RF):** Distance travelled by a solute / distance travelled by solvent
11. **Retention volume:** Volume of mobile phase required to elute a particular solute (compound)
12. **Tailing:** compound or solute is eluted in several fractions (Disadvantage of chromatography)
13. **Visualization:** Making the colourless bands or spots visible (detection of the separated spots)

A) Adsorption mechanism:

1- Column chromatography

It represents the most common type on the level of isolation and purification of compounds from natural or synthetic sources.



Stationary phase: It is the static phase in chromatographic process.

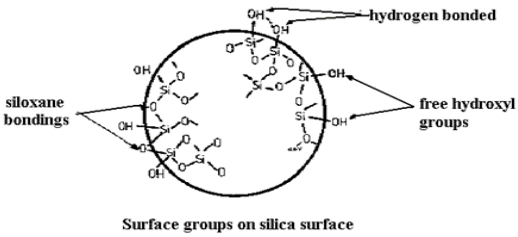
Characters of stationary phase:

- Inert, not to react with column constructing material, mobile phase or components to be separated
- Insoluble in mobile phase.
- Small particle size in case of adsorption mechanism. As the particle size decrease, the surface area for separation increase leading to good separation.
- Colorless or white.

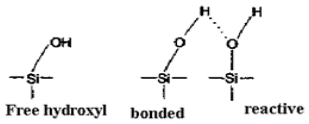
Examples for stationary phase in adsorption mechanism:

1- Silica gel:

- The most common stationary phase.
- Its chemical structure is $SiO_2(H_2O)_n$.
- To be active as adsorbent, silica gel is heated for 2 hours at 190˚C to yield silanol groups. Overheating must be avoided as it leads to inactive siloxane formation.



Surface groups on silica surface



2- Alumina:

- Common in separation of alkaloids.
- Its chemical structure is Al_2O_3.
- To be active as adsorbent, alumina is heated for 12 hours at 200-400˚C.
- Alumina has the disadvantage of catalyzing some reactions as saponification of esters, polymerization of olefins, condensation reaction of aldehydes and ketones.

Grades of alumina:

Grade 1 - holding no water.

Grade II- holding 3% water.

Grade III- holding 6% water.

Grade IV- holding 10% water.

Grade V- holding 15% water.

Alumina can be treated to separate different classes of compounds as following:

- At neutral pH (7): alumina is used for separation of non-polar compounds such as steroids.
- At acidic pH (4) (treatment with HCl then water wash): alumina is used for separation of acidic compounds e.g. carboxylic acid.
- At alkaline pH (10) (treatment with NaOH then water wash): alumina is used for separation of basic compounds e.g. alkaloids.

3- **Polyamide:** Common is separation of phenolic compounds such as falvonoids, tannins, anthraquinones and anthocyanins.

4- **Diatomes:** Prepared from siliceous skeleton remains of microscopic marine animals.

5- **Charcoal:** Not used due to its black colour and non-selectivity.

6- **Reversed phase silica:** It is silica particles impregnated with hydrocarbon on the outer surface (C6 to C18), so it is non-polar.

Mobile phase: It is the moveable phase in chromatographic process.

Characters of mobile phase:

- Inert, not to react with column constructing material, stationary phase or components to be separated
- Low boiling point to ease its evaporation.
- Low viscosity to ease its movement in the system.
- Low toxicity, low inflammability and not explosive.

Examples of mobile phase:

Water as well as several organic solvents represents examples for mobile phases. The choice of one or mixture of them depends on the polarity and nature of the compounds to be separated.

The following table summarize common solvents that used in chromatographic separations with their arrangement in the eluotropic series:

Solvent	Polarity	Boiling point, ^{O}C
Pet. ether	**0**	**36**
Cyclohexane	**0.04**	**69**
CCL_4	**0.18**	**77**
Toluene	**0.29**	**111**
Diethyl ether	**0.38**	**35**
Chloroform	**0.40**	**62**
Dichloromethane	**0.42**	**40**
Acetone	**0.56**	**56**
Ethyl acetate	**0.58**	**77**
Acetonitrile	**0.65**	**82**
2-propanol	**0.82**	**82**
Ethanol	**0.88**	**78**
Methanol	**0.95**	**64**
Water	**1.00**	**100**

Eluotropic series: It is the arrangement of solvents according to their polarities in an ascending order.

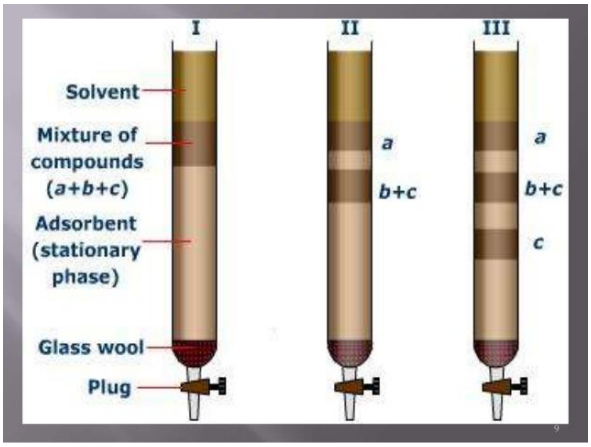
Columns and their construction:

Columns can be made up from glass, inert types of polymers or stainless-steel.

The first step in column construction is the **selection of a suitable kind** with **suitable dimensions** according to the components to be separated and their weight. (Columns can range from 1 mm in height and diameter to 100 cm in height and 10 cm in diameter or even more).

The second step in column construction is **packing process**:

It is the filling up of column with stationary phase. The amount of stationary phase used should be 15-50 times the weight of the sample to be chromatographed.



Packing is achieved by one of the following methods:

Dry packing: The stationary phase is poured into the column and the mobile phase is allowed to pass through it.

Wet packing: The stationary phase is suspended in the first mobile phase and the formed **slurry** is poured into the column.

The third step in column construction is **sample loading:**

Sample loading could be:

Dry method: By adsorbing the sample on small amount of the stationary phase and then apply this mixture to the top of the column.

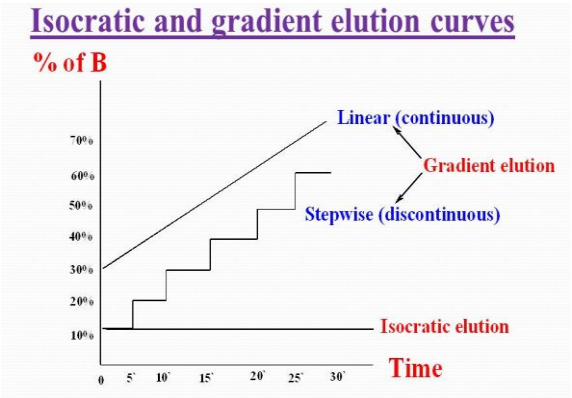
Wet method: By dissolving the sample in a small volume of a suitable solvent then apply it to the top of the column.

Disc method: By adsorbing the liquid or solubilized sample on small discs of filter paper then apply these discs to the top of the column.

Now the column is ready for development process which can be isocratic or gradient.

Development: Defined as passage of mobile phase through the column for the purpose of separation.

Isocratic development: The same mobile phase (single or mixture of solvents) is used through the whole process without any change in polarity.



Gradient development: The polarity of the mobile phase is changed with time in a predetermined or specific manner either **stepwise** (at predetermined

time intervals) or **gradient** (continuous change over the time). It could be either increase in polarity in the case of normal phase separation or decrease in polarity in the case of reversed phase separation.

During the development, fractions come out of the column are collected and subjected to visualization process to detect their constituents. The fractions collection may be at predetermined specific volume or time, it may be manual or automated process.

Detection or visualization of composition in each fraction can based on:

- Naked eye depending on the colors.
- Ultra violet detection.
- TLC results.

From the above process we can say that, column chromatographic process is affected by:

1- Column dimensions: Narrow and long columns give better separation than wide and short ones.

2- Type and amount of stationary phase used.

3- Polarity and physical characters of mobile phase.

4- Sample amount and purity. Overloading and impurities may interfere with the separation process.

5- Temperature and pressure as they can affect the rate of flow of the mobile phase.

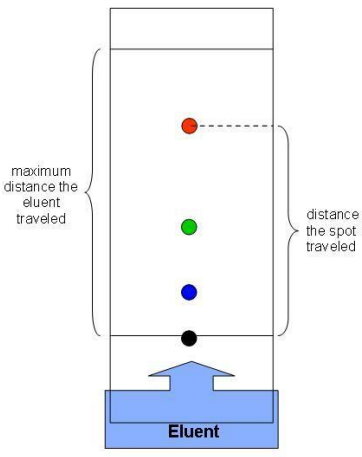
Applications of Column Chromatography:

1- Isolation and purification of natural products.
2- Isolation and purification of synthetic products.
3- Preparation of samples before further analyses.

2- Planar Chromatography

- **Thin layer chromatography (TLC)**

TLC represents the most common chromatographic technique for detection and identification.



Stationary phase: All kinds of stationary phases in column chromatography can be used in TLC but with a very small particle size (1-25 um) and in

presence of binder like gypsum CaSO4 or starch to adhere it to the backing material (support). The support can be glass, aluminum or plastic material.

TLCs can be **prepared at laboratory (what is so called home made TLC)** by spreading a layer of stationary phase for TLC over the supporting material then keeping it for drying and activating it at 120˚C for 2 hours or bought as **ready plates (what is known as pre-coated TLC)**.

Mobile phase: The selection here is a trial and error till finding the most suitable one for separating the compounds under investigation.

Sample application:

Using capillary tubes, samples can be applied as spots or bands on a predetermined start line at a distance of 1 cm from the bottom of the plate and at least 0.5 cm from the edges.

Development:

Before putting TLC in the tank for development (jar), the mobile phase is allowed to stand alone in the tank for about 5 minutes to make homogenous distribution (saturation) of the tank's atmosphere with the vapor of the mobile phase. This process is called **equilibration**.

After the equilibration, we put TLC in the tank and allow the mobile phase to pass through it in order to isolate the sample's constituents into single spots.

Actually, there are different modes for development that can be summarized in the following:

Ascending development: The mobile phase is moved from the bottom to the top.

Descending development: The mobile phase is moved from the top to the bottom. In such case, the start line must be at the top.

Horizontal development: The mobile phase is moved from side to another side.

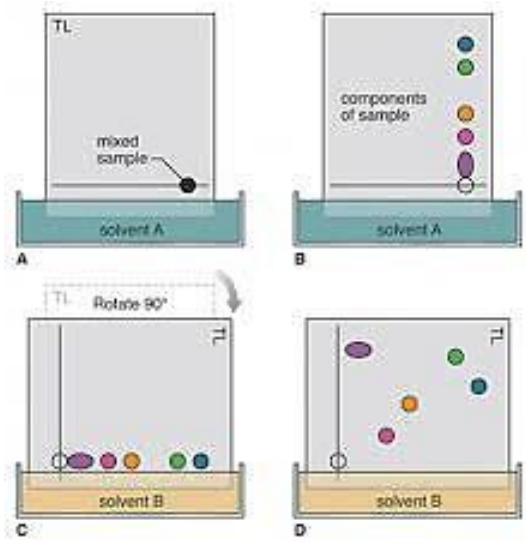
Radial development: The mobile phase is moved from the center to the periphery.

Special modes of development that participate in separating closely related spots:

Continuous development: The mobile phase is allowed to evaporate at the solvent front in the case of ascending mode or drained out in the case of descending mode to allow the passage of large volume of the mobile phase which helps in the separation process of close spots.

Multiple developments: After the first run (development), the mobile phase is dried and a second development is started with the same mobile phase or different mobile phase. The process is repeated several times that is why it is called multiple.

Two dimensional development: After the first run (development), the mobile phase is dried, the plated is inverted 90 degree and allowed to develop again in the same or new mobile phase.



Visualization (detection):

Based on the properties of the separated substances and the purpose from the process, a suitable visualizing agent must be selected.

Physical visualization:

- Naked eyes can detect colored spots or bands.
- Ultraviolet can be used for detecting unsaturated compounds without any destruction for the separated substances.

Chemical visualization:

This method is depends on the reaction of the isolated substance (in-situ) with a general (like sulfuric acid) or specific reagent to convert it from non-colored substance to a colored one.

The following table contains examples for different chemical visualizing reagents and their applications:

Spray reagent	Composition	Detected compounds Produced color
Aniline phthalate	Aniline (0.93g) + 1.66 phthalic acid in 100ml n – butanol sat with water	**Reducing sugars**
Antimony (III) chloride**	22% antimony (III) chloride in chloroform	**Steroids glycosides, and related compounds, (red – to- blue)**
Bial`s reagent	Add 10 ml 10% H_2SO_4 w/v (containing 1 g $FeCl_3$)to 1ml 6% orcinol in acidified ethanol	**Sugars and glycosides**
Dragendorff,s reagent	0.1 / MKI 0.6 Mm basic bismuth sub-nitrate in 3.5 M acetic acid.	**Alkaloids , and quat nitrogen compounds (orange – red)**
Iron (III) chloride	50% ferric chloride in 0.5 NHCL	**Phenolics**
Iodoplatinate	0.15 % w/v potassium chloro palatinate + 3% KI in dil. HCl	**Alkaloids, amines, and org nitrogen compounds (violet)**
Ninhydrin	0.2 % w/v in ethanol	**Amino acids, amines and amino sugars, (blue or purple)**
Phosphomolybdic acid**	10% w/v in ethanol	**Lipids, steroids, keto (hydroxyl acids)**
P – Anisaldehyde-sulfuric acid**	**1 ml conc. Sulfuric +50 ml acetic acid containing 0.5ml P – anisaldehyde.**	**Terpenes, steroids**

Biological visualization:

Based on a biological property of the separated substances.

e.g. Inhibition of bacterial growth in the case of separation of anti-bacterial agents.

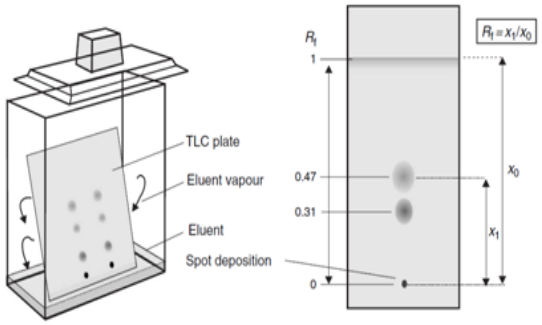
e.g. Blood hemolysis in the case of separation of hemolytic agents like saponins.

Enzymatic visualization:

Based on detecting a specific property for an enzyme substrate interaction.

After the visualization process, identification of the separated substances can take place depending on the color or property of the visualized spot, comparison to a standard and/or by Rf calculation.

Retardation factor (Rf): is defined as the **distance travelled by substance/ distance travelled by solvent.** It is never more than one and must be differentiated from **Rst** which is defined as the **distance travelled by substance/ distance travelled by standard.**



Rf can be affected by:

Stationary phase, mobile phase, sample volume, tank dimension and temperature.

Applications of Thin Layer Chromatography:

1- Natural and synthetic compounds detection and monitoring.
2- Screening of natural products.

- **Chromatotron (Centrifugal development):**

Chromatotron is an apparatus used for separation of compounds based on centrifugal chromatography. It consists of a circular glass plate (acts as supporting material for stationary phase) with a central clear zone and a central hole. The glass plate is mounted on a rotor. Sample is applied at the center of the plate and mobile phase is

pumped and rotated with the sample in the form of bands due to the spinning of the rotor. Separation will take place according to the affinity of the sample components to either stationary or mobile phase. The separated components are eluted and collected in a special channel.

The process is fast and can separated large volume compared to other chromatographic techniques.

- **Preparative thick layer chromatography:**

- It is similar to TLC with the difference of stationary phase layer thickness; it could be 3 mm to allow addition of large volume of the sample.
- The sample is applied in the form of band.
- The separated bands are detected by non-destructive agent or edge spray technique must be used.
- Separated bands are scraped and de-adsorption of isolated substances from the stationary phase is performed by their solubilization in a suitable solvent and filtration to get rid of the stationary phase.

B- Partition and partition chromatography:

Partition is defined as the distribution of a solute between two immiscible solvents. Fractionation represents an example for partition process.

This distribution depends on the distribution coefficient (K).

K = concentration of solute in lighter solvent/concentration of solute in heavier solvent.

In partition chromatography, one of the immiscible solvents (phases) must be liquid and stationary while the other one could be a liquid or a gas.

In order to make a liquid stationary, it must be adsorbed to the surface of a support. A common example for that is filter paper. It is well known that filter papers consist of cellulose fibers which can act as a support for water adsorbed from the surrounding atmosphere (moisture). Other examples include, cellulose powder deactivated silica and diatomites.

- **Column partition chromatography:** it is similar to the column in the adsorption mechanism except in the type of stationary phase.
- **Thin layer partition chromatography:** it is similar to TLC in the adsorption mechanism except in the type of stationary phase and the process of visualization; here corrosive visualizing reagents cannot be used in case of using cellulose as a stationary phase as it will be destructed.

Paper Chromatography:

- Based on the purpose from the process and the amount of the sample, different **filter papers** can be used.
- **Whatman No 1** filter papers are used for analytical purpose.
- **Whatman No 3** filter papers are used for preparative purpose.

Mobile phase:

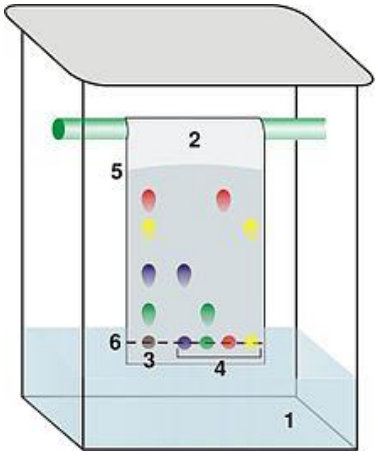
Its composition depends on the substances to be isolated; using organic solvents saturated with water is common in paper chromatography.

Sample loading:

It is similar to that in TLC, previous purification from phenolic compounds and colouring matter is required.

Development:

- Previous equilibration is required.
- The same TLC modes of development can be applied in paper chromatography.

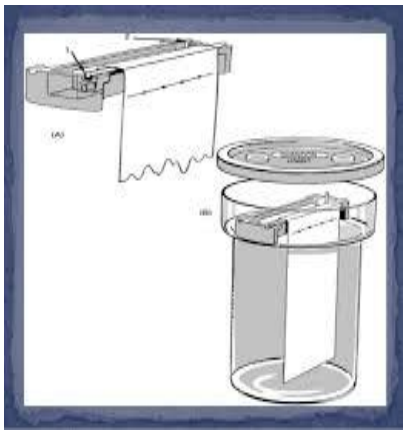


- **Ascending development:**

The paper can be stand in vertical position by hanging in a hock or bending it into a cylindrical form with edges hold with clips.

- **Descending development:**

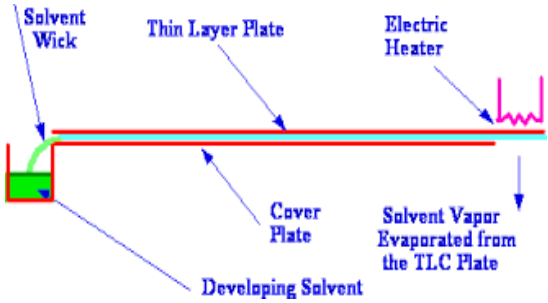
- In this mode of development; a jar designed with a trough for mobile phase and anti-siphon glass rod to hold the paper away of the trough wall is used, the sample is loaded 2 cm after the anti-siphon glass rod. After jar equilibration, development is started and continue till reaching the lower edge or even to drip out of the paper in case of using continuous development with serrated margin.



- This technique is faster than ascending one and allows long distance development (contrast for ascending one; maximum 50 cm).

- **Horizontal development:**

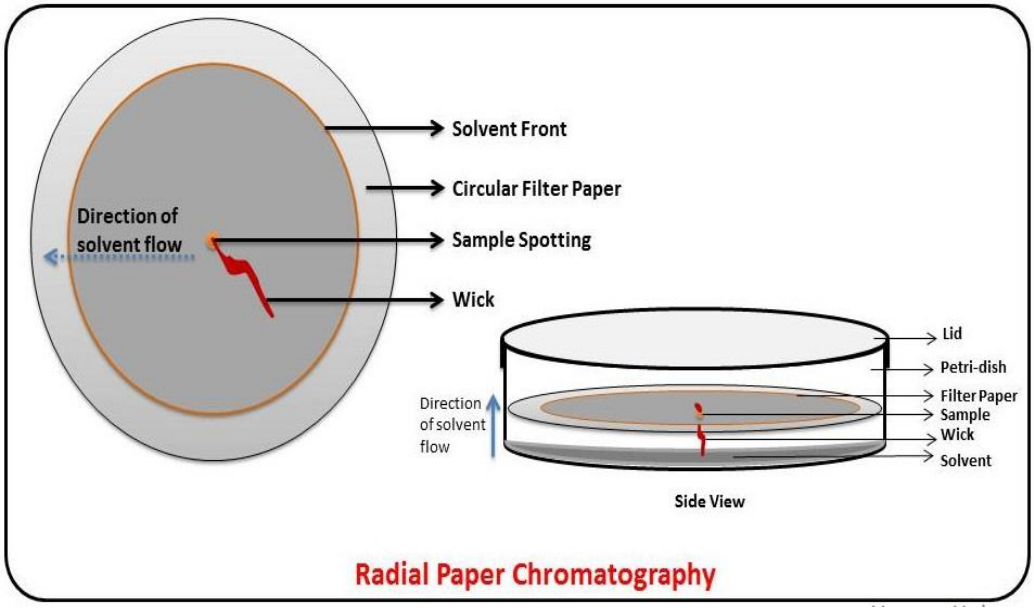
The paper is kept in horizontal position on glass rods with one end dipped in the mobile phase. The sample is loaded about 1 cm from the edge immersed in the mobile phase.



- **Radial development:**

In this technique; the constituents of the sample moved from the center of the paper to the periphery. Two tops or bottoms of petri-

dishes are used as a chromatographic chamber, the mobile phase is moved from the lower part of petri-dish through a wick-like tail to the center where it meets the sample and starts the separation process in form of concentric rings toward the periphery.



Reversed phase partition:

- A paper impregnated with non polar liquid (e.g. paraffin) is used; where the paper acts as a support and the non-polar liquid acts as the stationary phase. A polar mobile phase must be used for the development.
- Cellulose acetate represents an example for stationary phase in reversed phase partition chromatography.

Preparative Paper Chromatography:

It is the same idea like in preparative TLC, but cutting the paper parts containing the bands by a scissor is done instead of scrapping and the separated substances are solubilized using a suitable solvent.

- **Visualization:** It is similar to that in TLC but without using corrosive reagents.

Quantitative analysis in TLC and paper chromatography:

This can be achieved by several methods like:

Spot size, spot weight, spot area, spot density or spectrometric method.

Application of paper chromatography:

1- Identification and purification of compounds.

2- Detection of impurities.

3- Separation of carbohydrates.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It is characterized by a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

HPLC is also known as High Speed Liquid Chromatography (HSLC) and High Resolution Liquid Chromatography (HRLC)

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

Why high pressure?

In HPLC the stationary phase has two characters:

- Has small particles size (5- 10 µm).
- Packed under high pressure.
- Reduction of the particle size of the stationary phase leads to leaving less space for the mobile phase to pass through.
- Decrease the flow rate of the liquid mobile phase.
- The pressure ranging from 1000 to 5000 psi, pound per square inch (68 to 400 atm.) is applied to overcome the obstructive effect of the fine particles.

Classification of HPLC

I-Types of HPLC According to the Mechanism of Separation

1- Adsorption Chromatography

The stationary phase is an adsorbent and the separation is based on adsorption-desorption steps.

a- Normal Phase Chromatography

The stationary phase is strongly polar (e.g. silica gel) and the mobile phase is nonpolar such as (hexane). Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

b- Reversed Phase Chromatography

The stationary phase is strongly non polar (e.g. silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol or acetonitrile.

In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or

methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that the polar molecules will travel through the column more quickly.

Reversed phase HPLC is the most commonly used form of HPLC.

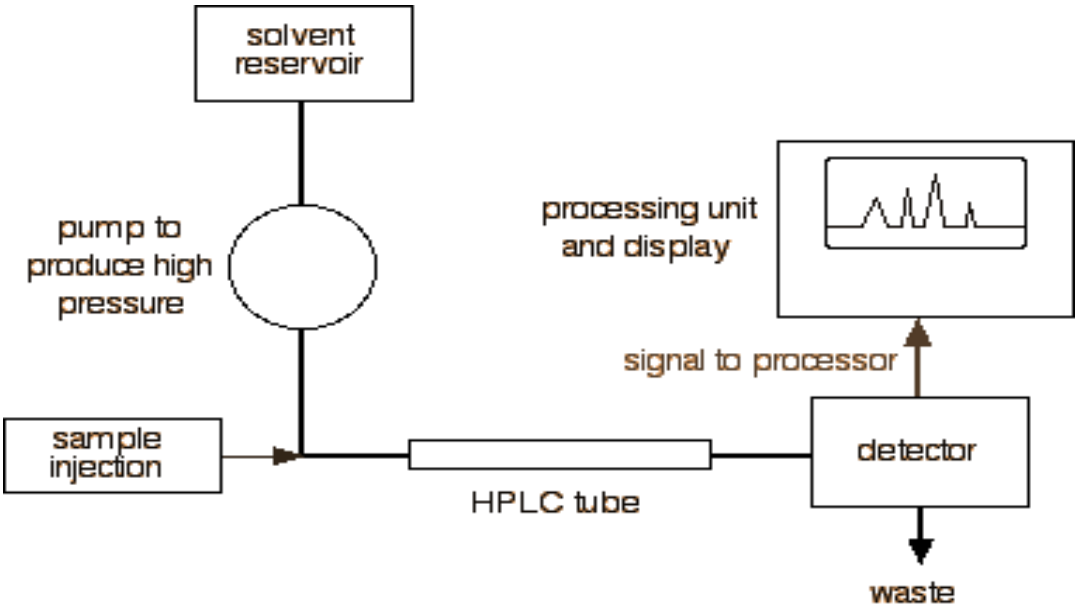
II- HPLC Can Be Divided Into Two Main Types According to the Uses:

1- Analytical Type:

This is used in identification of the components in a mixture and screening the number of components in a mixture.

2- Preparative or semi preparative type: used in isolation and purification.

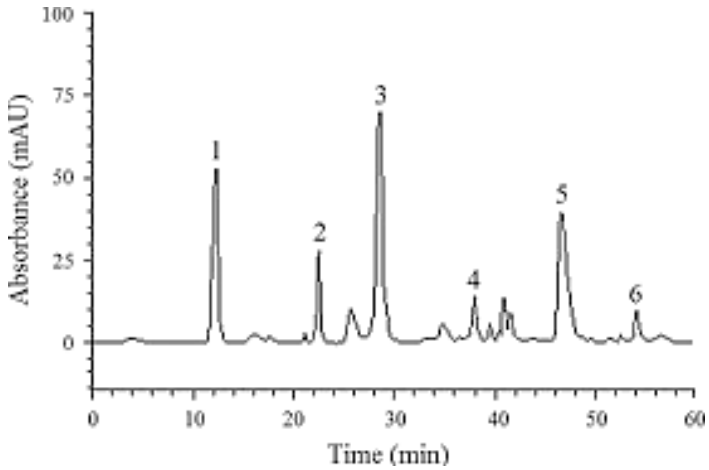
	Analytical HPLC	Preparative HPLC
Internal diameter of the column	1-6 mm	up to 6 cm
Flow rate of mobile phase	1 to 10 ml/min	up to 100 ml/min
Injected volume of the sample	20 uL to 1 mL	1 ml to 5 ml or more



HPLC Graph

The Process Begins By:

- Injecting the solute onto the column (zero time).
- The separation occurs as the analyte and mobile phase are pumped through the column
- Detection of components by detector is displayed on a chart or computer screen (chromatogram).
-



The Advantages of HPLC:

— 1- High speed

— 2- High resolution

— 3- High sensitivity

— 4- Re-usable column

— 5- No destruction of the components

— 6- The instrumentation are automatic, computerized

— 7- Sample is recovered completely

— 8- Quantitative work is more easily and most sensitive

Instrumentation of HPLC

HPLC instrument includes:

1- Reservoir for solvents (mobile phase)

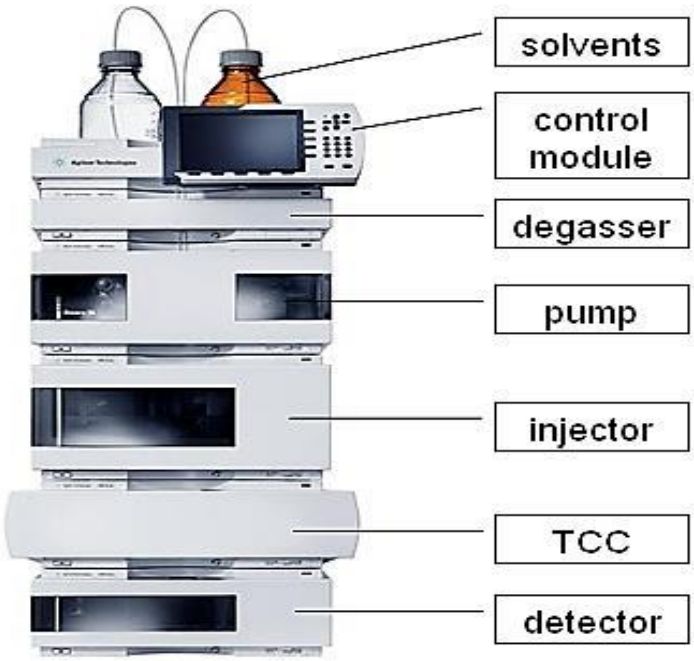
2- High pressure pump

3- Sample inlet device

4- Column

5- Detector

6- Recorder

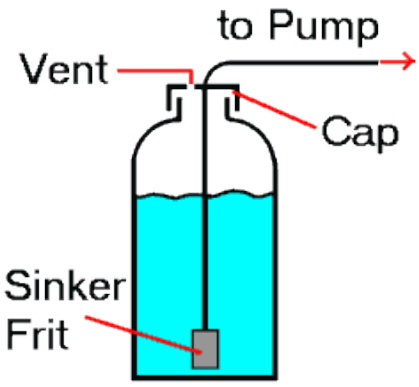


HPLC machine

1- Reservoir for solvents (Mobile Phase):

Mobile phase is usually organic or aqueous or mixture of both.

Mobile phase is placed in bottles of glass.



HPLC solvent reservoirs

Characters of Mobile Phase:

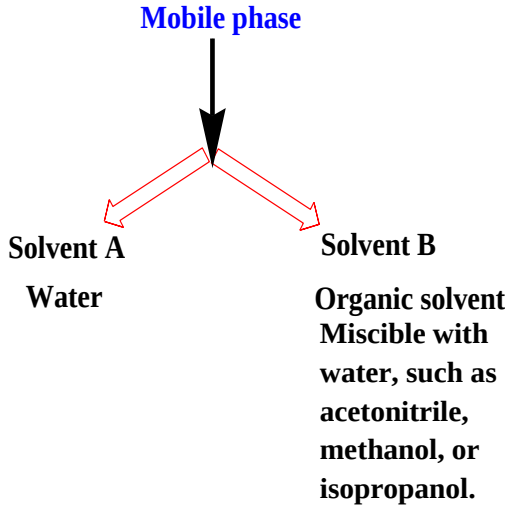
1-Pure 2 - Low viscosity

3- Chemically inert

4- Low price

5-Compatible with detector

6-Solubility of the sample



Elution Techniques (Programinig)

1- Isocratic Elution:

The mobile phase composition remains constant throughout the separation procedure.

2- Gradient Elution:

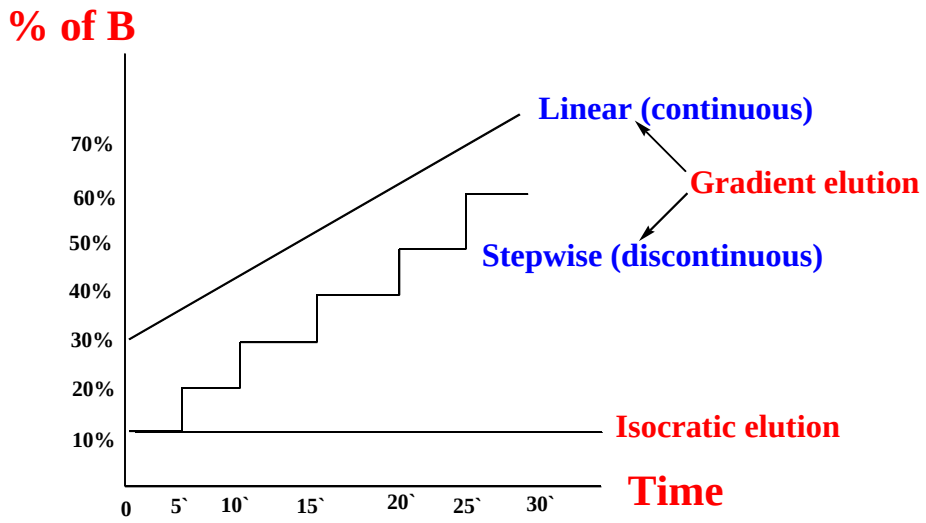
The mobile phase composition is changed during the separation process.

Gradient Elution Is Divided Into Two Types:

A. Continuous (linear)

B. Discontinuous (stepwise)

Isocratic and Gradient Elution Curves



Advantages of Gradient Elution Technique

1- Shortening the time of analysis.

2- Reduces tailing, gives sharp peak.

3- Increases the sensitivity of analysis.

4- Decreases the retention of the later-eluting components so that they elute faster.

Pre- Treatment of Mobile Phase:

1- Filtration before entering the column.

2- Degassing using degasser by:

i- Heating with stirring

ii- Applying vacuum,

iii- Passing nitrogen or helium

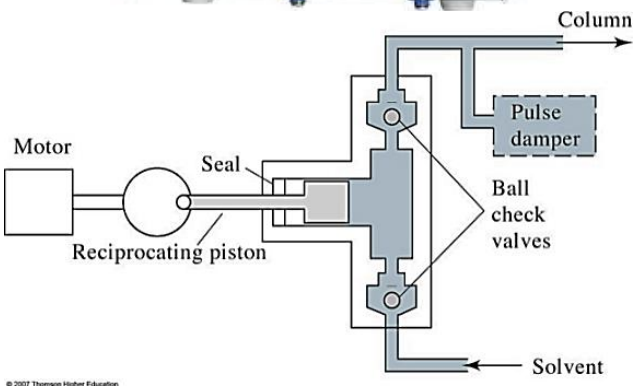
iv- Ultrasound

3- Pre-saturation with the stationary phase in case of liquid-liquid chromatography.

2- Pump

Function of the Pump:

Pump is used for forcing the mobile phase through the column.



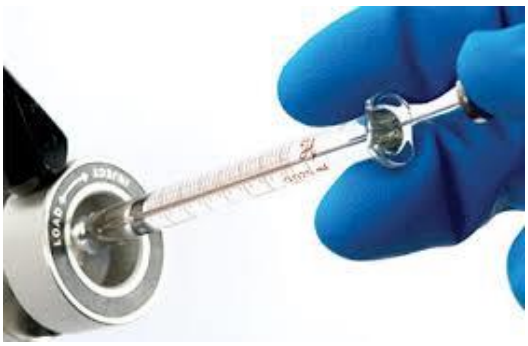
The Main Criteria for the Pump

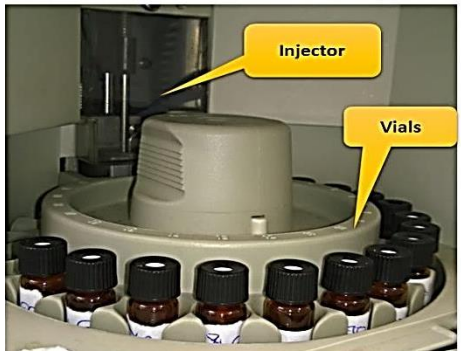
The pump should be capable of delivering:

a. Accurate and pulse free flow rate (e.g. 5 ml/min).
b. High volume of solvent.
c. High pressure up to 5000 psi.

3- Sample Inlet Device (Injection Port)

1- Manual injection **2-Automated injection**





The injection port consists of

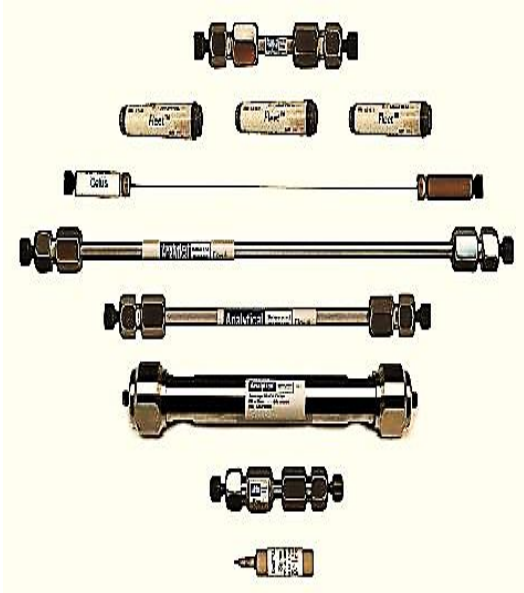
a- The injection valve. b- The sample loop.

Manual Injection

1- The sample is typically dissolved in the mobile phase.

2- It is drawn into a syringe and injected into the loop via injection valv

4- The column



Column in HPLC is either:

1- Analytical, 1-6 mm (i. d.)

2- Preparative up to 6 cm (i. d.)

Made from: Stainless

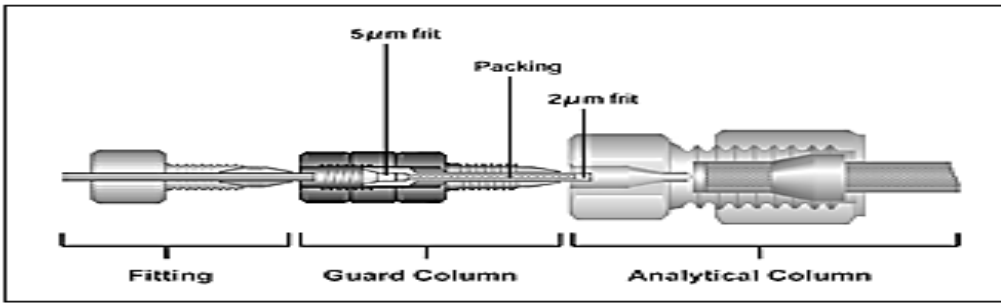
Shape: Straight

Length: Variable

Guard Column:

1- Protect the analytical column

2- Organization of separation in HPLC



5- Detectors (Brain of HPLC)

Characters of Detector

1-High sensitivity 2-Low noise (straight base line)

3-Wide range of response to different compounds

4- Unaffected by temperature or mobile phase

5- None destructive to the compounds

6- Provides qualitative and quantitative information about the detected sample

Types of Detectors

1. Ultra-violet (UV)Detector :

It is the most sensitive, sensitive to ng of compound, and the most widely used. It measures the UV absorption of the solute. Many organic compounds absorb UV light of various wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time. There are many types of UV detectors:

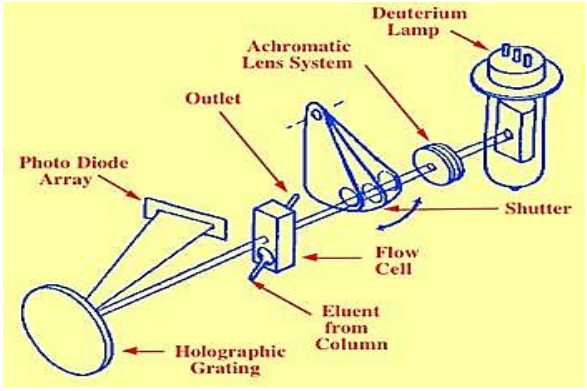
A. Fixed wavelength →measure at 254nm
B. Variable wavelengths→detect over wide range of wavelengths
C. Diode-Array: measure the spectrum of wave lengths simultaneously. Sensitivity 10^{-8}----10^{-9} gm /ml

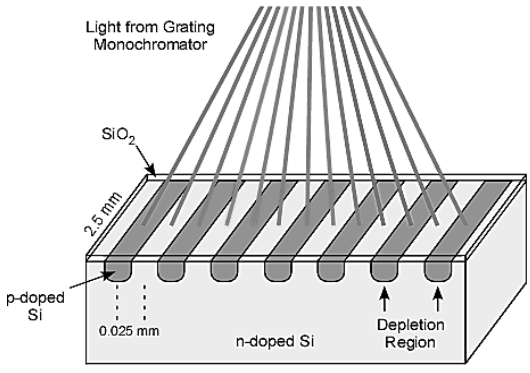
Photodiode Array Detector:

Consists of 200 detectors mounted in $1cm^2$ silicon ship, each detector receiving single wave length and the absorbance is measured simultaneously

The light source from the deuterium lamp →sample cell →diffraction grating→surface of detectors

Each diode will receive light of a different wavelength to that received by its neighbor (210 nm-330 nm)→ photocurrent →processed into spectrum in a second.





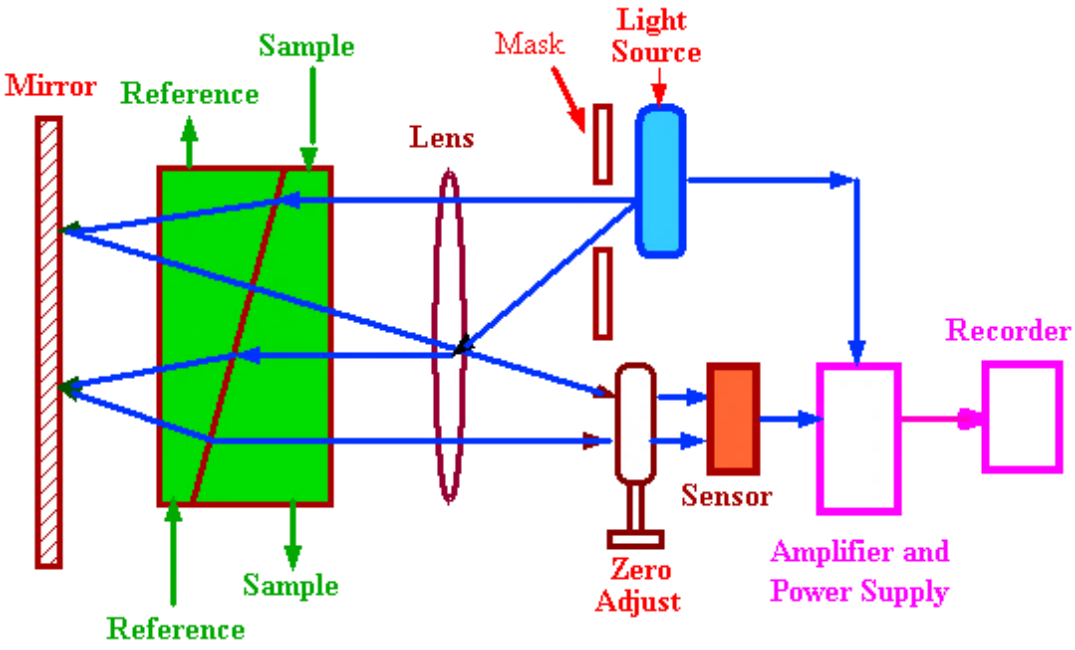
Photodiode Array Detector

2- Refractive Index Detector

- Not used in case of gradient elution
- Less sensitive

Measure the ability of the sample to bend or refract light. Light passes through a bilocular flow- cell to a photodetector. On channel is for the mobile phase coming out from the column and the other for the pure mobile phase.

The detector subtract the reading due to the pure mobile phase from the reading of mobile phase +sample →reading due to sample .Detection occurs when the light is bent due to the sample eluting from the column



Refractive Index Detector

3- Mass Spectrometer Detector

Coupling HPLC to a mass spectrometer (HPLC-MS) is an advanced technique. When the detector is showing a peak, some of what is passing through the detector at that time can be diverted to a mass spectrometer. There it will give a fragmentation pattern which can be compared against a computer database of known patterns. That means that the identity of a huge range of compounds can be found without having to know their retention times.

It is used with capillary column in analytical HPLC to give information about nature of the material by giving the mass spectrum of the material.

The sample is ionized→pass to mass analyzer→the ion current is detected.

Methods of Ionization:

a- Electron Impact (EI): An electron current created under high electric potential is used to ionize the sample eluted from the column

b- Chemical ionization: Ionized gas is used to remove electron from the compound eluting from the column.

c- Fast atom Bombardment (FAB): Xenon gas is propelled at high speed to ionize the sample eluted from the column. Sensitivity 10^{-8} to 10^{-10} gm /ml

4- Fluorescence Detector:

More sensitive than UV detector (1000 fold as UV)

It is used with compounds which are naturally fluorescent or compound which can be converted to fluorescent derivative.

Measure the ability of a compound to absorb light at a given wavelength and then emit it at longer wave length

The light from excitation source → flow cell → photo detector →Monochromator measure the emission wavelength

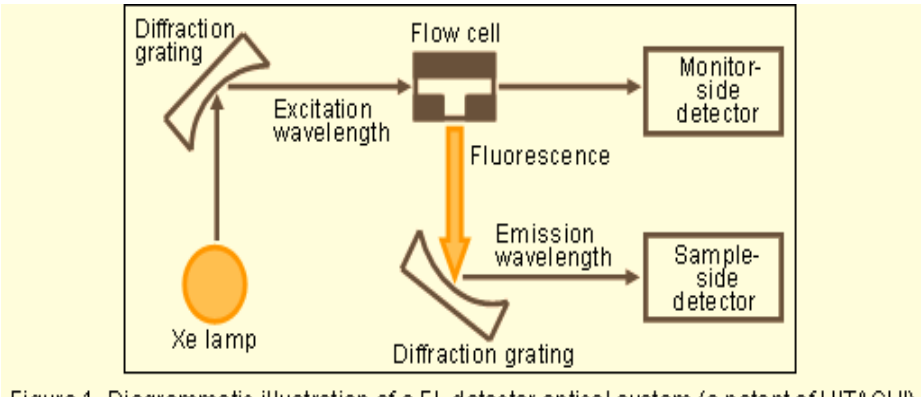


Figure 1. Diagrammatic illustration of a FL detector optical system (a patent of HITACHI)

Advantages and Applications of HPLC

1- High speed

2- High resolution

3- High sensitivity

4- Re-usable column

5- No destruction of the components

6- The instrumentation are automatic, computerized

7- Sample is recovered completely

8- Quantitative work is more easily and most sensitive

9-Can be applied to a wide variety of constituents (no derivatization as in GC).

10-Analysis of most plant constituents: flavonoids, terpenes, steroids, glycosides, alkaloids and antibiotics.

Applications of HPLC

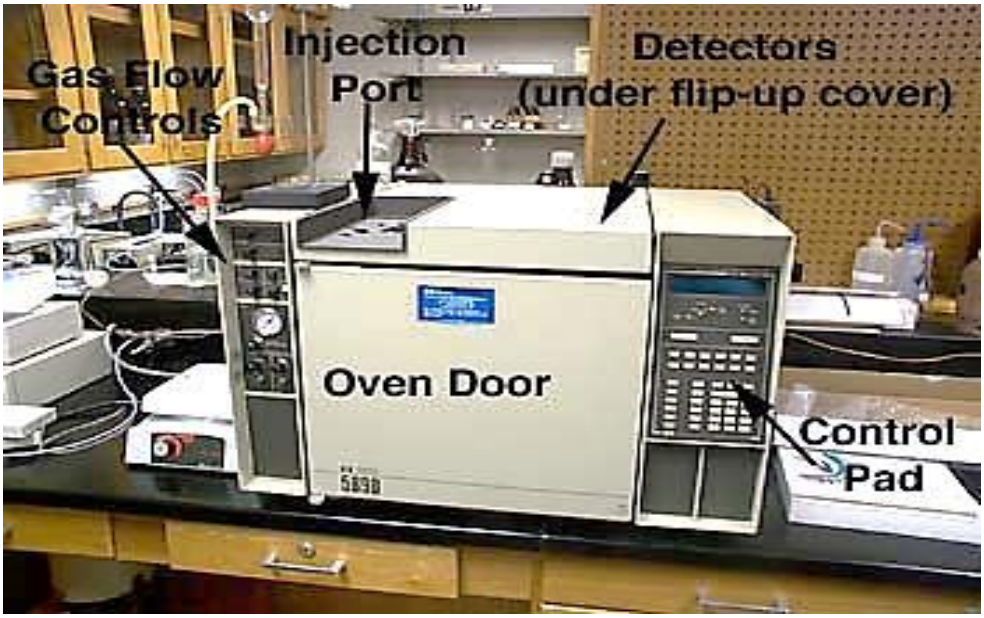
- HPLC play an important and critical role in the field of pharmaceutical industries and analysis, since it is used to test the products and to detect the raw ingredient used to make them i.e., qualitative and quantitative analysis. Moreover, the importance of HPLC uses in these fields falls under the stringent regulations established by the U.S. Food and Drug Administration (FDA). This obligates all pharmaceutical companies to detect the quality of their products by using the HPLC before allowing them to sell it in the global market.
- Pharmacokinetics study: Pharmacokinetic study comprises the measurement of drug metabolites concentration in body fluids, absorption, bioavailability and elimination of drugs. HPLC determines the drug and its metabolites in one step.
- Stability test: rapid method of analysis in stability test.
- The most important benefits gain from the uses of HPLC technique in the industrial and analytical field that it is help in structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations for the synthetic and herbal medicine too.
- Control the microbiological process. Used for separation of antibiotic from broth mixture
- Biosynthesis study: Detection of biogenetic intermediates and enzymes involved.
- Control of synthetic reactions: Identification of intermediates and target compound.
- Common applications in foods including determination of vitamins, residual pesticides, antioxidants, sugar, cholesterol, dyes, mycotoxins such as Aflatoxins, amino acids, residual antibiotics, aspartame and many other food ingredients.

Gas Chromatography

Gas chromatography is a common type of chromatography used analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition.

Gas Chromatography (GC or GLC) is a commonly used in many research and industrial laboratories for quality control as well as identification and quantitation of compounds in a mixture. GC is also a frequently used technique in many environmental and forensic laboratories because it allows for the detection of very small quantities. A broad variety of samples can be analyzed as long as the compounds are sufficiently thermally stable and reasonably volatile.

GC Instrument



GC Machine

GC is divided into two types:

1- Gas – solid chromatography when the stationary phase is solid
2- Gas – liquid chromatography when the stationary phase is liquid

Principle of GC:

Like for all other chromatographic techniques, a mobile and a stationary phase are required for this technique. The mobile phase (=carrier gas) is comprised of an inert gas i.e., helium, argon, or nitrogen. The stationary phase consists of a packed column in which the packing or solid support itself acts as stationary phase, or is coated with the liquid stationary phase (=high boiling polymer). Most analytical gas chromatographs use capillary columns, where the stationary phase coats the walls of a small-diameter tube directly (i.e., 0.25 µm film in a 0.32 mm tube).

The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase ("like-dissolves-like"-rule). The stronger the interaction is, the longer the compound interacts with the stationary phase, and the more time it takes to migrate through the column (=longer retention time)

Detection is carried out by a very sensitive detector and then transferred to recorder to draw the separated peaks.

Advantages of GC

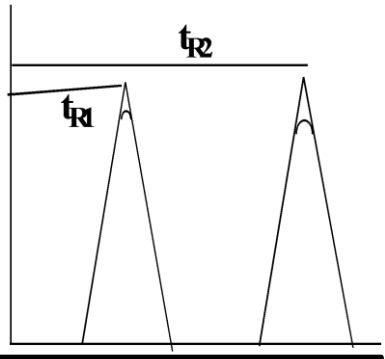
1. Capable of resolving more than 150 mixed compounds in one experiment
2. Time of analysis of any sample takes less than five minutes
3. Qualitative and quantitative analysis could be

   Obtained
4. Can be used for analysis of most of the natural products in the volatile form
5. Very small sample size always required less than 0.1 mg

Retention Time is the time required for maximum of the solute peak to reach the detector

Resolution Factor is a measure of the degree of separation of adjacent peaks and is determined from measurement taken from the peak pair by using the next equation

$$\text{Resolution factor Rs} = \frac{t_{R2}-t_{R1}}{1/2\,(W_1-W_2)}$$



Resolution Power of Column

Relates the width of the eluted peaks to the distance between maxima

Retention Volume is the volume of mobile phase required for the maximum of the solute peak to reach the detector

Instrumentation

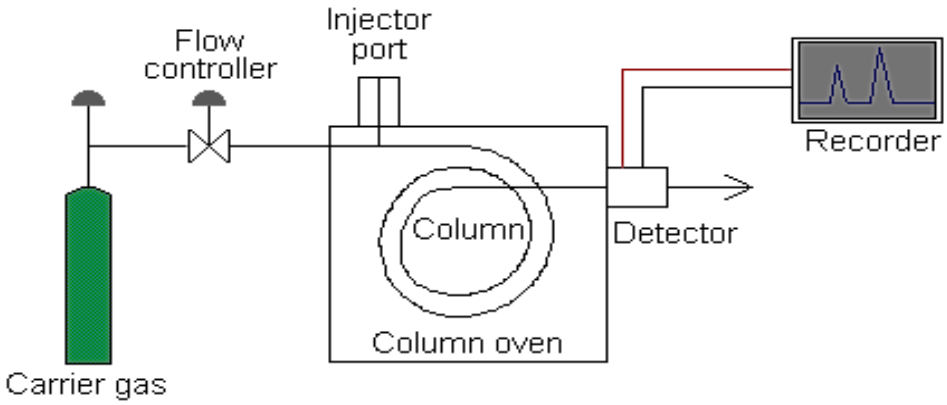
1-Sample inlet system

2-Carrier gas source

3- Column and oven

4- Detector

5-Recorder and amplifier



GC Graph

1- Sample Introduction System

Sample is liquid or solid dissolved in ether or chloroform is introduced by a micro-syringe (10µl-100 µl).

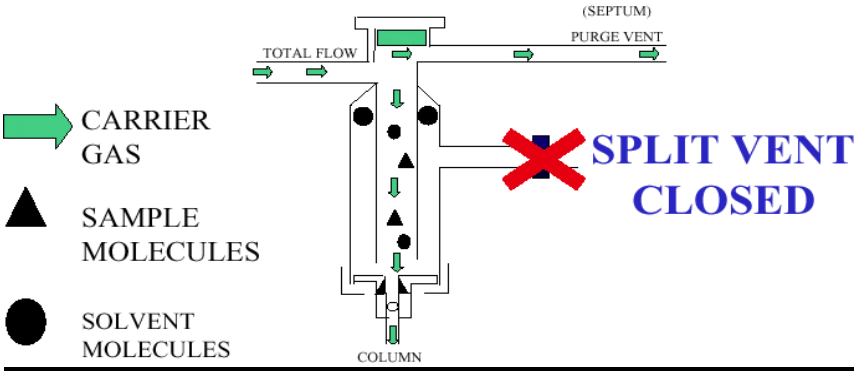
Ideally, the peaks in the chromatogram display a symmetric shape (Gaussian curve). If too much of the sample is injected, the peaks show a significant tailing, which causes a poorer separation. Most detectors are relatively sensitive and do not need a lot of material in order to produce a detectable signal. Strictly speaking, under standard conditions only 1-2 % of the compound injected into the injection port passes through the column because most GC instruments are operated in split-mode to prevent overloading of the column and the detector. The splitless mode will only be used if the sample is extremely low in concentration in terms of the analyte.

5-10 µl for thermal conductivity detector

1 µl for other high sensitivity detector

The sample is injected within 0.1 second longer time e.g. one second causes several feeding (multiple feeding) to the column with the same sample which will give multiple appearance of the same peak.

Split and splitless injection



Inlet Temperature

The temperature of the injection port is usually about 50°C higher than the boiling point of the least volatile component of the sample

The injection port has heater this heater to gives 5 -10 ^{o}C over the temperature of the column

The flow rate of the gas is 10 -100 ml gas /min it is measured by flow meter while the pressure is measured by mercury manometer.

2- The Mobile Phase (Carrier Gas)

Commonly used gases includes nitrogen, helium, argon, hydrogen, and carbon dioxide

Choice of carrier gas depend on types of detector

Characters of Carrier Gas

1- Very low viscosity

Low viscosity afford high flow rate (hydrogen)

2- High thermal conductivity

For thermal conductivity detector e. g. hydrogen, helium

3- Diffusability

- Gases with high diffusion in the stationary phases such as H_2 and helium cause lowering of the column efficiency.
- Nitrogen and other heavy gases are used to give high efficiency

4- Ionization

Gas as H_2 has very high ionization (requiring least energy to ionization) this is useful when the detectors are of ionization type.

5- Compressibility

When temperature increases, the viscosity of gas increases and this causes decrease in velocity (decrease the flow rate) so gas must be compressible to overcome this problem

6- Safety requirement

H_2 is flammable and He in non flammable

7- High purity of the gas

- From water so dried by molecular sieve tube in front of the gas and just before the column
- From dust and other impurities using filter before the column
- From oxygen by using copper oxide tube before the column

8- Chemical inertness

3- Columns

Packed Columns **Capillary Columns**

Types of columns used in GC

a- Packed column

1.5-10 m in length , 2-6 mm in diameter Stainless or glass

- Contains finely divided inert solid support material e.g. diatomaceous earth or active adsorbent material like alumina
- These columns work by adsorption or may be coated with a liquid phase
- Efficiency (30.000 theoretical plates)
- For packed columns, sample size ranges from tenths of a microliter up to 20 microliters.

Advantages: used for large size of sample without overloading

b- Capillary Column (Golay column) or (open tubular)

- Capillary columns have an internal diameter of a 0.2-1 mm diameter
- Column efficiency = 200.000 plates
- Capillary columns need much less sample, typically around 10^{-3} mL. and split/splitless injection is used.

They can be one of two types:

a) Wall-Coated Open Tubular (WCOT)

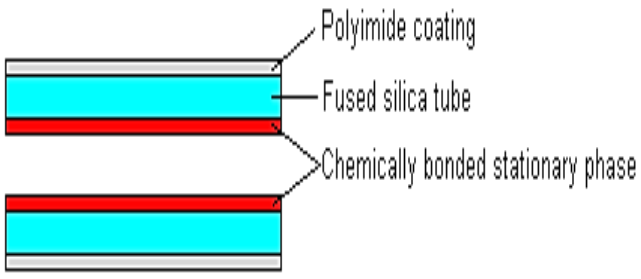
Consist of a capillary tube whose walls are coated with liquid stationary phase

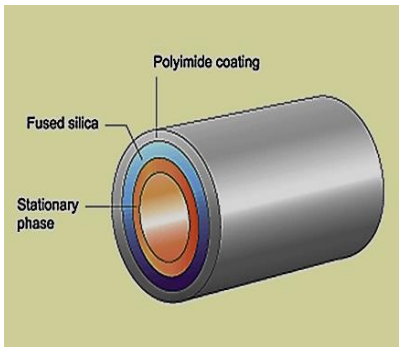
b) Support-Coated Open Tubular (SCOT).

The inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, through which the stationary phase has been adsorbed.

- SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns.
- In 1979, a new type of WCOT column was devised - the *Fused Silica Open Tubular* (FSOT) column
- These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating.
- These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity.

Cross section of a Fused Silica Open Tubular Column





Column Temperature

For precise work, column temperature must be controlled to within tenths of a degree.

The optimum column temperature is dependant upon the boiling point of the sample.

- As a rule of Thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes.
- Minimal temperatures give good resolution, but increase elution times.
- If a sample has a wide boiling range, then temperature programming can be useful.
- The column temperature is increased either continuously (linear) or in steps as separation proceeds

Column Performance Is Measured By

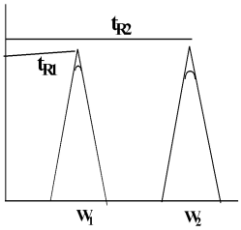
1- Column efficiency

It is the number of theoretical plates

$$N = 16\left(\frac{t}{W}\right)^2$$

2-Measurement of resolution factor for two closely eluting peaks

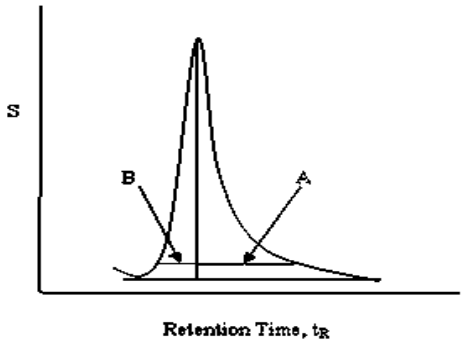
$$\text{Resolution factor Rs} = \frac{t_{R2}-t_{R1}}{1/2\ (W_1-W_2)}$$



t is a measure of the degree of separation of two adjacent peaks

3- Measurement of peak asymmetry (Ideal value is 1)

Peak asymmetry factor (As) = CB/AC



Factors Affecting the Column Performance:

1- Type of stationary phase.

2- Particle size.

3- Reduction in sample size.

4- High affinity of solute in carrier gas.

5- Velocity of the carrier gas.

6- Column diameter.

7- Column temperature.

8 Column length.

The GC Stationary Phase

I- Solid Stationary Phase

Characters:

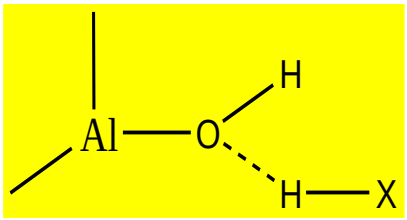
- Chemically inert
- Porous
- High thermal conductivity
- Good mechanical resistance
- No tendency for solute

Types of Stationary Phase:

Charcoal, silica gel, alumina, molecular sieves, porous polymer, chromosorb-w and chromosorb-p.

1- Alumina:

Al_2O_3 is powerful adsorbent; it can form hydrogen bond through hydroxyl groups formed on its surface by hydration



2- Carbon Black

Used for gas solid chromatography

3- Zeolites

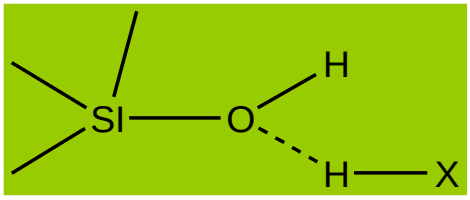
These are the original alumino-silicate molecular sieve

It is powerful adsorbent

Adsorb water and CO_2 so it must be protected from the atmosphere

4- Silica Gel:

OH group is the main site of adsorption



5– Porous Polymer:

Styrene can polymerise to give porous beads

6- Chromosorb-W (white):

This is a mixture of sod. carbonate and diatomite ignited at 900 ^{0}C to produce iron-sodium-silicate.

7-Chromosorb –P (pink):

Diatomites and clay, heated at 900 ^{0}C afford silicates with excess of iron oxide**.**

II - Liquid Stationary Phase

-Liquid is directly packed in capillary column

- Adsorbed on inert solid support

Liquid phase should have the following characters:

1- Non volatile

Bleeding is the volatilization of liquid phase

2- High thermal stability

3-Chemically inert

4- Having low viscosity

Temperature Choice and Control

The control of temperature is very important as high temp. Cause bleeding and affects column separation.

The column operates more efficiently at higher temp. There is much less tailing, few poorly shaped peaks because of volatilities of the components.

At high temperature the bleeding or volatilization of liquid stationary phase will destroy the column.

4-Detectors

- There are many detectors which can be used in gas chromatography.
- Different detectors will give different types of selectivity.
- A *non-selective* detector responds to all compounds except the carrier gas
- A *selective detector* responds to a range of compounds with a common physical or chemical property and a *specific detector* responds to a single chemical compound.
- Detectors can also be grouped into *concentration dependant detectors* and *mass flow dependant detectors*.
- The signal from a concentration dependant detector is related to the concentration of solute in the detector, and does not usually destroy the sample

- Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector.

Characters of ideal GC detector:

1. Highly sensitive (10^{-7}µg)
2. Fast responsibility to any change in eluted components.
3. Linearity to concentration (The intensity of the response or the reading should bear some linear qualitative relationship to the concentration of a component of the effluent).
4. Simplicity.
5. Stability (Withstand the large temp. Range during the operation).
6. Inert response.
7. Complete release of the gas.

Types of Detectors

1. Mass Spectrometer (GC/MS)
2. Thermal Conductivity Type (TCD)
3. Flame Ionization Detector (FID)
4. Electron Capture Detector (ECD)
5. Alkali Flame-Ionization Detector (AFID)
6. Flame Photometric Detector (FPD)

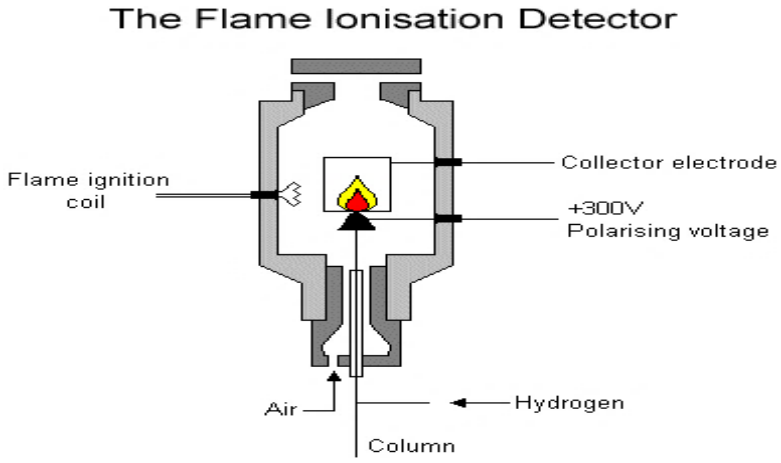
1- Mass Spectrometer (GC/MS)

Many GC instruments are coupled with a mass spectrometer, which is a very good combination. The GC separates the compounds from each other, while the mass spectrometer helps to identify them based on their fragmentation pattern (see Mass Spectrometry chapter).

2- Flame Ionization Detector (FID)

This detector is very sensitive towards organic molecules (10-12 g/s = 1 pg/s, linear range: 106-107), but relative insensitive for a few small

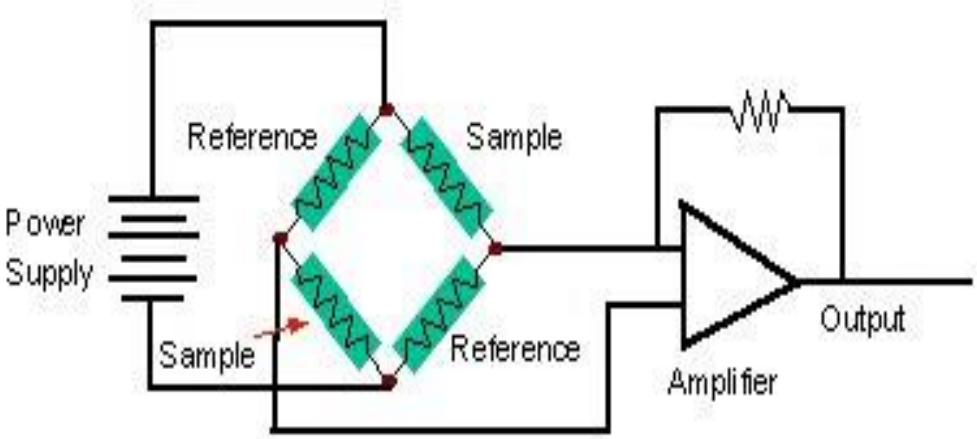
molecules i.e., N2, NOx, H2S, CO, CO2, H2O. If proper amounts of hydrogen/air are mixed, the combustion does not afford any or very few ions resulting in a low background signal. If other carbon containing components, are introduced to this stream, cations will be produced in the effluent stream. The more carbon atoms are in the molecule, the more fragments are formed and the more sensitive the detector is for this compound. Unfortunately, there is no direct relationship between the number of carbon atoms and the size of the signal. As a result, the individual response factors for each compound have to be experimentally determined for each instrument. Due to the fact that the sample is burnt (pyrolysis), this technique is not suitable for preparative GC. In addition, several gases are usually required to operate a FID: hydrogen, oxygen (or compressed air), and a carrier gas.



3. Thermal Conductivity Detector (TCD):

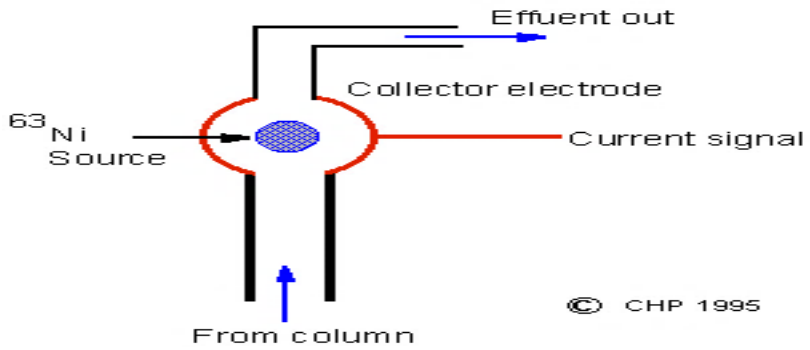
This detector is less sensitive than the FID (10-5-10-6 g/s, linear range: 103-104), but is well suited for preparative applications, because the sample is not destroyed. The detection is based on the comparison of two gas streams, one containing only the carrier gas, the other one containing the carrier gas and the compound. Naturally, a carrier gas with a high thermal conductivity i.e., helium or hydrogen is used in order to maximize the temperature difference (and therefore the difference in resistance) between two filaments (= thin tungsten wires).

The large surface-to-mass ratio permits a fast equilibration to a steady state. The temperature difference between the reference and the sample cell filaments is monitored by a Wheatstone bridge circuit.



4. Electron Capture Detector (ECD):

This detector consists of a cavity that contains two electrodes and a radiation source that emits -radiation (i.e., ^{63}Ni, ^{3}H). The collision between electrons and the carrier gas (methane plus an inert gas) produces a plasma-containing electrons and positive ions. If a compound is present that contains electronegative atoms, those electrons will be "captured" to form negative ions and the rate of electron collection will decrease. The detector is extremely selective for compounds with atoms of high electron affinity (10^{-14} g/s), but has a relatively small linear range (~10^{2}-10^{3}). This detector is frequently used in the analysis of chlorinated compounds i.e., pesticides (herbicides, insecticides), polychlorinated biphenyls, etc. for which it exhibits a very high sensitivity.



Programming of GLC

1-Isothermal Techniques:

In this method the temperature is fixed at suitable temperature. The resolution is modified by several trials,

2-Programmed- Temperature Techniques:

The temperature is changed in two ways:

a- Non linear :temperature is changed by time; every 5, 10, 20 minutes
b- Linear: The temperature is changed at uniform rate after the injection time.

3- Programmed Pressure

In this case the temp is fixed and the change in the flow rate only

Advantages

Low temp. can be used , it is suitable for unstable compounds

pressure is more controlled than temp

Sample Preparation for GC

It must be volatile (Volatile oils)

Non volatile compounds can be converted into volatile by

- 1- Methylation
- 2- Acetylation
- 3- Silylation

Sample Preparation for GC:

If the components to be analyzed are not volatile, these samples have to be converted to volatile by derivatization

This can be carried out by one of the following methods:

1-Methylation:

The methyl ether ($R\text{-}OCH_3$) or the methyl ester ($R\text{-}COOCH_3$) of many compounds are found to be volatile, thus converting these compounds into their methyl derivatives by the addition of: Methanol/ sulfuric acid

Compounds with R-COOH or R-OH or different chemical skeletons are refluxed in methanol/ sulfuric acid mixture for 2-4 hours. The resulting methyl derivative is then distilled off and analyzed by GC.

R-COOH + MeOH / H →R-COOCH3

2-Acetylation:

This method is used to convert some amines, alcohols and phenols to the volatile acetate derivatives

The reaction is carried out by stirring a solution of the mixture in pyridine and acetic anhydride for few hours.

Compounds with R-COOH or R-OH or different chemical skeletons are refluxed in methanol/ sulfuric acid mixture for 2-4 hours. The resulting methyl derivative is then distilled off and analyzed by GC.

R-COOH + MeOH / H →R-COOCH3

The acetate is extracted with chloroform, evaporation affords the acetate, dissolved in ether and injected to the GC,

3-Silylation:

Used for derivatization of acid, alcohol, amines, all types of carbohydrates, glycosides, alkaloid, phenol, amino acids

Reagents used: Trimethyl chlorosilane

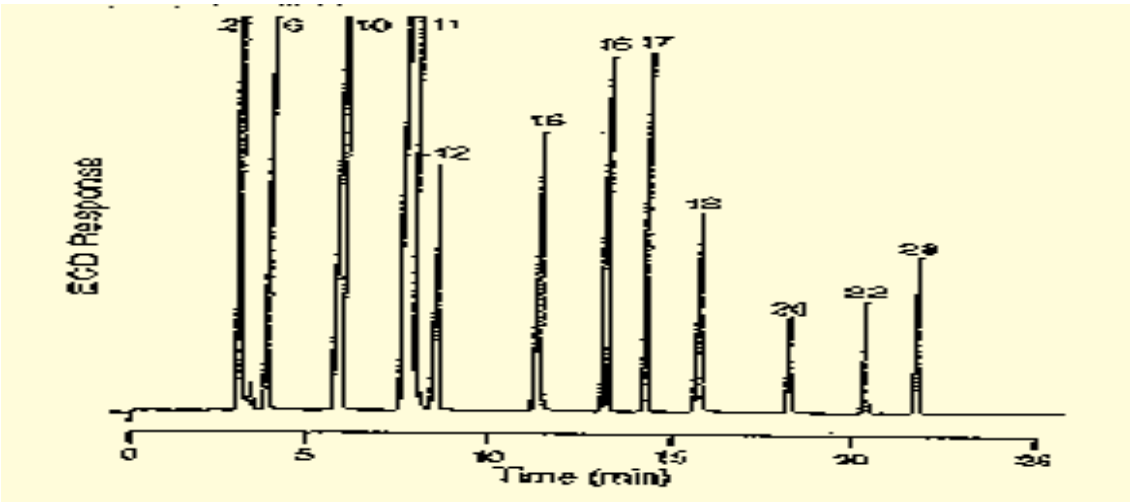
Method:

The sample is dissolved in pyridine then adds the reagent

The reaction mixture is stirred at room temperature for few minutes

The mixture is then filtered off, dissolved in chloroform and injected into GC.

Example of GC Chromatogram



Factors Affecting Separation on GC:

1- Sample (solvent used, concentration and weight)

2-Column (length, inner diameter, type of tubing and shape)

3-Packing (nature and particle size)

4- Detector (nature and characteristic)

5- Injection chamber (ratio of splitting in relation to gas flow rate)

6-Recorder (Response time, chart speed)

7-Temperature (of the injector, column and detector)

8- Carrier gas (nature, inlet and outlet pressure and flow rate).

9-Programming temperature

10- Flow rate

Applications of GC

1- Free fatty acid (Fixed Oil Analysis)

2- Fatty alcohols, Ethers and Ketones

3- Fatty acids methyl ester

4- Amines and Amino acids

5- Hydrocarbons and Sterols

6- Carbohydrates

7- Flavours and fragrances

8- Pesticides determination

9- Food analysis

10- Drug analysis

11- Environmental analysis

12-In forensic analysis of blood and urine alcohol (ethanol) levels

Ion –Exchange Chromatography (IEC)

An ion exchange material consists of an insoluble polymer, organic or inorganic in nature of high molecular weight.

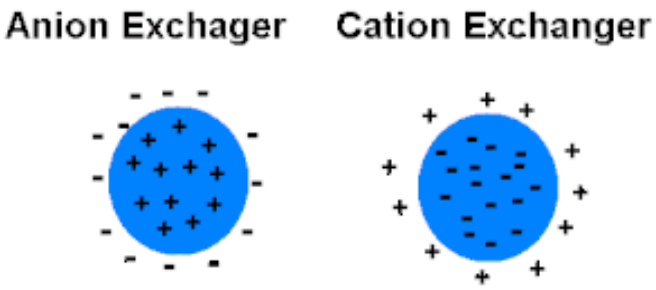
The polymer matrix carries fixed charge positive or negative which is balanced by opposite counter ion charge.

The counter ion is loosely attached to the matrix and can change places with similar ion in the solution.

Therefore, substance forming ions in aqueous solutions (electrolytes), when come into contact with the bead of ion exchanger, exchange occurs

The ion exchanger expels the counter ions and will bind ions of the same signs from the electrolytes.

The beads of the ion exchanger are the stationary phase and the solution flowing through it is the mobile phase.

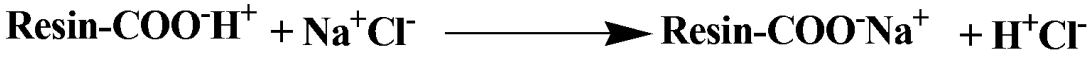


Types of Exchanger:

1-Cationic Exchanger: e.g.

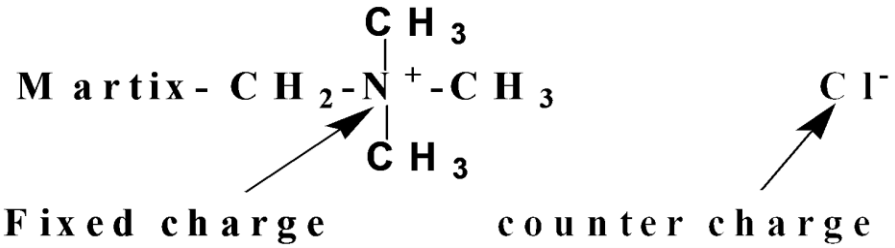
a- Sulphonic acid resin (counter ion H^+)

b- Carboxy methyl (counter ion Na^+)



2- Anionic Exchanger: e.g.

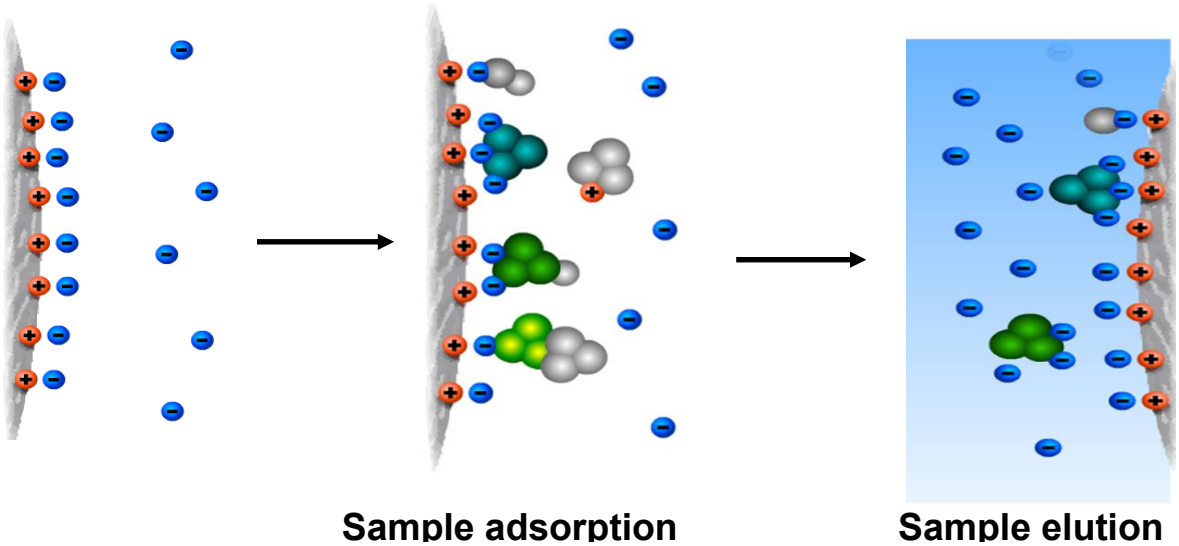
Quaternary ammonium group (Anion)



Summary of ion exchange Procedure:

1. Set up (column, ring stand, pump, chart, recorder, fraction collector) and perform calibration steps)

2. Equilibration
3. Loading sample
4. Washing unbound
5. Elution
6. Regeneration



Equilibration:

1. Calculate the amount of ion exchange resin you need, based on the amount of protein (solute) you have.

The resin used is Diethyl amino ethyl with Cl^- counter ion

The resin becomes pre –swollen in an ethanol/ water mixture that must be removed and replaced with buffer

An aliquot of resin (the resin should be about 75% of the total volume of the final slurry) is placed in a column, so the resin will be settled, leaving the ethanol at the top

Pour off excess ethanol and add buffer

Check the PH with PH paper

Begin equilibiration of the column with the buffer for about 30 minutes

The column is equilibrated if the PH reading of the buffer going into the column is the same as the pH coming out of the column

At this point, the column is ready for loading sample

Loading the Sample:

Close off the column outlet, remove excess buffer from the top of the column and pipette the sample into the column

Then connect the buffer reservoir to the top of the column and open the column outlet.

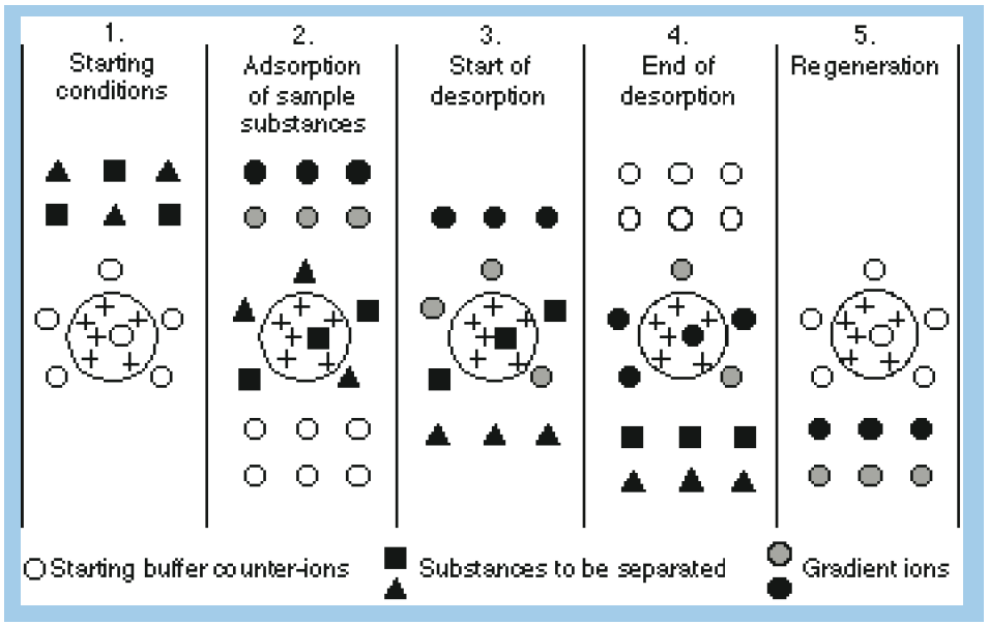
Washing the Column:

Start running the column with buffer at a flow rate 1ml/minute to wash out the bound protein

Collect the effluent in a beaker and save it (this depends on measuring the absorption).

Elution:

After the unbound protein has been removed from the column, then begin elution with the gradient.



Application of IEC

A. Analytical Application :

1-Water softening (Ion exchange resin is used to replace the magnesium and calcium ions found in hard water with sodium ions

2- Water purification (Ion exchange resin is used to remove poisonous e.g. copper and lead ions from solution, replacing them with sodium and potassium ions.

3-Production of high purity water:

Water of highest purity is required for electronics, production of superconductor and for nuclear industry.

Such water is produced using ion exchange processes.

Cations are replaced with hydrogen ions using cation exchanger

Anions are replaced with hydroxyls using anion exchanger resins

The hydrogen ions and hydroxyls ions are recombine producing water molecules. Thus no ions remain in the produced

4. Catalysis (catalyze organic reactions.)

5. Juice purification (used to remove bitter tasting components and improve the flavor)

6. Sugar manufacturing: (used to decolorize and purify sugar syrups).

B. Application Of IEC In Pharmacy :

1- Separation of antibiotics

2- Separation of vitamins

3- Separation of Amino acids and peptides

4- Separation and purification of alkaloids

C. Medical And Therapeutic Applications:

1- Obstructive jaundice------cholestyramine (strong basic ion exchange resin)

2-Odema (Hyperkalemia): increase in K ion ---------- sodium polystyrene sulfonate, a strong acidic resin

3-Diarrhea: food poisoning---- polymixin resin + sulpha resin

4- Enteric coating of tablets

5-Deodorant

6-Skin diseases

Gel Chromatography

Gel Permeation

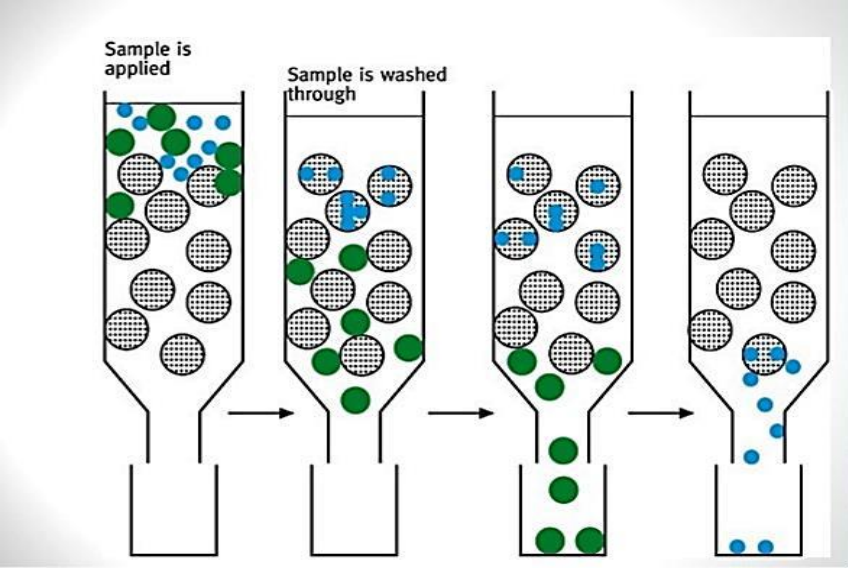
Molecular Sieving

Size Exclusion= Steric Exclusion

Principle of Separation

Organic polymer such as sephadex are capable of separating large molecules up to 150,000,000 Dalton from smaller molecules less than 2000 by virtue of their molecular size mass and molecular shape

The particles of gel posses internal pores formed by the molecular structure. When the gel is packed into a column and percolated with a solvent, it permits the large molecular weight compounds to pass rapidly without penetration of the pores



On the other hand the small molecules enter the pore and distributed across the column and pass down more slowly

Thus large molecules elute first and small after them.

Gel chromatography could be mentioned as:

- **Gel filtration chromatography**

- When an aqueous solution is used as a mobile phase

- Used for fractionation of proteins & other water-sol. polymers

- **Gel permeation chromatography**

- When an organic solvent is used as a mobile phase

- Used to analyze mol. weight of organic-soluble polymers

Nature of the Gel

1- Chemically inert

2- Mechanically stable

3- Ideal porous structure (wide pore size give low resolution)

4- Uniform particle size

Types of Gel:

1- Sephadex

A 1-6-polymer of glucose is prepared by microbial fermentation of sucrose (glucose + fructose)

The resulting glucose provides the required α1-6 glucosan polymer called dextran

The resulting dextran is treated with epichlorohydrin to give several types of crossed linked dextran (Sephadex)

Sephadex is obtained in different degrees depending on the pore size

High percentage of epichlorohydrin gives high degree of cross linking (small pore size)

Lower percentage produces sephadex with large pore size

Characters of sephadex:

1- Highly stable gels

2- Stable at PH 2-12

3- Their particles are free from ions

4- Insoluble in water and organic solvent

5- They swell in water and other hydrophillic solvent

6- They require bactericidal such as Hg acetate

Other Type of gel

A- Organophillic gel

Organophillic gel such as acrylamide polymers, **Bio-Glass,** are used with high efficiency using n-heptane as solvent for isolation of plant extract, alkaloids, flavonoids

1- styragel-II (mol.wt. Range of 1600-40,000

2- Sephdex-LH-20 which is hydroxypropyle derivative of sephadex (mol. Wt. Range 10,000)

B- Agarose gel

(Bio-gel-A-150 Sepharose B Sepharose 2B)

Obtained from agar and composed of alternating units of 1,3 linked β-D-gal and 1,4 linked 3,6-anhydro-α, L-galactose

This was subjected to epichlorohydrin to give sepharose

Characters:

1- It dissolves in H2O at 50 c and on cooling form gel

2- Insoluble below 40 c

3- Freezing destroys the gel

C- Acrylamide gels (synthetic gel)

It is not dextran polymer

It is polymerized acrylamide or methylen-bis-acrylamide

D- Glycol methacrylate

E- Polyvinyl acetate

F- Polystyrene

G- Inorganic gels

Column Packing:

1- Gel is mixed with solvent for 3 hrs to swell

2- Pack the column

3- Sample should be solution

4- Not to allow dry

Application of Gel Filtration Chromatography

1- Separation of large molecular weight compound as protein, carbohydrate, peptides, nucleic acids

2- Desalting of colloids

Small size of contaminating salt will allow them to diffuse inside the gel particles

E.g. desalting of albumin from 25% ammonium sulphate

3- Molecular weight determination

A linear relationship exists between the logarithm of the molecular weight and the elution volume

By determination of the elution volume of series of proteins one can determine the molecular weight of unknown protein.

4- Separation of monosaccharides from polysaccharides

Affinity Chromatography

Affinity Chromatography: is a method of separating biochemical

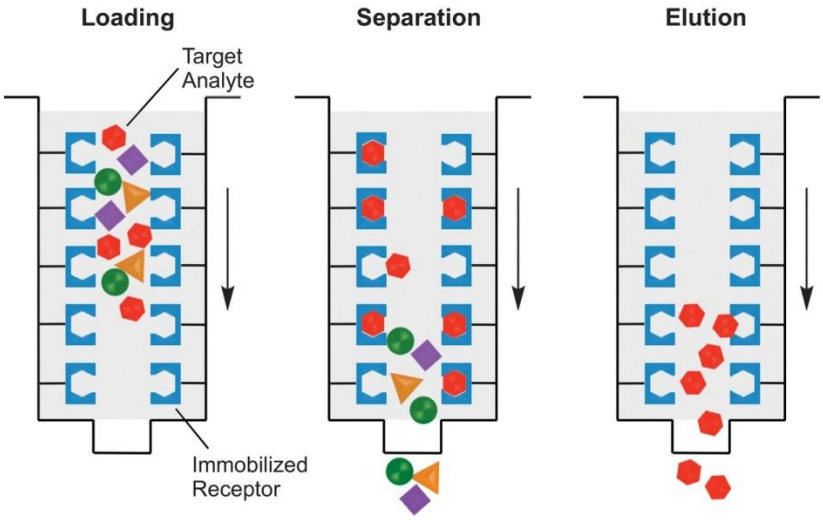
mixtures and based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand.

Operates by using immobilized ligand that have a specific affinity to the compound of interest

Separation occurs as the mobile phase and sample pass over the stationary phase

The compound of interest is retained as the rest of impurities and the mobile phase pass through.

The compound is then eluted by changing the mobile phase



For example, the antibodies in a serum sample specific for a particular antigen can be isolated by the use of affinity chromatography

Step 1:

An immunoadsorbent is prepared. This consists of a solid matrix to which an antigen (shown in blue) has been coupled. Agarose, sephadex, derivatives of cellulose, or other polymers can be used as the matrix.

Step 2:

The serum is passed over the immunoadsorbent. As long as the capacity of the column is not exceeded, those antibodies in the mixture specific for the antigen (shown in red) will bind and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass.

Step 3: Elution.

A reagent is passed into the column to release the antibodies from the immunoadsorbent.

Buffers containing a high concentration of salts and/or low pH are often used to disrupt the interactions between antibodies and antigen.

Step 4: Dialysis.

The eluate is then dialyzed against, for example, buffered saline in order to remove the reagent used for elution.

Electrophoresis

Electrophoresis is the movement of an electrically charged substance under the influence of an electric field

In electrophoresis, ionic substances (positively or negatively charged) are separated along a strip of filter paper immersed in a buffer solution in which the ends of electric poles are connected.

Once the current is put on, the compounds migrate towards the electrodes in such a manner, the positively charged travels towards the negative pole and the negatively charged travel to the positive pole.

1- Stationary phase (Carrier)

Electrophoresis is performed on buffered solid carriers such as paper, gels and thin layers.

2- Selection of the suitable voltage

The voltage selected should be high enough to bring about maximum separation in the shortest time possible, but should be sufficiently low to avoid excessive heat

Two types of electrophoretic procedures are distinguished:

A-Low Voltage Electrophoresis (LVE) (2-10 V/CM):

Used for analysis of peptides and proteins.

B-High Voltage Electrophoresis (HVE) (30-100 V/CM):

The resolution is highly improved and the time of analysis reduced.

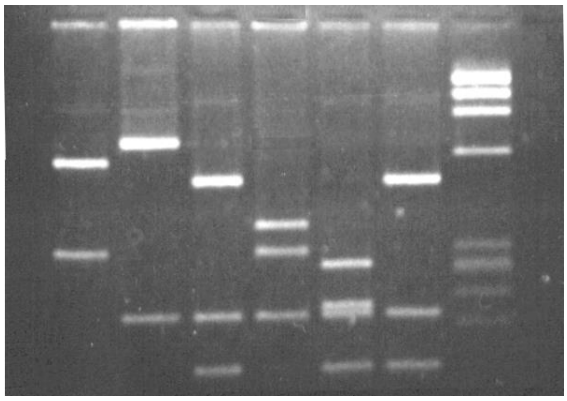
Used for separation of small ions such as amino acids and small peptides.

3- Selection of the buffer

The buffer may have a considerable effect especially in case of **amphoteric substances** which carry positive and negative centers such as amino acids and proteins.

- Therefore, such compounds may migrate to the anode or to the cathode or remain at the origin by the selection of the suitable buffer.

Paper Electrophoresis



Types of ionic molecules:

1- Amino acids, peptides, serum plasma

2-Hormones, enzymes and globulins ionized in PH of a buffer.

3- Compounds convered into ionic molecule e.g. carbohydrates →borates.

Factors Affecting Separation by Electrophoresis:

1-Types and number of charge

2- Types of strip used

3-Molecular weight of the compounds

4-Molecular shape and size

5- Voltage applied.

Supporting Media:

Whatman#1 #3 mm

Cellulose Acetate

Gel (sephadex, agarose, polyacryl amid)

Capillary Electrophoresis:

Instead of gel, a capillary tube of (50 µm I.D X300 µm O.D.) is filled with an electrolyte , then the sample is applied under high pressure injection and high voltage.

Process takes place within 10-30 minutes

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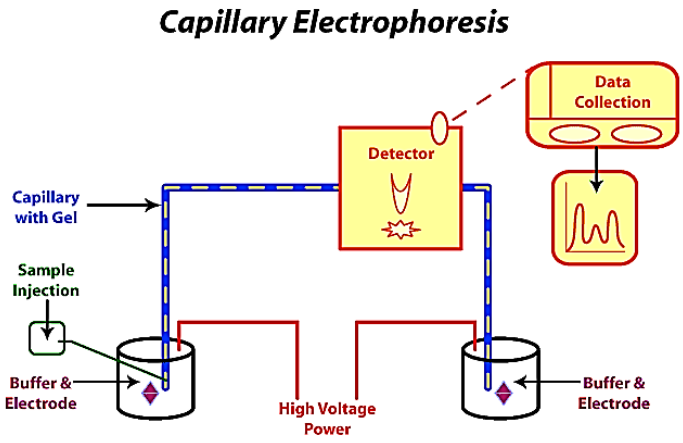


Applications

-Isolation of alkaloids, plant acids and anthraquinone derivatives

- Common (qualitative) technique in molecular biology

i.e.Nucleic acids and proteins

- Determine the isoelectric point of a compound

Electrophoretic mobility:

It is the motion of charged species in a fluid due to an external electric field

Migration velocity:

It is the distance moved by charged particle

Electrophoretogram:

It is the result of an electrophoresis. Which gives the movement of charged particles over time in a gel, Paper or another medium or it is the plot of fluorescence units over time

Part III

Quality Control of Natural Products

Quality control of natural products

Introduction:

Herbal medicine (phytomedicine) refers to using a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Herbalism has a long tradition of use outside of conventional medicine. It is becoming more main stream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in the treating and preventing disease.

Terms Relating to Herbal Medicines:

Pharmacognosy

Is the scientific study of drugs from natural products (plants, animals, minerals).

Herbs

Herbs include crude plant material such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.

Herbal materials

Herbal materials are either whole plants or parts of medicinal plants in the crude State. They include herbs, fresh juices, gums, fixed oils, essential oils, resins and Dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir baking with honey, Alcoholic beverages or other materials.

Herbal preparations

Herbal preparations are the basis for finished herbal products and may include Comminuted or powdered herbal materials, or extracts, tinctures and fatty oils, Expressed juices and processed exudates of herbal materials. They are produced with the aid of extraction, distillation, expression, fractionation, purification,

Concentration, fermentation or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic

Beverages and/or honey, or in other materials.

Finished herbal products or herbal medicinal products

These terms refer to products containing as active substances exclusively herbal drugs or herbal drug preparations. They may consist of herbal preparations made from one or more herbs. If more than one herb is used, the term “mixed herbal product” may also be used. They may contain excipients in addition to the active ingredients. In some countries herbal medicines may contain, by tradition, natural organic or inorganic active ingredients, which are not of plant origin (e.g. Animal materials and mineral materials). Generally however, finished products or mixed products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

Medicinal plant

A plant, either growing wild or cultivated, used for its medicinal purposes.

Ethnopharmacology

The scientific study of indigenous medicines – is an interdisciplinary science practiced all over the world.

Phytotherapeutic agents

Or phytomedicines are standardized herbal preparations that contain, as active ingredients, complex mixtures of plant materials in the crude or processed state.

Contamination

The undesired introduction of impurities of a chemical or microbiological nature, Or of foreign matter, into or onto a starting material, intermediate product or Finished herbal product during production, sampling, packaging or repackaging, Storage or transport.

Foreign matters:

Material consisting of any or all of the following:

- Parts of the herbal material or materials other than those named with the limits specified for the herbal material concerned;
- Any organism, part or product of an organism, other than that named in the specification and description of the herbal material concerned;
- Mineral admixtures such as soil, stones, sand, and dust; and glass, metal and plastics or any other extraneous materials. These may be loose or adhering to these herbal materials.

Marker compound:

It is chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not.

Advantages of herbal medicine

1. Herbal medicine have long history of use and better patient tolerance as well as acceptance.
2. Medicinal plants have a renewable source, which is our only hope for sustainable supplies of cheaper medicines for the world growing population.
3. Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity.
4. The cultivation and processing of medicinal herbs and herbal products is environmental friendly.

5. Prolong and apparently uneventful use of herbal medicines may offer testimony of their safety and efficacy.
6. Throughout the world, herbal medicine has provided many of the most potent medicines to the vast arsenal of drugs available to modern medical science, both in crude form and as a pure chemical upon which modern medicines are structured.

Limitations of herbal medicines

Like any other branch of science and technology, present scenario of herbal medicine has its own limitations arising out of its own technical constituents. The prominent limitations of herbal medicines can be summarized as follow:

1. Ineffective in acute medical care

As may be observed, herbal medicines are not varying effective to treat any acute illness. As most of the medicines are designed to work at molecular level of physiology, the drug takes its time to deliver the results. However there are few herbal medicines which work instantly in acute conditions like diarrhea. On the other hand, modern system of medicine has adequate paraphernalia for management of acute conditions. It has already been established by virtue of its efficacy. It may be a futile exercise to investigate and discover such methods of acute medicinal care within the framework of herbal medicines.

2. Inadequate standardization and lack of quality specifications

This is the most often criticized aspect of herbal medicines. One important fact is that a herbal preparation is administered for its holistic value. Each herbal ingredient in the herbal preparation has an array of chemical constituents with complex molecular formulae. This each herbal preparation is a source of polypharmacy within itself.

As results, standardization of herbal preparation or its ingredients become a highly complex issue. Standardization of herbal drugs by known marker compounds may not be complete answer. Therefore a consensus is being arrived at to incorporate the qualitative finger-printing together with other physicochemical parameters of quality protocols for herbal medicines is an ongoing process and this shortcoming could be overcome shortly.

3. Lack of scientific data

Literature on herbal medicines, lack of scientific data in support of the medicinal activity claimed and their safety and efficacy assumed. Hence there is a need to incorporate certain parameters of the pharmacological evaluation on modern lines. WHO guidelines clearly direct that it is not necessary to carry out detailed toxicological evaluation of herbs and herbal preparation originating from traditional system medicine.

4. Need of Standardizations

In recent years there is a spurt in the interest regarding survival of Ayurvedic forms of medication. In the global perspective, there is a shift towards the use of medicine of herbal origin, as the dangers and the shortcoming of modern medicine have started getting more apparent, majority of Ayurvedic formulation are prepared from herbs.

Preparation of Herbal Drugs:

Herbal therapies are usually prepared by grinding or steeping the parts of a plant that are believed to contain medicinal properties. The ground plant matter is called the **macerate**. The macerate is soaked in a liquid referred to as the **menstruum** in order to extract the active ingredients.

Dosage forms of herbal preparation:

Liquid dosage forms (infusions, decoctions, tinctures, and extracts) are widely used in traditional herbal medicine. They are readily absorbed; dosing is flexible; and they are easy to take. Disadvantages include a disagreeable or bitter taste, and small amount of alcohol in most tinctures and extracts (ethanol is used typically as a solvent). There is also some confusion about dosing and potency.

Water extraction of herbal drugs:

Infusion and decoctions are obtained by extraction of water-soluble or polar constituents such as tannins and glycosides, although water is not good solvent for many other active chemicals. They are the safest type of extract. Water extracts

have a short shelf-life (unless they contain antimicrobial additives) due to bacterial contamination, and thus should be refrigerated and discarded after a few days. They are also difficult to standardize, and often bitter or unpleasent tasting unless flavor additives are incorporated.

An infusion is another name for strong tea, and is the preferred extraction method for delicate plant parts such as leaves, flowers, soft stems, and fruits. Infusions are commonly prepared by pouring boiling water over an herb and allowing steeping or “brewing". Typically 8 ounces (ca 215 mL) of water is poured over 2-3 teaspoons of herb and allowed to steep for 10-15 minutes. The solid are strained out, and the result liquid is available for use.

A decoction is similar to boiled coffee or tea: the herb and water are boiled together. Decoction is generally more concentrated than infusion and the method is useful for fibrous plant material such as root, stem, rhizome and bark. Typically, 2-3 teaspoons of herb are placed in 12 ounces of cold water (ca 325 mL), which is brought to boil and simmered under cover for 5-15 minutes or longer. Again the solids are strained out.

Solvent (hydroalcoholic) extraction:

Tinctures and fluid extractions can be commercially prepared for retail scale because they have a long shelf-life. Alcohol-based solvent extracts generally have a shelf-life of 2-3 year. They are usually stronger than infusion or decoction as alcohol can extract additional constituents that are lipophilic or water insoluble. Technically there is a difference between a tincture and fluid extract.

A tincture is generally defined as a solvent (e.g. alcohol) extraction in a 1:5 (1 g of herb is contained in 5 mL liquid) or weaker ratio (e.g. 1: 10). Tinctures were traditionally made by placing the herb in an organic solvent (ethanol) and leaving it to soak for days to weeks. For many herbs a typical tincture dose is several mL, which is equivalent to about 1 g of the crude or dried herb.

A fluid extract is similar to a tincture, but by definition is a 1:1 or 1:2 ratio. The solvent is usually a 30 – 70 % ethanol and water mixture. It is supposed to be about

5-10 times more potent than tincture, and smaller dose are used (often dose in drops).

The resulting therapies may also come in several forms, including oral tablets, capsules, gel caps. Solid or powdered extracts are prepared by evaporation of the solvents used in the process of extraction of the raw material. Some phytotherapeutic agents are greatly concentrated in order to improve their therapeutic efficacy. In this process, it is possible to remove some secondary metabolites present in the plants, which may produce undesirable side effects. The extracts also contain marker compounds which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not.

Bases of Quality Control of Herbal Drugs:

Quality control for efficacy and safety of herbal products is of paramount importance. It can be defined as **the status of a drug that is determined by identity, purity, content, and other chemical, physical, or biological properties, or by the manufacturing processes.** Quality control is a term that refers to processes involved in maintaining the quality and validity of a manufactured product. For the quality control of a traditional medicine, the traditional methods are procured and studied, and documents and the traditional information about the identity and quality assessment are interpreted in terms of modern assessment.

In general, all medicines, whether they are of synthetic or of plant origin, should fulfill the basic requirements of being efficacious and safe, and this can be achieved by suitable clinical trials.

Quality criteria are based on clear scientific definitions of the raw material. The term “herbal drugs” denotes plants or plant parts that have been converted into phyto-pharmaceuticals by means of simple processes involving **harvesting, drying, and storage (Manufacture practice)**. Hence they are capable of variation. This variability is also caused by differences in growth, geographical location, and time of harvesting (Agriculture practice). Herbal drugs in combinations with chemically defined active substances or isolated constituents,

and homeopathic preparations which frequently contain plants, are not regarded as herbal medicines. Their production is already based on adequate quality control of the respective starting materials. The following paragraphs will focus on quality control of herbal drugs in compliance with the above definition.

In general, quality control is based on three important pharmacopeial definitions e.g.

• ***Identity***: Is the herb the one it should be?

Identity can be achieved by macro- and microscopical examinations. The correct identity of the crude herbal material, or the botanical quality, is of prime importance in establishing the quality control of herbal drugs. Voucher specimens are reliable reference sources. Outbreaks of diseases among plants may result in changes to the physical appearance of the plant and lead to incorrect identification. At times an incorrect botanical quality with respect to the labeling can be a problem. For example, in the 1990s, a South American product labeled as "Paraguay Tea" was associated with an outbreak of anticholinergic poisoning in New York. Subsequent chemical analysis revealed the presence of a class of constituents that was different from the metabolites normally found in the plant from which Paraguay tea is made.

• **Purity**: Are there contaminants, e.g., in the form of other herbs which should not be there?

Purity is closely linked with the safe use of drugs and deals with factors such ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. However, due to the application of improved analytical methods, modern purity evaluation also includes microbial contamination, aflatoxins, radioactivity, and pesticide residues. Analytical methods such as photometric analysis, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) can be employed in order to establish the constant composition of herbal preparations. Depending upon whether the active principles of the preparation are known or unknown, different concepts such as "normalization versus standardization" have to be applied in order to establish relevant criteria for uniformity.

Content or assay is the most difficult area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes markers can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the percentage extractable matter with a solvent may be used as a form of assay. The choice of the extracting solvent depends on the nature of the compounds involved, and might be deduced from the traditional uses. For example, when a herbal drug is used to make a tea, the hot water extractable matter, expressed as milligrams per gram of air-dried material, may serve this purpose.
A special form of assay is the determination of essential oils by steam distillation. When the active constituents (e.g. sennosides in Senna) or markers (e.g. alkyl-amides in Echinacea) are known, a vast array of modern chemical analytical methods such as ultraviolet/visible spectroscopy (UV/VIS), TLC, HPLC, GC, mass spectrometry (MS), or a combination of GC and MS (GC/MS), or HPLC and MS (LC-MS) can be employed.

• **Content or assay** of active constituents within the defined limits?
It is obvious that the content is the most difficult one to assess, since in most herbal drugs the active constituents are unknown. Concerning the evaluation of the active constituents of herbal drugs two type of standardization:
True standardization, a definite phytochemical or group of constituents is known to have activity. Ginkgo with its 26% ginkgo flavones and 6% terpenes is a classic example.
Marker substances, when the active principles are unknown, markers can be used which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not (e.g. alkyl-amides in Echinacea).
marker substance(s) should be established for analytical purposes and standardization of the finished product which based on manufacturers guaranteeing.

To prove identity and purity, criteria such as type of preparation sensory properties, physical constants, adulteration, contaminants, moisture, ash content and solvent residues have to be checked.

The following factors can greatly affect the quality, and hence the therapeutic value of herbal medicines:

- The use of fresh plants;
- Age and part of plant collected;
- Period, time and method of collection;
- Temperature of processing, exposure to light;
- Availability of water, nutrients;
- Drying, packing, transportation of raw material and storage.

Adulteration of Herbal Drugs:

Direct or intentional adulteration of drugs usually includes practices in which a herbal drug is substituted partially or fully with other inferior products. Due to morphological resemblance to the authentic herb, many different inferior commercial varieties are used as adulterants. These may or may not have any chemical or therapeutic potential.

The adulteration is done deliberately but it may occur accidentally in condition such as deterioration, admixture, sophistication, substitution, inferiority and spoilage.

Deterioration is impairment in the quality of drug.

Admixture is addition of one article to another due to ignorance or carelessness or by accident.

Sophistication is the intentional or deliberate type of adulteration.

Substitution occurs when some totally different substance is added in place of original drug.

Inferiority refers to any substandard drug.

Spoilage is due to attack of microorganisms.

Substitution by "exhausted" drugs entails adulteration of the plant material with the same plant material devoid of the active constituents.

Also, substitution or adulteration as a result of ignorance or carelessness or fraud should be identified because many of the crude vegetable drugs are collected by independent and unorganized collectors, they are generally more interested in quantity than quality. However, the local buyers are not always sufficiently critical with regard to identity and quality of drugs, and drugs of unsatisfactory quality sometimes reach the market. Although impurities, such as elements from other plants or some worthless portions from the same plant are present and a lowering of the quality results, the impurities may not be harmful. But when poisonous plants are mixed with nonpoisonous drugs the situation become dangerous.

Many methods are still used for the adulteration of crude drugs and adulteration occurs when a drug is:

1- **Scare**
2- **Its price is normally high**
3- **When certain drugs are in short supply.**

An adulterated drug is that which does not comply with pharmacopeial requirements. The adulterant is usually some material which is both cheap and available in fairly large amount.

Adulteration involves:

1- Manufacture of constituents

Materials are artificially manufactured so as to simulate the general form and appearance of various drugs e.g.

- Paraffin wax colourd yellow has been substituted for beeswax.
- Artificial invert sugar has been substituted for honey.
- Cotton thread colourd orange has been substituted for saffron.

2- Substitution of exhausted drugs:

Here the same plant material is mixed which is having no active medicinal components as they have already been extracted out. Sometimes as in the

preparation of volatile oils from clove, ginger or umbelliferous fruits. Partially or completely exhausted powder was mixed with potato starch and capsicum powder to provide pungent taste. Also, exhausted gentian has sometime had its bitterness restored by adding aloes to it.

3- Substitution of superficially similar but cheaper natural substances; usually having no relation to genuine article e.g. peach kernels and apricot for almonds; saffron is admixed with dried flowers of *Carthamus tinctorius*; or clove stalk and mother cloves are often mixed with cloves.

4- Substitution of inferior commercial varieties;

Indian senna substituted with Arabian senna, and dog senna. Medicinal ginger replaced by it is inferior verities viz. African, Japanese and Cohin ginger.

5- Addition of worthless heavy materials;

Large masses of stones has been found in a bale of liquorice. Addition of sand to many drugs are other examples.

6- Addition of synthetic principles to fortify inferior products so as to contain a proper percentage of the active constituents used in official drug e.g. vanillin to vanilla pods or citral to lemon oil.

7- The presence of adventitious matter naturally occurring with the drug, if in excessive amount, also, constitutes an adulteration e.g. clove stalk with clove, cork in cinnamon powder or senna stalk my present also with senna leaf.

8- Addition of waste products of suitable colour and density to powdered drugs e.g. powdered olive stones are added to powdered liquorice and powdered Hazel-nut shells are added to cinnamon.

9- Admixture may occur through faulty collection and collection the drug at the improper time or collecting other less valuable parts of the genuine plant or collecting other plants.

10 - In correct storage condition

11-Abstraction or destruction of the therapeutically active constituents of drugs by extraction or distillation, aging, moisture, heat, light, fungi, micro-organisms, insects.

Factors such as geographical sources, growing conditions, processing, and storage are all factors that influence the quality of the drug. Deterioration may contribute to indirect adulteration, and crude drugs are often prone to deterioration, especially during storage, leading to the loss of the active ingredients, production of metabolites with no activity and, in extreme cases, the production of toxic metabolites. Physical factors such as air (oxygen), humidity, light, and temperature can bring about deterioration directly or indirectly. Oxidation of the constituents of a drug can be brought about by oxygen in the air, causing some products, such as essential oils, to resinify or to become rancid. Moisture or humidity and elevated temperatures can accelerate enzymatic activities, leading to changes in the physical appearance and decomposition of the herb.

Dried herbs are particularly prone to contamination with spores of bacteria and fungi present in the air. Bacterial growth is usually accompanied by the growth of molds, whose presence is evidenced by changes in appearance, break down of the plant material, and smell. Mites, nematode worms, insects/moths, and beetles can also destroy herbal drugs during storage.

Control measures to protect against deterioration include:

- Use of airtight containers made of materials that will not interact physically or chemically with the material being stored.
- Storage in ventilated, cool, dry areas.

- periodic spraying of the stored area with insecticides will help to prevent the spread of infestation.

Sterilization of crude drugs is achieved by treatment of bulk consignments with ethylene oxide, and methyl bromide under controlled conditions and complying with acceptable limits for toxic residues. World markets from time to time experience wild fluctuations in the price of herbals. One reason for this is indiscriminate harvesting which leads to the extinction of natural populations - still the only source of bioresources. This in turn encourages producers to replace the required herb with other supplements.

Parameters for Quality Control of Herbal Drugs

By using the following parameters, identification and standardization of crude drug is easily possible:

1- Sampling of drugs for evaluation:

Considerable care must be taken to ensure that a sample taken for evaluation truly represents the whole batch of the drug. Because of the specific characteristics of herbal materials, in particular their lack of homogeneity, special handling procedures are required in relation to sampling.

The following procedures should be observed when selecting and preparing an average sample from a batch of material.

Inspect each container or packaging unit for conformity with requirements regarding packaging and labeling. Check the condition of the package and note any defects that may influence the quality or stability of the contents (physical damage, moisture, etc.).

If initial inspection indicates that the batch is uniform, take samples as follows:

- When a batch consists of five containers or packaging units, take a sample from each one.
- From a batch of 6–50 units, take a sample from five.

- In the case of batches of over 50 units, sample 10%, rounding up the number of units to the nearest multiple of 10. For example, a batch of 51 units would be sampled as for 60 - i.e. take samples from six packages.

After opening, inspect the contents of the units selected for sampling for:
- organoleptic characteristics (colour, texture and odour);
- presentation of the material (raw, cut, crushed, compressed);
- the presence of admixtures, foreign matter (sand, glass particles, dirt), mould or signs of decay;
- the presence of packaging material originating from poor or degraded containers.

From each container or package selected, take three original samples. Samples should be taken from the ***top***, ***middle*** and ***bottom*** of the container. Samples of seeds should be withdrawn with a grain probe. The three original samples should then be combined into a pooled sample which should be mixed carefully.
The average sample is obtained by quartering. Form the pooled sample, adequately mixed, into an even and square-shaped heap, and divide it diagonally into four equal parts. Take two diagonally opposite parts and mix carefully. Repeat the process as necessary until the required quantity, to within ± 10%, is obtained (100–200 g for flowers and up to 10 kg for certain roots). Any remaining material should be returned to the batch.

Using the same quartering procedure, divide the average sample into four final samples, taking care that each portion is representative of the bulk material. The final samples are tested for the following characteristics:
* degree of fragmentation (sieve test);
* identity and level of impurities;
* moisture and ash content;
*level of active ingredients, where possible.
A portion of each final sample should be retained to serve as reference material, which may also be used for re-test purposes, if necessary.

Procedures of sample weight

1- Samples of vegetable drugs, in which the component part is 1 cm or less in any dimension and for all powdered or ground drugs, use a sampler which removes a core from the top to the bottom of the container, not less than to cores in opposite directions.

a- When the total weight of the drug to be sampled is less than 100 kg., at least 250 g should constitute an official sample.

b- Samples of crude drugs , in which the total weight more than 100 Kg, take repeated samples, not less than one-tenth in number of the packages to be sampled. Mix and quarter, reject two of the diagonal quarters, combine the remaining two quarters and carefully mix them and again subject the mixture to quartering process in the same manner until two of the quarters weigh at least 250g. which should constitute the official sample of the drug.

2- Entire drugs, in which the component parts are over 1 cm in any dimension are taken by hand in different parts of the container or containers

a- When the total weigh of the drug to be sampled is less than 100 kg at least 500 g should constitute an official sample.

b- When the total weigh of the drug to be sampled is more than 100 kg., repeated samples, not less than one-tenth in number of the packages to be sampled are taken, mixed and quartered as before to get 500g sample.

c- When the total weigh of the drug to be sampled is less than 10 kg the final official sample should be not less than or at least 250 g.

Macroscopic and microscopic examination:

Herbal materials are categorized according to:

- Sensory,
- Macroscopic characteristics
- Microscopic characteristics.

An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials, and should be carried out before any further tests are undertaken. Wherever possible, authentic

specimens of the material in question and samples of pharmacopoeial quality should be available to serve as a reference.

Sensory characters ***(***visual inspection) provides a supplementary way in evaluation of drugs and considered as the simplest and quickest means by which to establish identity, purity and - possibly - quality. If a sample is found to be significantly different from the specifications in terms of colour, consistency, odour or taste, it is considered as not fulfilling the requirements.

Macroscopic identity of herbal materials is based on shape, size, colour, surface characteristics, texture, fracture characteristics and appearance of thecut surface. However, since these characteristics are judged subjectively and substitutes or adulterants may closely resemble the genuine material, it is often necessary to substantiate the findings by microscopy and/or physicochemical analysis.

Once the material has been examined and classified according to external characteristics, inspection by microscopy can be carried out as the next step.

Microscopic inspection of herbal materials is indispensable for the identification of broken or powdered materials; the specimen may have to be treated with chemical reagents. microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present. For instance, pollen morphology may be used in the case of flowers to identify the species, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used. Although this may seem obvious, it is of prime importance, especially when different parts of the same plant are to be used for different treatments e.g stinging nettle (*Urtica urens*) is a classic example where the aerial parts are used to treat rheumatism, while the roots are applied for benign prostate hyperplasia. An examination by comparison with a reference material will often reveal characteristics not described in the requirements which might otherwise have been attributed to foreign matter, rather than normal constituents. For this purposes, it is necessary to know the microscopical characters of genuine materials and then, if foreign structures are detected in the sample, adulteration would be suspected and frequently a knowledge of the microscopical characters of

common adulterants will lead to identification of the foreign matter. For example rhubarb and ginger are characterized by their non-lignified vessels.

Any additional useful information for preparation or analysis should also be included in the test procedures for individual plant materials - for example the determination of vein-islets and the palisade ratio.
microscopy alone cannot always provide complete identification, though when used in association with other analytical methods it can frequently supply invaluable supporting evidence.

Histochemical detection of cell walls and contents:

Reagents can be applied to a powdered sample or a section on a slide and the progress of the reaction may be observed under a microscope to detect the following:

Cellulose cell walls

Add 1–2 drops of iodinated zinc chloride TS and allow to stand for a few minutes; alternatively, add 1 drop of iodine (0.1 mol/l), allow to stand for 1 minute, remove excess reagent with a strip of filter-paper and add 1 drop of sulfuric acid TS; cellulose cell walls are stained blue to blue-violet. On the addition of 1–2 drops of cuoxam TS, the cellulose cell walls will swell and gradually dissolve.

Lignified cell walls

Moisten the powder or section on a slide with a small volume of phloroglucinol TS and allow to stand for about 2 minutes or until almost dry. Add 1 drop of hydrochloric acid TS and apply a cover-glass; lignified cell walls are stained pink to cherry red.

Suberized or cuticular cell walls

Add 1–2 drops of sudan red TS and allow to stand for a few minutes or warm gently; suberized or cuticular cell walls are stained orange-red or red.

Aleurone grains

Add a few drops of iodine/ethanol TS; the aleurone grains will turn yellowish-brown to brown. Then add a few drops of ethanolic trinitrophenol TS; the grains will turn yellow. Add about 1 ml of mercuric nitrate TS and allow to dissolve; the colour of the solution turns brick red. If the specimen is oily, render it fat-free by immersing and washing it in an appropriate solvent before carrying out the test.

Calcium carbonate

Crystals or deposits of calcium carbonate dissolve slowly with effervescence when acetic acid TS or hydrochloric acid TS is added.

Calcium oxalate

Calcium oxalate is best viewed after the sample has been clarified (e.g. with chloral hydrate TS).

Fats, fatty oils, volatile oils and resins

Add 1–2 drops of sudan red TS and allow to stand for a few minutes or heat gently, if necessary. The fatty substances are stained orange-red to red.

Hydroxyanthraquinones

Add 1 drop of potassium hydroxide TS; cells containing 1,8-dihydroxyanthraquinones are stained red.

Inulin

Add 1 drop each of 1-naphthol TS and sulfuric acid TS; spherical aggregations of crystals of inulin turn brownish red and dissolve.

Mucilage

Add 1 drop of Chinese ink TS to the dry sample; the mucilage shows up as transparent, spherically dilated fragments on a black background. Alternatively, add 1 drop of thionine TS to the dry sample, allow to stand for about 15 minutes, then wash with ethanol TS; the mucilage turns violet-red (cellulose and lignified cell walls are stained blue and bluish violet, respectively).

Starch

Add a small volume of iodine; a blue or reddish blue colour is produced. Alternatively, add a small volume of glycerol/ethanol TS and examine under a microscope with polarized light; birefringence is observed giving a Maltese cross effect with the arms of the cross intersecting at the hilum.

Tannin

Add 1 drop of ferric chloride TS; it turns bluish black or greenish black.

Microscopical numerical values:

There are certain numerical values which are used for identification purposes and are particularly useful in determining the purity and botanical sources of certain powdered drugs specially when possessing a close similarities to other drug derived either from related species or from substitutes which have external resemblance to the genuine drug.

I. Microscopical linear measurements

Measurements of the most prominent or unusual structures in powdered drugs such as stone cells (sclerides), fibers, pollen grains or cell-contents such as starch granules and crystals of calcium oxalates. For examples:

A- Rio or Brazilian ipecacuanha root (*Cephaelis ipecacuanha*), contains starch granules which never exceed 15µ in diameter and is thus distinguished from Cartagena ipecacunha (*Cephaelis acuminate*) and has starch grains many of which measure between 17-20µ.

B- American podophyllum rhizome (*Podophyllum peltatum*) and certain cells of its parenchyma contain large cluster crystals of calcium oxalate up to 100 µ in diameter and often over 60 µ. Indian Podophyllum (*Podophyllum hexandrum*) also has cluster crystals of calcium oxalate in its parenchyma but they are usually 30-40 µ in diameter and very rarely exceed 60 µ.

C- Cassia bark is distinguished also from cinnamon by the wider phloem fibers which often measure from 30-45 µ in width compared with those of cinnamon which rarely exceed 30 µ in width.

II. Ratio values

Two of the important numerical values and are termed Palisade ratio and stomatal index. Those two values are based upon the microscopical structures of leaves only. They are fairly constant for the leaf of any particular plant. So, it can be used to differentiate between closely related species (Indian and Alexandrian Senna leaves).

1- Palisade ratio:

It is the number of palisade cells under one epidermal cell, using 4 continuous epidermal cells for the count. The palisade cells bear a definite relation to the epidermal cells in each species. For counting use clarified leaves mounted in chloral hydrate for 30 min.

2- Stomatal index:

It is the percentage of the number of stomata to the total number of epidermal cells including the stomata, each stomata being counted as one cell.

$$\text{Stomatal index} = \frac{S}{E+S} \times 100$$

Where S = the number of stomata per unit area

E = The number of epidermal cells in the same unit area.

The figure obtained is constant for any species and can be used for the differnation between the closely related species.

3- Vein islets

The mesophyll of a leaf is divided into small portion of photosynthetic tissue by the anastomosis the veins and veinlets. The small area of green tissue outlined by the veinlets are termed vein-islets. Frequently a small vein tip runs out from the surrounding veinlets into the center of each islet.

Simple maceration of the leaf in hot solution of chloralhydrate for 03-60 min, on water bath to clarify the surfaces. The difficulty are abundance of calcium oxalate, colouring matter and presence of mucilage in the epidermis. Mucilaginous epidermis if present, must be removed by soaking the leaf in water and stripping the epidermis. Colouring matter may be removed by soaking for 6-24 hrs in solution of chlorinated soda followed by washing with water. The leaf after this is soaked with hydrochloric acid (10%) to dissolve calcium oxalate followed by washing with water. The treated leaf is the placed in hot chloral hydrate until clear. The cleared leaf can be stained with phloroglucinol and hydrochloric acid (veins acquired red colour) the mounted in glycerin before making the count of vein-islets.

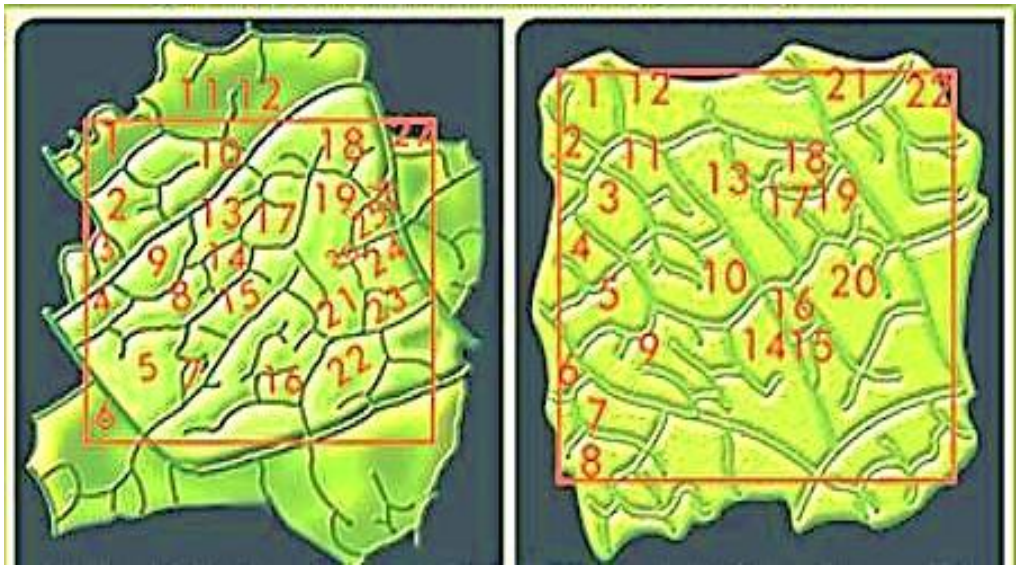
4-

a- Vein-islet number

It is the number of vein-islets per square mm. of leaf surface. This value has been shown to be constant for any species and unaffected by the age of the plant or the size of the leaves. A differentiation between *Cassia acutifolia* (Alexandrian senna) and *Cassia angustifolia* (Indian senna) is a good example for using of vein islet number in identification of closely related drug species.

b- Veinlet termination number

Is the number of veinlet termination per square mm of leaf surface. A vein termination is the ultimate free termination of a veinlet or branch of a veinlet. It can be also distinguish between leaves of closely related species



5. Cells per unit area

It is the number of cells of certain type per unit area of a tissue. It is useful qualitative diagnostic character based on number and area. This method is particularly applicable to layers one cell-thick as sclerides of cardamom.

Determination of moisture content:

An excess of water in herbal materials uneconomical and will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis (in conjunction with suitable temperature will lead to the activation of enzymes). Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water.

Many methods are now available for moisture determination, many being employed in industries will also contribute to the weight loss.

1. Loss on drying (gravimetric determination)

Although the loss in weight in the sample so tested principally is due to water, small amounts of other volatile materials will also contribute to weight loss. Dry the sample by one of the following techniques:

a- **Drying using direct heat;** by direct drying a accurately known weight of the material in oven at 105 ^{o}C to constant weight the loss of weight is the water content. (thus, this methods is not suitable for plant material containing volatile constituents).

b- **Vacuum oven method;** used mainly for drugs containing volatile constituents or which liable to decomposition by normal drying method (vacuum help moisture to loss or evaporate at low temp.)

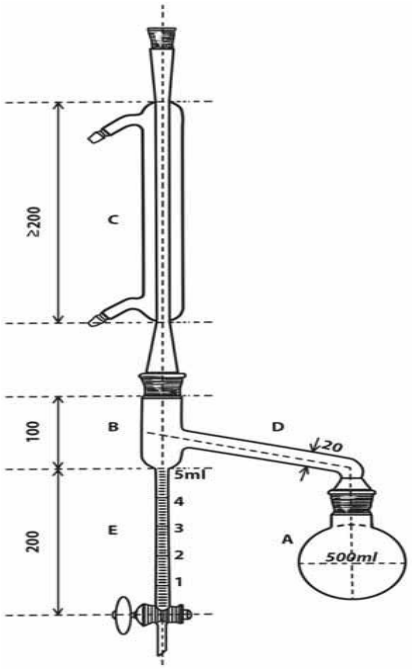
c- **Drying over sulphuric acid**; employed for drugs with high content of volatile oils. The drying process is performed by spreading thin layers of weighed drugs over glass plates and placing in a desicator containing conc. sulphuric acid which absorb moisture from the drug.
(Dry until two consecutive weighs do not differ by more than 5 mg, unless otherwise specified in the test procedure. Calculate the loss of weight in mg per g of air-dried material).

2- Separation and measurements of moisture

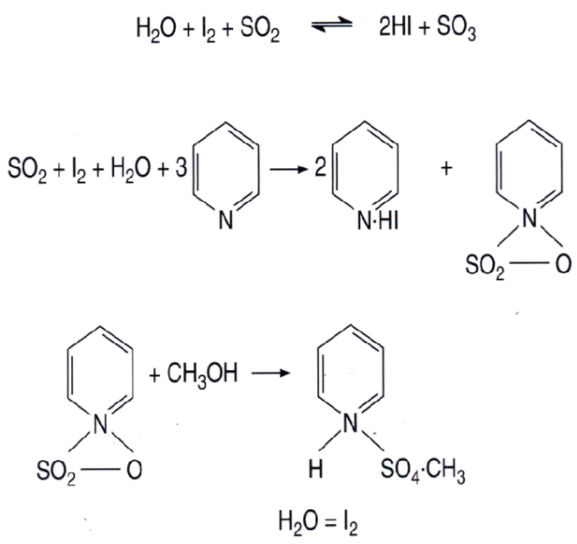
This method gives a direct measurement of the water present in the material being examined using the following techniques:

a. Azeotropic method (Toluene distillation)

When the sample is distilled together with an immiscible solvent, such as toluene or xylene, the water present in the sample is absorbed by the solvent. The water and the solvent are distilled together and separated in the receiving tube on cooling. The apparatus consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1-ml divisions so that the error of readings does not exceed 0.05 ml. The preferred source of heat is an electric heater.



b. Karl Fischer procedure

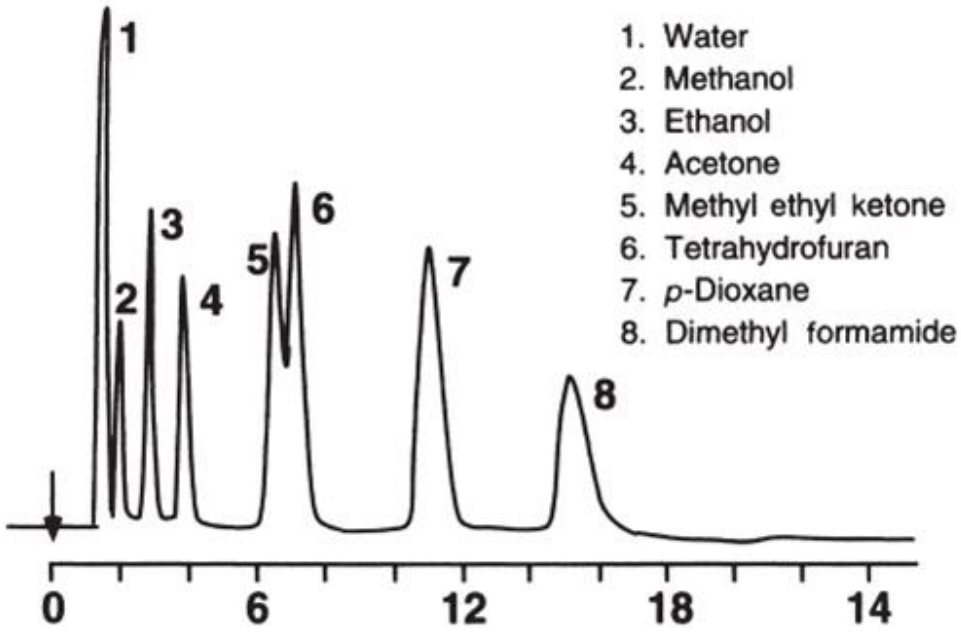


This is a chemical method for water determination which detect very small quantity of water (10 µg – 10 mg). The Karl Fischer reagent consists of iodine, sulphur dioxide and pyridin in dry methanol. This is titrated against a sample containing water , which causes loss of the dark brown colour. At the end end-point, when no water is available, the colour of the reagent persists. The basic reaction a reduction of iodine by sulphur dioxide in the presence of water. The reaction goes to completion by the removal of sulphur trioxide as pyridin sulphur trioxide, which in turns reacts with the methanol to form the pyridin salt of methyl sulphate.

This method is more specific for determination of water by separating and evaluating the water obtained from a sample.

3. Gas chromatographic method:

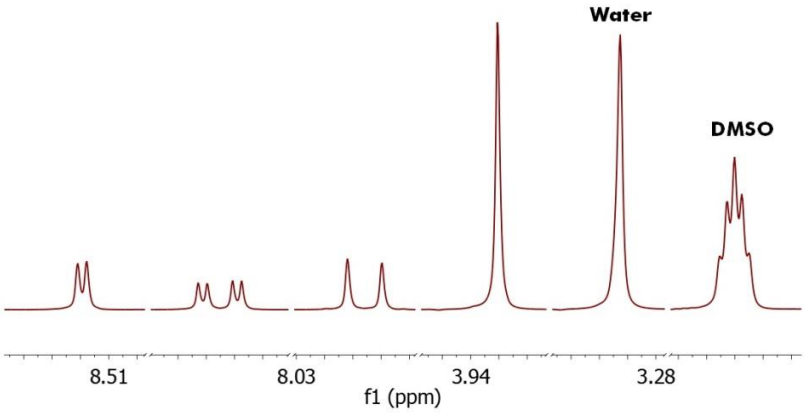
This method becomes increasingly important for moisture determination because of their specificity and efficiency. The water in a weighed powder sample can be extracted by dry methanol and an aliquot submitted to chromatography on either 10% carbowax on Fluro-pack 80 or Porapak column. The water separated by this means is readily quantitatively determined from the resulting chromatogram using standard calibration curve.



Separation of water in a mixture of polar solvents on Porapak®

4. Spectroscopic method:

Nuclear magnetic resonance (NMR) spectroscopy has been employed for the determination of moisture in starch, cotton and other plant products.



Determination of volatile oils

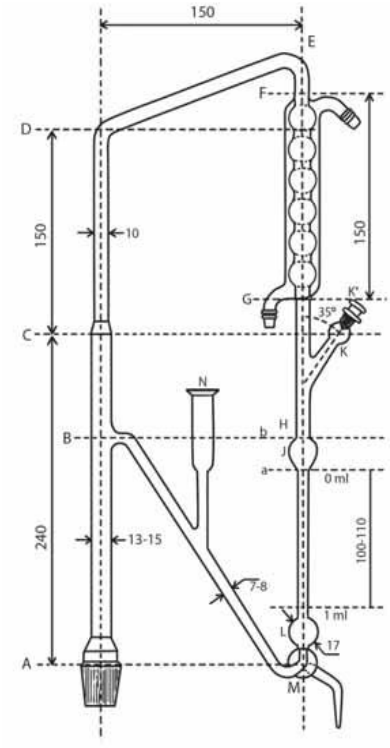
Volatile oils are characterized by their odour, oil-like appearance and ability to

volatilize at room temperature. Chemically, they are usually composed of mixtures of, for example, monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. Minimum standard for the percentage of volatile oil present in a number of drugs are prescribed by many pharmacopoeias.

In order to determine the volume of oil, the plant material is distilled with water

and the distillate is collected in a graduated tube. The aqueous portion separates

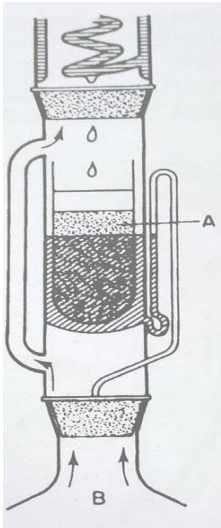
automatically and is returned to the distillation flask. If the volatile oils possess a mass density higher than or near to that of water, or are difficult to separate from the aqueous phase owing to the formation of emulsions, a solvent (e.g. ether) with a low mass density and a suitable boiling-point may be added to the measuring tube. The dissolved volatile oils will then float on top of the aqueous phase.



Apparatus used to determine volatile oils (dimensions in mm)

Determination of extractable values

The determination of water-soluble or ethanol-soluble extractive is used as a means of evaluating drugs the constituents of which are not readily estimated by other means. In certain cases extraction of the drug by maceration (cold), in others by continuous extraction process by boiling the solvent (soxhlet extractor). Examples are gentian and liquorice (percentage of water-soluble extractive), ginseng, ginger (percentage of ethanol-soluble extractive), crushed linseed (percentage of ether-soluble extractive), colocynth (limit of light petroleum extractive).



Soxhlet continuous extraction apparatus

A: powdered drug for extraction in thimble,

B: Flask containing boiling solvent.

Determination of ash

When herbal drugs are incinerated, they leave an ash which is varies and of importance and indicates to some extent the amount of care taken in the preparation of the drugs (e.g. rhubarb rhizome, peeled and unpeeled liquorice).

The ash remaining following ignition of herbal materials is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash.

The **total ash** method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived

from the plant tissue itself (e.g. scleride cells, organic acids and salts), and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.
Place about 2- 4 g of the ground air-dried material, accurately weighed, in a previously ignited and tared crucible (usually of platinum or silica). Spread the material in an even layer and ignite it by gradually increasing the heat to 500–600 °C until it is white, indicating the absence of carbon. Cool in a desiccator and weigh. Calculate the content of total ash in mg per g of air-dried material.

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

To the crucible containing the total ash, add 25 ml of hydrochloric acid TS, cover with a watch-glass and boil gently for 5 minutes. Collect the insoluble matter on an ashless filter-paper and wash with hot water until the filtrate is neutral. Transfer the filter-paper containing the insoluble matter to the original crucible, dry on a hotplate and ignite to constant weight. Allow the residue to cool in a suitable desiccator for 30 minutes, then weigh without delay. Calculate the content of acid-insoluble ash in mg per g of air-dried material.

Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water.
To the crucible containing the total ash, add 25 ml of water and boil for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper. Wash with hot water and ignite in a crucible for 15 minutes at a temperature not exceeding 450 °C. Subtract the weight of this residue in mg from the weight of total ash. Calculate the content of water-soluble ash in mg per g of air-dried material.

Determination of crude fiber:

This method gives the crude fiber content of the sample after it has been digested in sulphuric acid and sodium hydroxide solutions and the residue calcined. The difference in weight after calcination indicates the quantity of fiber present.

Crude fiber determination are used to detect excessive amounts of woody matter in powdered drugs such as sclereids, vessels and fibers, cellulose and cork. The detection of adulterants such as: clove stalk in cloves, mustard hulls, olive stones, coco-nut shells in powdered drugs is much more facilitated by preparing the crude fiber. The techniques involves defatting (ether or petroleum ether), the powder and boiling in turn with standard acid and alkali with suitable washing of the insoluble residue obtained at the different stages with water, dried and weight. The residue represents the crude fibers and minerals which are insoluble in the acid and alkali. The residue is incinerated and the ash left is weighted again.

Calculations

$$\text{Crude fibre content (\%)} = 100\,\frac{A - B}{C}$$

Where:

A = weight of dry residue after acid and alkali treatment (g)
B = weight of ash (g)
C = weight of sample (g)

Determination of foreign matters:

Herbal materials should be entirely free from visible signs of contamination by moulds or insects, and other animal contamination, including animal excreta.
No abnormal odour, discoloration, slime or signs of deterioration should be detected. It is seldom possible to obtain marketed plant materials that are entirely free from some form of innocuous foreign matter. However, no poisonous, dangerous or otherwise harmful foreign matter or residue should be allowed.
During storage, products should be kept in a clean and hygienic place, so that no contamination occurs. Special care should be taken to avoid formation of microbial contaminants e.g. moulds, since they may produce aflatoxins.

Generally foreign matter is material consisting of any or all of the following:

- parts of the herbal material or materials other than those named with the limits specified for the herbal material concerned;

- Any organism, part or product of an organism, other than that named in the specification and description of the herbal material concerned;
- Mineral admixtures not adhering to the herbal materials, such as soil, stones, sand and dust.

Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. However, microscopy is indispensable for powdered materials. Any soil, stones, sand, dust and other foreign inorganic matter must be removed before herbal materials are cut or ground for testing.

Foreign matter in whole or cut herbal materials

Spread it in a thin layer (accurate weighted plant material) and sort the foreign matter into groups either by visual inspection, using a magnifying lens (6× or 10×), or with the help of a suitable sieve, according to the requirements for the specific herbal material. Sift the remainder of the sample through a No. 250 sieve; dust is regarded as mineral admixture. Weigh the portions of this sorted foreign matter to within 0.05 g. Calculate the content of each group in grams per 100 g of air-dried sample.

Furthermore, when foreign matter consists, for example, of a chemical residue, TLC is often needed to detect the contaminants.

Determination of heavy metals:

Small quantities of trace elements are invariably present in plant materials and, indeed some such as zinc, copper and molybdenum appear to be necessary microcomponents of a normal diet. However, contamination by heavy metals such as mercury, lead, cadmium, and arsenic in herbal remedies can be attributed to many causes, including environmental pollution (atmosphere, nature of the soil and water of irrigation), which impose serious health risks to consumers. It is critical to analyse source materials for heavy metals in order to ensure that their concentrations meet the related standards or regulations limiting their concentrations in herbal medicines.

Contamination by toxic metals can either be accidental or intentional. The potential intake of the toxic metal can be estimated on the basis of the level of its presence in the product and the recommended or estimated dosage of the product. Limitation of particular metals are placed on some products that have been chemically manipulated for examples: nickel in hydrogenated soya and arachis oils; iron, chromium and zinc in gelatin; cadmium in linseed oil.
Several commonly used and sensitive analytical techniques, including atomic absorption spectrometry, inductively coupled plasma optical emission spectrometry or mass spectrometry, X-ray fluorescence spectrometry, high-performance liquid chromatography, differential pulse polarography, neutron activation analysis and anodic stripping voltammetry.

A simple, straightforward determination of heavy metals can be found in many pharmacopeias and is based on color reactions with special reagents such as thioacetamide or diethyldithiocarbamate, and the amount present is estimated by comparison with a standard. Instrumental analyses have to be employed when the metals are present in trace quantities, in admixture, or when the analyses have to be quantitative. The main methods commonly used are atomic absorption spectroscopy after acid digestion of the sample with concentrated HNO_3, HCL and H_2SO_4. Absorption is measured at the following wavelengths:

Heavy metal	Absorption wavelength (nm)
Cd	**228.8**
Cu	**324.8**
Fe	**248.3**
Ni	**232**
Pb	**283.5**
Zn	**213.9**
As	**193.7**
Hg	**253.7**

Determination of microbial contaminant and aflatoxins:

Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. This contamination

exerts an important impact on the overall quality of herbal products and preparations. Microbial contaminants often originating in the soil, poor methods of harvesting, cleaning, drying, handling, and storage. *Escherichia coli* or *Salmonella* spp. Are the most common microbial contaminant. However, a large range of bacteria and fungi are from naturally occurring microflora, aerobic spore forming bacteria frequently predominate.

The total viable aerobic count (TVC) of the herbal material being examined is determined, , using one of the following methods:

- membrane-filtration,
- plate count or
- serial dilution.

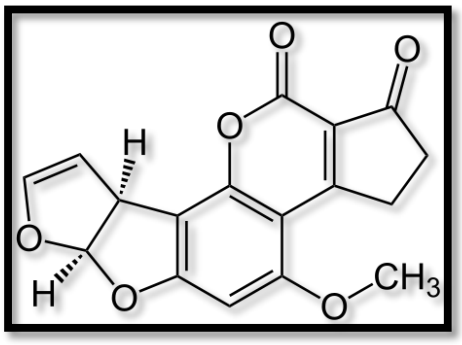
Aerobic bacteria and fungi (moulds and yeasts) are determined by the TVC. Usually a maximum permitted level is set for certain products, but when the TVC exceeds this level then it is unnecessary to proceed with determination of specific organisms; the material should be rejected without being subjected to further testing.

Laboratory procedures investigating microbial contaminations are laid down in the well known pharmacopeias, as well as in the WHO guidelines. Limit values can also be found in the sources mentioned. In general, a complete procedure consists of determining the total aerobic microbial count, the total fungal count, and the total *Enterobacteriaceae* count, together with tests for the presence of *Escherichia coli*, *Staphylococcus aureus*, *Shigella*, and *Pseudomonas aeruginosa* and *Salmonella* spp. The European Pharmacopoeia also specifies that *E. coli* and *Salmonella* spp. should be absent from herbal preparations. However it is not always these two pathogenic bacteria that cause clinical problems. For example, a fatal case of *listeriosis* was caused by contamination of alfalfa tablets with the Gram-positive bacillus *Listeria monocytogenes*.

Materials of vegetable origin tend to show much higher levels of microbial contamination than synthetic products and the requirements for microbial contamination in the European Pharmacopoeia allow higher levels of microbial contamination in herbal remedies than in synthetic pharmaceuticals. The allowed contamination level may also depend on the method of processing of the drug. For

example, higher contamination levels are permitted if the final herbal preparation involves boiling with water.

The mycotoxins produced by various (*Aspergillus*, e.g *A. flavus* and *A. parasiticus* and *Fusarium* species are termed aflatoxins, all having a coumarin nucleus fused to a bifuran unity and possessing in addition a pentnone ring (B-series) or six-membered lactone (G series). They have been circumstantially implicated in the death of children in a number of countries. Aflatoxins are potent toxins as carcinogens, as teratogens and as mutagens.



Aflatoxin B1

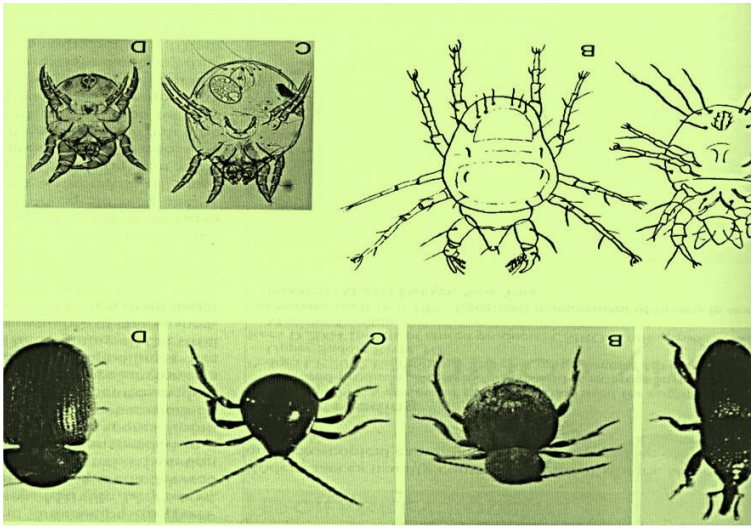
The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflotoxins which can be dangerous to health even if they are absorbed in minute amounts. In addition, certain plant constituents are susceptible to chemical transformation by contaminating microorganisms. Withering leads to enhanced enzymic activity, transforming some the constituents to other metabolites not initially found in the herb. These newly formed constituent(s) along with the molds such as *Penicillium nigricans* and *P. jensi* may then have adverse effects.

Methods of assay for these compounds (aflatoxins) have been developed in recent year and include high-performance liquid chromatography (HPLC) using fluorescence detection to determine aflatoxins B1, B2, G1 and G2 and thin layer chromatography (TLC) along with Standard solutions of aflatoxin B1, B2, G1 and G2 (2.5 ng/ml).

Determination of insect contamination and pesticide residues:

The detection, prevention and eradication of mite and insect infestation is an important hygienic and economic consideration for all who have occasion to store and use crude drugs. To keep drug without or with minimal infection need:

1- Good hygiene in the warehouse (removal of spillages, old debris and packing materials, elimination of sources of infection).
2- Effective stock control (regular inspection, rotation of stock, early recognition of infestations).
3- Optimum storage condition (maintenance of cool and dry environment).
4- Good packaging (woven sacks and bags, multiply paper sacks, polyethylene film … etc.).



Insect pests and Arachnids found in crude drugs.

Microscopical examination could be used as effective way for detection of the life or died insect parts.

Chemical examination for chitin which constitute the major part of cell wall of crustaceans, insects and many fungi (e.g. ergot).
Test for chitin $(C_8H_{13}O_5N)$ n:

When heated with 50% potash at 160-170 ◦C for 1 hour, it is converted to chitosan ($C_{14}H_{26}O_{16}N_2$), ammonia and acids such as acetic and oxalic. The mass may be dissolved in 3% acetic acid and the chitosan reprecipited by addition of slight excess alkali. Chitosan gives a violet colour when treated with a 0.5% solution of iodine in KI, and then 1% H_2SO_4.

Pesticide

Pesticides are defined as any substance intended for preventing, destroying, attracting, repelling, or controlling any pest - including unwanted species of plants or animals - during production, storage, transport, distribution and processing. The term includes substances intended for use as a plant-growth regulator, defoliant, desiccant, fruit thinning agent, or sprouting inhibitor and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. The term normally excludes fertilizers and plant nutrients.

Pesticide residue

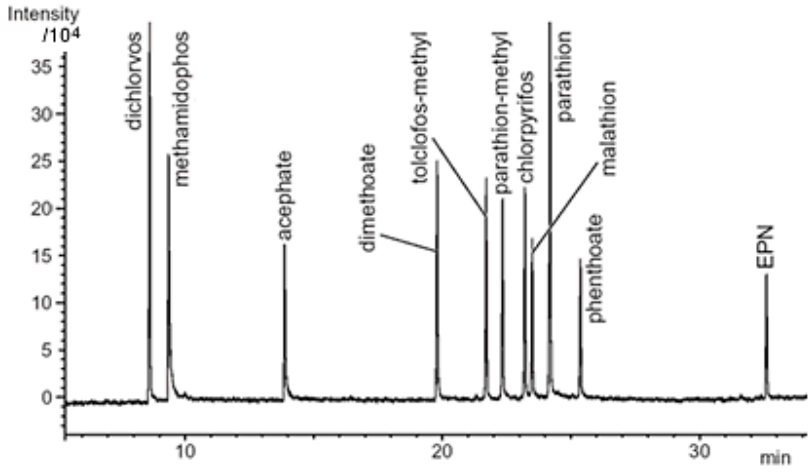
A pesticide residue is any specified substance in food, agricultural commodities or animal feed resulting from the use of a pesticide. The term includes any derivatives of a pesticide, such as conversion products, metabolites, reaction products and impurities considered to be of toxicological significance.

Even though there are no serious reports of toxicity due to the presence of pesticides and fumigants, it is important that herbs and herbal products are free of these chemicals or at least are controlled for the absence of unsafe levels. Herbal drugs are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administering of fumigants during storage. However, it may be desirable to test herbal drugs for broad groups in general, rather than for individual pesticides. Many pesticides contain chlorine in the molecule, which, for example, can be measured by analysis of total organic chlorine. In an analogous way, insecticides containing phosphate can be detected by measuring total organic phosphorus. whereas pesticides containing arsenic and lead can be detected by measurement of total arsenic or total lead, respectively. Samples of herbal material are extracted by a standard procedure, impurities are removed by partition and/or adsorption. Chromatography

(mostly column and gas) is recommended as the principal method for the determination of pesticide residues. These methods may be coupled with mass spectrometry (MS). Some simple procedures have been published by the WHO and the European Pharamacopoeia has laid down general limits for pesticide residues in medicine.

The following are the most common organophosphorus insecticides:

Dichlorvos, parathion, fonofos, chlorpyrifos, diazinon, methidathion, parathion-methyl, ethion, chlorpyrifos-methyl, carbophenothion, pirimiphos-methyl, azinphos-methyl,malathio, and phosalon.



Gas chromatograph (GC) with flame photometric detector (FPD) chromatogram for organophosphate compounds.

The most common organochlorine and pyrethroid insecticides:

α- hexachlorocyclohexane, *p,p'*-DDE, hexachlorobenzene, *o,p'*-DDD, β-hexachlorocyclohexane, endrin, lindane, α-endosulfan, δ-hexachlorocyclohexane, *o,p'*-DDT, ε- hexachlorocyclohexane, carbophenothion, heptachlor, *p,p'*- DDT, aldrin, *cis*-permethrin, *cis*-heptachlor-epoxide, *trans*-permethrin, *o,p'*-DDE, cypermethrina, β-endosulfan, fenvaleratea, dieldrin and deltamethrin.

Limit test for arsenic

Arsenic is abundant in nature and its presence in herbal materials should be no Different to its wide occurrence in foods. A popular test method relies on the digestion of the herbal material matrix followed by subjection of the digestate to a comparative colorimetric test in a special apparatus.

The test method depends on uses colorimetry and does not use toxic mercuric bromide paper. The method uses *N*-*N*-diethylmethyldithiocarbamate in pyridine and it reacts with hydrogen arsenide to afford a red–purple complex. The limit is expressed in terms of arsenic (III) trioxide (As2O3).

Determination of radioactive contamination:

Following a severe nuclear accident, the environment may be contaminated with airborne radioactive materials. These may deposit on the leaves of medicinal plants. Their activity concentration and the type of radioactive contamination can be measured by the radiation monitoring laboratories of most of the WHO Member States.

There are many sources of ionization radiation, including radionuclides, occurring in the environment. Hence a certain degree of exposure is inevitable. Dangerous contamination, however, may be the consequence of a nuclear accident. The WHO, in close cooperation with several other international organizations, has developed guidelines in the event of a widespread contamination by radionuclides resulting from major nuclear accidents. These publications emphasize that the health risk, in general, due to radioactive contamination from naturally occurring radio nuclides is not a real concern, but those arising from major nuclear accidents such as the nuclear accident in Chernobyl, may be serious and depend on the specific radionuclide, the level of contamination, and the quantity of the contaminant consumed. Taking into account the quantity of herbal medicine normally consumed by an individual, they are unlikely to be a health risk. Therefore, at present, no limits are proposed for radioactive contamination.

Analytical Methods for Active Constituents:

Content or assay is the most difficult area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes markers

can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the percentage extractable matter with a solvent may be used as a form of assay, an approach often seen in pharmacopeias.

All plant material used should be properly authenticated as much time and money can be wasted on the examination of material of doubtful origin. The choice of extraction procedure depends on the nature of the plant material and the components to be isolated and might be deduced from the traditional uses. Dried materials are usually powdered before extraction, whereas fresh plants (leaves. etc) can be homogenized or macerated with a solvents such as alcohol. The latter is also particularly useful for stabilizing fresh leaves by dropping them into the boiling solvent (stop enzymatic effects). Alcohol is a general solvent for many plant constituents (most fixed oils excepted) and as such may give problems in the subsequent elimination of pigments, resins, etc. Water immiscible solvents are widely used-light petroleum (essential and fixed oils. steroids), ether and chloroform (alkaloids' quinones). The extraction of organic bases (e.g. alkaloids) usually necessitate basification of the plant material if a water-immiscible solvent is to be used. For aromatic acids and phenols acidification may be required. A special form of assay is the determination of essential oils by steam distillation.

Extraction of crude drugs:]

The method used for extraction and isolation depends upon the nature of the compounds present in the drug:

1- Alkaloids are commonly extracted either directly by alcohol, by dil. acids or by organic solvents as chloroform and ether after conversion to their bases.
2- Volatile and fixed oils are commonly extracted by non-polar solvents as light petroleum and hexane.
3- Glycosides, saponins and tannins are commonly extracted by polar solvents as ethanol, methanol or aqueous alcohol.
4- Steroids and triterpenoids are extracted by light petroleum, hexane or ether.
5- Carbohydrates are extracted by aqueous alcohol.

6- Specific class could be extracted according to their polarities, other materials which are not belonging to specific class could be extracted according to their polarities.

Extraction method depends on:

1. Texture and water content of the plant material.
2. Type of substance that is being isolated e.g. volatile oils, lipids, alkaloids, sterols, saponins, flavonoids and or glycosides etc.

Methods of extraction

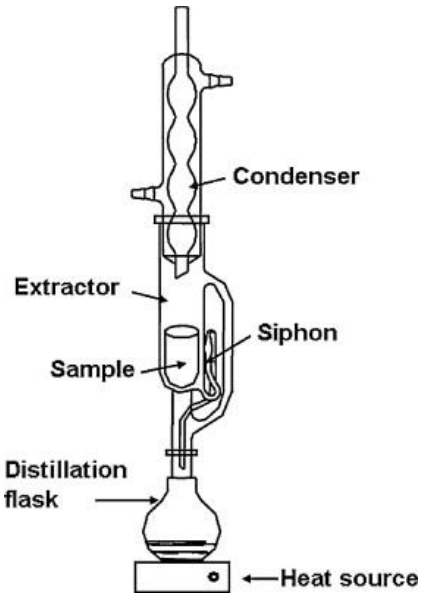
The following are the most common extraction methods for herbal plant materials:

1- Alcohol extraction or percolation
2- Soxhlet extractor
3- Successive extraction
4- Supercritical liquid extraction

Alcohol extraction

Alcohol (ethanol) in any case is a good all-purpose solvent for preliminary extraction by percolation of the homogenized fresh or dried powdered plant material several times till exhaustion. The obtained extract is filtered and then concentrated in *vacuo* by a rotary evaporator which will concentrate bulky solutions down to small volumes at temperature between 30-40^{o}c. Also, alcohol is desirable to kill the plant tissues preventing enzymatic oxidation or hydrolysis of the plant constituents in fresh materials.

Soxhlet extraction



A schematic representation of a Soxhlet extractor

Successive extraction of powdered plant material

The air-dried powdered plant is successively extracted till exhaustion in continuous extraction

apparatus (soxhlet) with the following solvents:

1- Light petroleum
2- Diethyleher
3- Chloroform
4- Ethyl alcohol (95%)

The powder, after each extraction, is freed from the solvent before the next one; the extract is filtered and the solvent is distilled off under reduced pressure in *vacuo*. The reminder residue is dried to constant weight, screened for physical properties and chromatographic analysis.

Supercritical extraction

This advanced extraction process yields a superior herbal extract that is highly concentrated "supercritical" extraction process.

This extraction process yields a superior herbal extract that is highly concentrated. The supercritical process does not use any chemical solvents at all, so the resulting extract is absolutely free of chemical solvents such as hexane or acetone. The supercritical process also produces an extremely broad representation of the herb's lipophilic (oils, fatty acids, etc.) constituents.

Those lipophilic constituents are often some of the most precious and necessary plant ingredients in such important herbs as ginger, St. John's Wort, evening primrose, saw palmetto, kava, valerian, rosemary, and many others. In the past these herbs were commonly extracted by the use of chemical solvents. Not so with supercritical extraction. Supercritical extracts are super potent, super pure, and broad spectrum with a representative composition very near to the botanical raw material.

The conventional process, then, creates a lipophilic extract that often

1. has some chemical solvent residue,
2. has been temperature stressed,
3. can distort or alter the nature of the delicate plant constituents, or
4. can create an extract that is biologically unstable.

Characters of super critical fluid extract:

No pollution, no heat stress or damage, no solvent residue and virtually complete representation of the plant's lipophilic constituents.

Not every herb or plant constituent is suitable for supercritical extraction, but for those that are it clearly yields the most concentrated, broad-spectrum, and pure extract possible. For some herbal constituents, like for certain phytochemicals in green tea, a water extract is preferred.

Methods of identification and separation of crude drug constituents:

The choice of technique depends on the solubility properties, polarity and volatilities of the compounds to be separated e.g.:

1. **Colour reaction of different classes of secondary metabolites**

   The class of the compound is usually clear from its response to color test: the details of the experimental procedure of the following testes will prescribed in the laboratory section.

   a- Test for glycosides and /or carbohydrates.
   b- Test for glycosidic linkage.
   c- Test for cardiac glycosides (Baljet's test, Kedd's test, Legal's test and Raymond's test).
   d- Test for flavonoids (aglycone, glycosides)
   e- Test for unsaturated sterols and triterpenoids
   f- Test for saponin
   g- Test for oxidase enzyme
   h- Test for anthraquinone (free, glycoside, dimmer).
   i- Test for tannins
   j- Test for alkaloids

2. Solubility

3. UV Spectral characteristics

4. Biochemical test (e.g. presence of glucoside moiety can be confirmed by hydrolysis with glucosidase enzyme).

5. Complete identification within the class depends on measuring other properties which may be:

1. Melting point (for solid)

2. Boiling point (for liquids)
3. Optical rotation (for optically active compounds)
4. R_f (under standard conditions)
5. Spectral characteristics (include UV, IR, NMR and mass spectral measurements) and then comparing between these data with those in the literature.

6. Direct comparison with authentic material (if available), should be carried out as final confirmation.

7. Identification of new compound can be done by X-ray crystallography together with other chromatographic data.

For dealing with mixtures of drugs greater skills and practice are required. When tentative identification has been made, further confirmatory observation and chemical tests can be performed.

Chromatography and Chemical Fingerprints of Herbal Medicines:

It is a technique in which the separation of molecules is based on molecular structure and molecular composition. Two phases are involved in chromatography method. The one is stationary phase and the other is mobile phase. The sample molecules show different interactions with the stationary phase. Thus, the separation is based on the distribution of solute between mobile phase and the stationary phase.

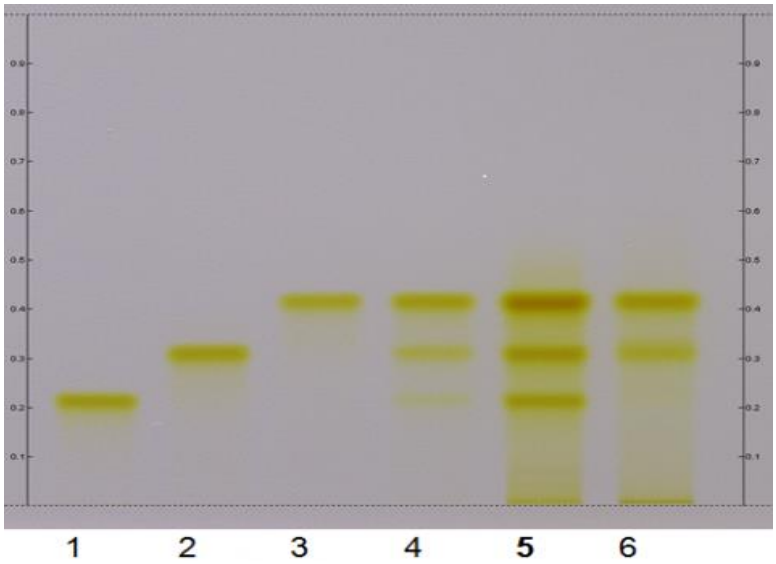
The various types of chromatographic technique are used and the methods are based on the supports which use in separations such as silica gel, glass plates, volatile gases, paper and liquids etc. The different types of chromatographic techniques are given as below.

- **Adsorption Chromatography**
- **Partition Chromatography**
- **Affinity Chromatography**
- **Gas Chromatography**
- **Liquid Chromatography**
- **Column Chromatography**

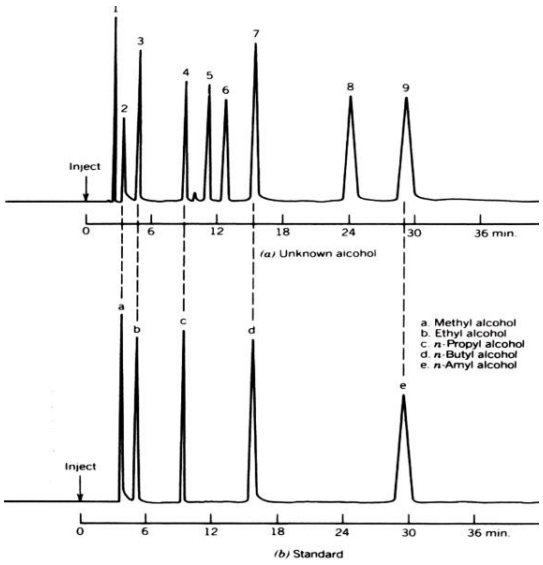
- **Paper Chromatography**
- **Flash Chromatography**
- **Reverse phase Chromatography**
- **Ion exchange Chromatography**

A- TLC fingerprinting

Is of key importance for herbal drugs made up with complex mixtures of constituents. It is a powerful and relatively rapid solution to distinguish between chemical classes, where macroscopy and microscopy will fail. Chromatograms of essential oils, for example, are widely published in the scientific literature, and can be of invaluable help in identification. The instruments for UV-VIS determinations are easy to operate, and validation procedures are straight forward but at the same time precise. Although measurements are made rapidly, sample preparation can be time consuming and works well only for less complex samples, and those compounds with absorbance in the UV-VIS region.



TLC plate

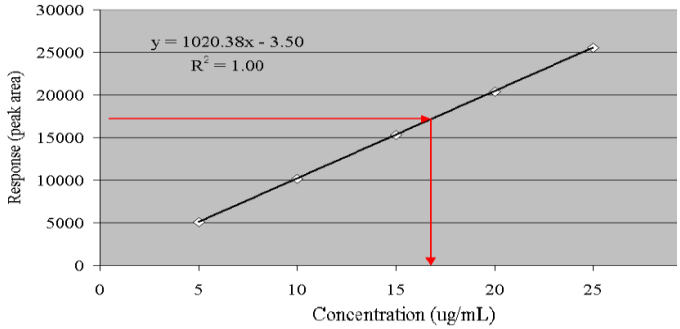


B- Gas Chromatography:

Identification of unknown by retention time in gas chromatography using standard

Computing integrators automatically determine the area under each peak which is proportional to the amount of the analyte present.

Concentration of the eluted compounds can be calculated using a calibration curve created by finding the response for a series of concentrations of analytes, or by determining the response factor of an analyte. In most modern GLC –MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.



Calibration curve peak area *vis*-concentration

C. High-performance liquid chromatography (HPLC).

HPLC can give much improved rapid qualitative and quantitative analysis in particular non-volatile polar compounds. Detection of the often very small quantities of solute in the eluate is possible by continuous monitoring of UV

absorption, mass spectra (LC-MS), refractive index, fluorescence and magnetic resonance can be now be added to this list.

HPLC is the preferred method for quantitative analysis of more complex mixtures. Though the separation of volatile components such as essential and fatty oils can be achieved with HPLC, it is best performed by GC or GC/MS.

D- Hyphenation procedures

The hyphenated technique is developed from the coupling of a separation technique and an on-line spectroscopic detection technology. The remarkable improvements in hyphenated analytical methods over the last two decades have significantly broadened their applications in the analysis of biomaterials, especially natural products. The recent advances in the applications of various hyphenated techniques, e.g.:

- GC-MS,
- LC-MS,
- LC-Fourier-transform infrared (LC-FTIR),
- LC-NMR,
- Capillary electrophoresis (CE)-MS.

The coupling of separation and detection techniques can involve more than one separation or detection techniques, e.g:

- LC-PDA (photodiode array UV- vis absorbance or fluorescence emission)-MS.
- LC-MS-MS.
- LC-NMR-MS.
- LCPDA-NMR-MS.

In the context of pre-isolation analyses of crude extracts or fraction from various natural sources, isolation and on-line detection of natural products, chemotaxonomic studies, chemical fingerprinting, quality control of herbal products, dereplication of natural products, and metabolomic studies could be performed.

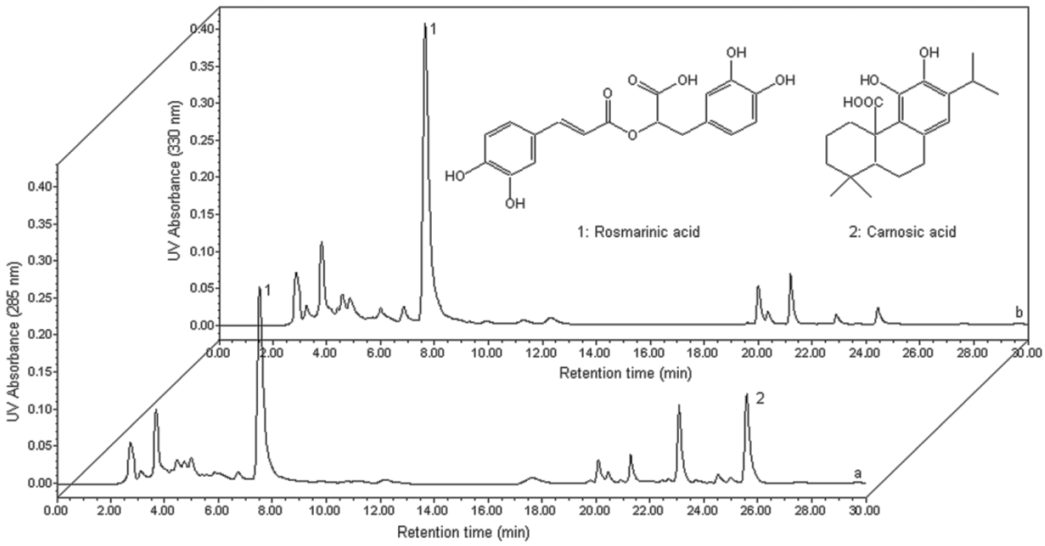
Quantitative evaluation

The quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. Classification of plants and organisms by their chemical constituents is referred to as chemotaxonomy. Qualitative chemical examination is designed to detect and isolate the active ingredient(s). TLC, GC and HPLC (used alone, or in combinations such as GC/MS, LC/MS, and MS/MS) are the main analytical techniques commonly used. In cases when active ingredients are not known or too complex, the quality of plant extracts can be assessed by a “fingerprint” chromatogram. The results from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant or extract.

Based on the concept of photo-equivalence, the chromatographic fingerprints of herbal medicines can be used to address the issue of quality control. Methods based on information theory, similarity estimation, chemical pattern recognition, spectral correlative chromatograms (SCC), multivariate resolution, the combination of chromatographic fingerprints and chemometric evaluation for evaluating fingerprints are all powerful tools for quality control of herbal products.

Profile Chromatograms

A profile chromatogram or, as it is more commonly known, a 'fingerprint' chromatogram, is a chromatographic profile of a botanical raw material, a preparation of a herbal material, or other substance that can be compared with that of a reference sample or standard.



High-performance liquid chromatography (HPLC) profile of target compounds in an plant extract

Validation

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories. Analytical methods need to be validated, verified, or revalidated in the following instances: Before initial use in routine testing When transferred to another laboratory Whenever the conditions or method parameters for which the method has been validated change (for example, an instrument with different characteristics or samples with a different matrix) and the change is outside the original scope of the method. Method validation has received considerable attention in literature from industrial committees and regulatory agencies. This chapter outlines how method validation helps to achieve high quality data.

Validation is the process of proving that an analytical method is acceptable for its intended purpose for pharmaceutical methods. Guidelines from the United States Pharmacopeia (USPC, 1994–2001), the International Conference on

Harmonization (ICH), and the US Food and Drug Administration (FDA) provide a framework for performing such validations.

Validation Characteristics

Although not all of the validation characteristics are applicable for all types of tests, typical validation characteristics are:

• ***Specificity (selectivity):*** "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc. Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength.

• **Linearity**; linearity and calibration curve of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

• **Accuracy**; the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found.

• **Precision;** the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

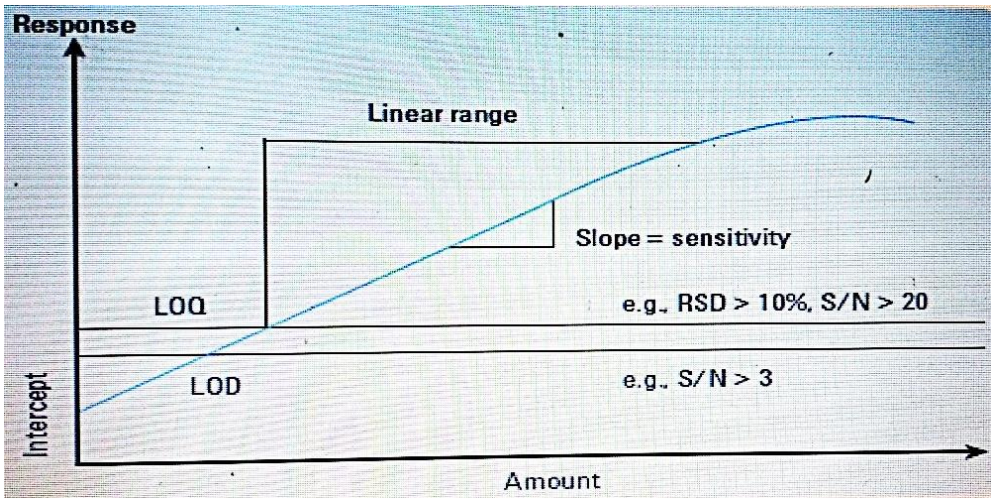
Repeatability expresses the precision under the same operating conditions over a short interval of time.

Intermediate precision expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth (Intermediate precision is determined by comparing the results of a method run within a single laboratory over a number of days.).

Reproducibility expresses the precision between laboratories (The objective of reproducibility is to verify that the method will provide the same results in different laboratories).

• Range;

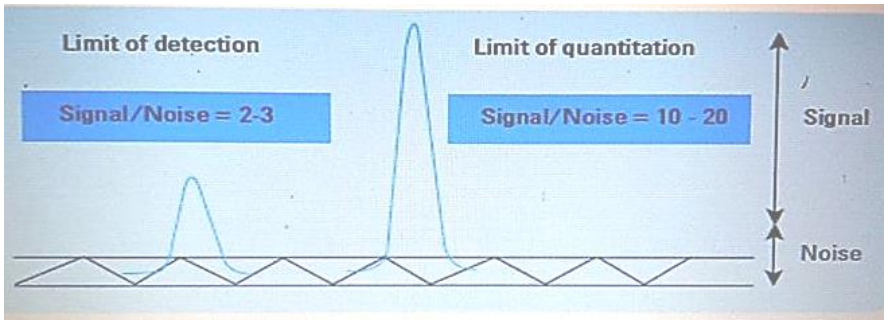
The range of an analytical procedure as the interval from the upper to the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.



Definition for linearity and range

• Quantitation limit;

The limit of quantitation (LOQ) of an individual analytical procedure as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation products.



Limit of detection and limit of quantitation via signal – to- noise

• Detection limit;

The detection limit of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Depending on whether the analytical method used is qualitative or quantitative. Also of utmost importance is the availability of standards. For macroscopic and microscopic procedures in general this means that reliable reference samples of the plant must be available. A defined botanical source (e.g. voucher specimens) will normally solve this problem. Standards for chromatographic procedures are less easy to obtain. Characteristic plant constituents, either active or markers, are seldom available commercially. Sometimes an LC/MS approach can be referred to as a mode of characterization. Going one step further, after isolation of such a compound, elucidations to prove its definite structure will not be easy. The method often employed is to use readily available compounds that behave similarly in the chosen chromatographic systems, and to calculate retention values and/or times towards these compounds as a standard.

Herbal Supplements

A botanical is a plant or part of a plant valued for its medicinal or therapeutic properties, flavor, and/or scent. Herbs are subsets of botanicals. To be classified as a dietary supplement, a botanical must meet the following criteria:

1. It is intended to supplement the diet.
2. It contains one or more dietary ingredients (including amino acids, vitamins, minerals, herbs, or other botanicals, etc.).
3. It is intended to be taken orally as a pill, capsule, tablet, or liquid.
4. It is labeled as being a dietary supplement.

Herbal supplement labeled “Natural” does not mean it is safe or without any harmful effects. Herbal products can act the same way as drugs. **Their safety depends on factors such as:**

- their chemical composition,
- how they work in the body,
- method of preparation,

- Dosage.

In the US, the FDA regulates herbal and other dietary supplements. This means that they have the following:

- do not have to meet the same standards as drugs and over-the-counter medications,
- They are not required to be standardized,
- No legal or regulatory definitions exist for standardization.

As a result, manufacturers are not required to demonstrate the safety and effectiveness of their products before they reach the market. In addition, they do not have to adhere to any of the quality control measures applicable to drugs; hence the composition may vary greatly from one batch to another.

The active ingredient(s) in many herbal supplements are not known, and some have been found to be contaminated with metals, unlabeled prescription drugs, and microorganisms. Under its current regulatory authority, the FDA can remove a herbal supplement from the market only after it has been shown to be unsafe. There has been an increase in the number of Internet websites that sell and promote herbal supplements. Unfortunately, some of them make inaccurate claims and statements regarding their products and claim unsubstantiated effects in curing disease and disease conditions. In the US, distributors of herbal products are under the jurisdiction of the Federal Trade Commission (FTC), which monitors advertising for truthful statements that do not mislead.

Contamination of Herbal Drugs and Herb–Drug Interactions

A recent study found that potent conventional synthetic pharmaceuticals such as synthetic corticosteroids, nonsteroidal anti-inflammatory had been deliberately added to herbal creams in order increase their efficacy. These “adulterated” herbal medicines sometimes result in serious ailments such as acute renal failure.

Many people, especially those living with HIV/AIDS, use both herbal medicines and prescription drugs. A number of clinically significant interactions between prescribed and herbal medicines have been identified. When these medications are used together, they can interact in the body, causing changes in the way the herbs and/or the drug works. Such changes are called herb–drug interactions.

Concurrent use of herbal or homeopathic remedies alongside prescribed or over the counter medicines are frequent, and may:

- *Mimic*,
- *Magnify*,
- *Oppose* the effect of the drug.

Herb–drug interactions are not chemical interactions between a drug and a herbal component to produce something toxic. Instead, the interactions generally cause either an increase or decrease in the amount of drug in the bloodstream. As with conventional medicines, herbal medicines interact with drugs in two general ways:

1. pharmacokinetically
2. and pharmacodynamically.

Pharmacokinetic interactions result in alterations in the absorption, distribution, metabolism, or elimination of the drug or natural medicine. These interactions affect drug action by quantitative alterations, either increasing or decreasing the amount of drug available to have an effect.

Pharmacodynamic interactions cause alterations in the way a drug or natural medicine affects a tissue or organ system. These actions affect drug action in a qualitative way, either through enhancing or antagonizing effects.

Herb–drug interactions change the effectiveness of the treatment, sometimes resulting in potentially dangerous side effects, possibly leading to toxicity, and/or reduced benefits. They can modify the mode of action of the drug, leading to unexpected complications or enhancement of the therapeutic effect, possibly leading to overmedication and an impact on health.

The risk of herb–drug interactions is not limited to synthetic drugs. Herbal supplements and certain foods can interact with medications. Unfortunately very little is known about these interactions and there is little available scientific research on herb–drug interactions. When combining herbal therapies with other medications, it is important to watch for potential symptoms and to inform health care providers. It is essential to train doctors to appreciate that drug interactions exist and to emphasize the importance of the need for physicians and naturopathic doctors to work together.

Until reports of interactions between St John's wort and drugs such as digoxin, warfarin, protease inhibitors and oral contraceptives began to appear, very few herb-drug interactions were documented. Garlic and ginger may increase bleeding, especially in patients already taking certain anti-clotting medications.

Controlled clinical studies are needed to clarify and determine their clinical importance and more research is required to define them.

Toxicity of Herbal Drugs

For several reasons it is not possible to establish absolute safety standards for herbal preparations based solely on epidemiological studies due to:

First, these types of studies would be costly.

Second, there is little published data in countries where the major use of medicinal plants occurs and thus general standards based on a limited number of reports would have little meaning.

Third, the exact identification of the products implicated in side effects claimed for medicinal plants is usually lacking.

In spite of these inadequacies, there are a number of general comments that can be made with regard to avoiding potential serious side effects from herbal medicines.

The definition of "toxic" is ultimately a matter of viewpoint. Traditionally, herbs and herbal products have been considered to be nontoxic and have been used by the general public and traditional medicinal doctors worldwide to treat a range of ailments. The fact that something is natural does not necessarily make it safe or effective. The active ingredients of plant extracts are chemicals that are similar to those in purified medications, and they have the same potential to cause serious adverse effects. Whilst the literature documents severe toxicity resulting from the use of herbs, on many occasions the potential toxicity of herbs and herbal products has not been recognized. In certain countries, such as Taiwan, herbs can be obtained from temples, night markets, street vendors, herbal stores, neighbor hoods, or relatives, and from traditional medicine practitioners. Ordinary people recommend the medicines to others without safety considerations. The general public and many practitioners also believe that the herbs are nontoxic.

Apparently, this cultural style/concept needs more attention in terms of drug safety education. Herbs and herbal preparations can cause:

- Toxic adverse effects,
- Serious allergic reactions,
- Adverse drug interactions,
- Can interfere with laboratory tests.

High-risk patients such as the elderly, expectant mothers, children, those taking several medications for chronic conditions, those with hypertension, depression, high cholesterol or congestive heart failure, should be more cautious in taking herbal medicine. It is axiomatic that pregnancy should be a time of minimal medical intervention, and herbalists in particular regard pregnancy as a "contraindication" to taking herbal medicines.

Two kinds of side effects have been reported for herbal medicines:
The first, considered to be intrinsic to herbal drugs themselves, is mainly related to predictable toxicity due to toxic constituents of the herbal ingredients and overdosage.
The second is allergy. Many cases of allergic reactions have been reported for herbal drugs. It is impossible to completely eliminate the possibility of any substance, including prescription drugs, herbal remedies, or cosmetics, producing an allergic response in people exposed to them.
Based on published reports, the side effects or toxic reactions associated with herbal medicines in any form are rare. This could be due to the fact that herbal medicines are generally safe, that adverse reactions following their use are under reported, or because the nature of the side effects or minor allergic reactions are such that they are not reported.
Perhaps the major problem with regard to the safety of herbal medicines is related to the manufacturing practice, including contamination, substitution, incorrect preparation and dosage, intentional addition of unnatural toxic substances, interactions involving synthetic prescriptions, drugs, and herbal medicines, either intentional or unintentional mislabeling, and the presence of natural toxic contaminants. Many ordinary foods contain constituents that could be regarded as poisonous e.g.
- Alpha gliadin produced by gluten in wheat, oats, and rye,
- the cyanogenic glycosides in many fruit skins and seeds can undergo gastric hydrolysis, resulting in the release of hydrogen cyanide.,
- thiocyanates of the brassica vegetables,
- and lectins of many pulses including soya and red kidney bean.

Viscotoxins, which are constituents of mistletoe, are both cytoxic and cardiotoxic. Nonetheless, these foods are generally regarded as safe. Similarly, both water and

oxygen can kill in excessive amounts! So quantity is often an important consideration.

In this context herbs can be broadly classified into three major categories:
• *The food herbs* - medicines such as peppermint, ginger, garlic, hawthorn, nettles, lemon, and balm are gentle in action, have low toxicity, and are unlikely to cause any adverse response. They can be consumed in substantial quantities over long periods of time without any acute or chronic toxicity. However they may bring about allergic reactions in certain individuals.

• *The medicinal herbs* – these are not daily "tonics" and need to be used with greater knowledge (dosage and rationale for use) for specific conditions (with a medical diagnosis) and usually only for a limited period. They have a greater potential for adverse reactions and in some cases drug interactions. They include aloe vera, black cohosh, comfrey, echinacea, ephedra, ginkgo biloba, ginseng, kava kava, milk thistle, and senna.

• *The poisonous herbs* have a strong potential for either acute or chronic toxicity and should only be prescribed by trained clinicians who understand their toxicology and appropriate use. Fortunately, the vast majority of these herbs are not available to the public and are not sold in health food or herbal stores. Aconite, Arnica spp., *Atropa belladonna*, digitalis, datura are some examples.
There are herbs such as Lobelia and Euonymus spp. that have powerful actions, often causing nausea or vomiting, although they are safe under appropriate conditions. There is also an idiosyncratic grouping of herbs that have been alleged, with some scientific support, to exhibit specific kinds of toxicity. The best known example is the hepatotoxicity of pyrrolizidine alkaloid-containing plants such as Symphytum (comfrey), Dryopteris (male fern), Viscum (mistletoe), and Corynanthe (Yohimbe).

Biological Screening of Herbal Drugs

Once the botanical identity of a herb is established, the next step is phytochemical screening, which involves bioassays, extraction, purification, and characterization of the active constituents of pharmaceutical importance. The herb or herbal drug

preparation in its entirety is regarded as the active substance. These constituents are either of known therapeutic activity or are chemically defined substances or a group of substances generally accepted to contribute substantially to the therapeutic activity of a herbal drug. In any program in which the end product is to be a drug, some type of pharmacological screening, or evaluation, must obviously be done.

Pharmacological screening programs are not without problems. Ideally the active principles should be isolated, preferably using bioassay guided isolation processes, which can be problematic. The ideal pharmacological screen would be to identify those extracts or pure compounds that are highly active and nontoxic. Such a screen is rare to find. Failure to duplicate pharmacological results is another problem. There are many pharmacological screening tests available:

1 - In the random selection program of the National Cancer Institute (NCI) in the US, plants are randomly selected, extracted, and the extracts are evaluated against one or more in vitro tumor systems and in vitro cytotoxicity tests. An extension of this procedure is to isolate metabolites or "active compounds" from the plant that had shown most promising activity and subject them to pharmacological tests.

2 - In another approach, plants containing specific types or classes of chemical compounds, for example alkaloids, are tested. Simple tests such as color reactions are carried out on various parts of the plant in the field, and assays are carried out in the laboratories. In terms of cost–benefit ratio, these "shotgun" approaches are considered to be very unsatisfactory.

3 - Another method involves random collection of plants and subjection of their extracts to several broad screening methods and pharmacological tests. The success of this method depends on the number of samples assayed, adequate funding, and appropriate predictable bioassay protocols. Broad-based empirical screening, which is time consuming and expensive, can detect novel activities but is not suited for screening large numbers of samples.

Diagnosis by observation, a method introduced by the "father" of medicine, Hippocrates, is still one of the most powerful tools of today's physicians. *In vitro* screening methods, though restricted to the detection of defined activities, are simpler and more useful. Recently, biochemical and receptor–ligand binding assays have gathered momentum. This has been made possible by the increasing

availability of human receptors from molecular cloning, and extracts and compounds can be tested for binding directly to the presumed therapeutic target protein. Clone receptors can be expressed in a functional state linked to receptor proteins in cells such as yeast, and this has been made possible by applications of molecular biology. Combined with automated instrumentation and computer databases, hundreds of such assays can be completed in relatively short periods of time. These screening processes are successfully used by international agencies such as the National Cancer Institute (NCI) in the United States and the Central Drug Research Institute in India.

The technology of plant medicinal screening processes has even advanced to enzyme isolation. The enzymes that cause the disease are first isolated and the plant extracts are tested to determine if they block enzyme action. An enzyme immunoassay for the quantification of femtomole quantities of therapeutically important alkaloids has been established. Ethanolic extracts, tinctures, and pure plant compounds from commercially available herbs have been analyzed for their in vitro cytochrome P450 3A4 (CYP3A4) inhibitory capability via a fluorometric microtiter plate assay. These studies indicate that high-throughput screening methods for assessing CYP3A4 inhibition by natural products have important implications for predicting the likelihood of potential herb–drug interactions.

Higher plants contain both mutagens and antimutagens and are susceptible to mutagenesis, but screening programs for the detection of antimutagenesis rarely employ higher plant systems. However, using modified screening tests to detect antimutagenic agents, higher plants have been shown to contain a variety of structurally novel antimutagenic agents. Short-term bacterial and mammalian tissue culture systems are the standard methods employed.

Labeling of herbal products

The quality of consumer information about the product is as important as the finished herbal product. Warnings on the packet or label will help to reduce the risk of inappropriate uses and adverse reactions. The primary source of information on herbal products is the product label. Currently, there is no organization or government body that certifies an herb or a supplement as being labeled correctly.

It has been found that herbal remedy labels often cannot be trusted to reveal what is in the container. Studies of herbal products have shown that consumers have less

than a 50% chance of actually getting what is listed on the label, and published analyses of herbal supplements have found significant differences between what is listed on the label and what is in the bottle. The word “standardized” on a product label is no guarantee of higher product quality, since there is no legal definition of the word “standardized.” Consumers are often left on their own to decide what is safe and effective for them and the lack of consistent labeling on herbal products can be a source of consumer frustration.

Certain information such as:

- the product has been manufactured according to Pharmacopoeia standards,
- listing of active ingredients and amounts,
- directions such as serving quantity (dosage),
- and frequency of intake of the drug, must be included on the labels of all herbal products and packages.

The label should also indicate:

- the method of extraction
- and relative amount of macerate and menstrum used,
- and possible side effects.
- It should indicate that the product’s content has been standardized to contain a particular amount of a specified biochemical constituent. This will also ensure that the correct herb has been used.

In addition to the above information, the label should include:

- the name and origin of the product,
- its intended use,
- net quantity of contents,
- other ingredients such as herbs and amino acids, and additives,
- storage conditions,
- shelf life or expiry date,
- warnings,
- and name and address of manufacturer, packer or distributor.

A herb categorized as a nutritional supplement cannot claim any health benefits or “disease claims” on the label, leaving the consumer with little information.

Marketing plays a big role in the use of herbal products and the media help significantly to provide information about natural health products. One of the

problems with mass media “propaganda” is scientific inconsistency. Unless the packaging contains a medical claim, herbal products are not reviewed by any government agency. FAD that regulate prescription drugs only review a herbal product if the item is suspected of being harmful or if the label contains medical claims. Scientists use several approaches to evaluate botanical dietary supplements for their potential health benefits and safety risks, including their history of use and laboratory studies using cell or animal models. Studies involving people can provide information that is relevant as to how botanical dietary supplements are used.

Policies and Regulations

It is a widely held myth that modern drugs are dangerous foreign chemicals with side effects, while herbals are natural, gentle and safe. The truth is that some herbs can be dangerous and can bring about serious diseases and even lead to death. Unlike conventional drugs, herbal products are not regulated for purity and potency and this could cause adverse effects and can even lead to drug interactions. There are fewer studies on herbal medicines than on conventional drugs, mainly because, unlike synthetic chemicals, herbs cannot be patented, so there is little money to be made by funding such research.

It is important that consumers are made aware of interactions herbs might have with other drugs they are taking. Unfortunately this information is not available with herbals. Herbals are also frequently adulterated with prescription drugs. In certain countries, herbal products used for diagnosis, cure, mitigation, treatment, or prevention of disease are normally treated as drugs, and hence regulated by legislation. However, in most countries, including the United States, such legislation does not exist and in fact, most botanical products are marketed as dietary supplements. Herbal products categorized as nutritional or dietary supplements are not regulated .

In many countries these medicines are not required to pass any regulatory analysis to be sold as health food supplements. It is clear that the herbal industry needs to follow strict guidelines and that regulations are needed. The FDA that regulate prescription drugs only review a herbal product if the item is suspected of being harmful or if the label contains a medical claim. Although research is being done, it is very limited and only a few herbal drugs have been studied adequately by

well-controlled clinical trials. Even though evidence should always be presented to support claims of products, most herbs are still marketed with little or no research. To be registered as drugs, these products need to be tested to prove their safety and clinical efficacy. However, so far, few programs have been established to study the safety and efficacy of herbal medicines as originally proposed in the WHO guidelines for the assessment of herbal medicines. The future of herbal drugs is overshadowed by the pervading lack of regulatory control.

In 1993, the WHO sponsored a symposium on the use of medicinal plants. The result was a standard guideline for the assessment of herbal medicines and a recommendation that governments of the world should:

- protect medicinal plants,
- improve regulation of herbal medicines,
- and respect traditional medicine approaches.

More recently the Health Directorate of Canada developed a new regulatory framework for natural health products, which came into effect in January 2004. Among other things, the new regulations call for:

- improved labeling,
- good manufacturing practices,
- product and site licensing,
- and provision of a full range of health claims that will be supported by evidence.

However, even in Canada, the only regulatory requirements enforced are that all products intended for medicinal use, including natural health products, are issued a Drug Identification Number (DIN). These numbers are not required for raw materials such as bulk herbs.

In the US, access to herbal medicines is restricted by FDA regulations. Before any new chemical or herbal drug is approved, research must prove that it is both safe and effective. As a result of these restrictions, packages of herbal medicines are labeled as food supplements, which do not require pre-approved testing. Food supplements cannot make any healing claims or issue warnings about potential risks. In the US, plant-based derivatives already appear in a quarter of the prescription medicines produced. However, many other plants with healing properties are shunned by the medical community despite scientific data from other countries showing their effectiveness. The misconception that herbs are old fashioned and unscientific has helped to promote a general distrust of phytotherapy. The American Botanical Council contends that, in many cases,

herbal medicines are safer than prescription drugs. According to the Council, herbal medicines react more slowly and often include their own antidotes to counteract any toxic effects. With proper enforcement of regulations, more products that are legitimate will enter the market and the consumers will see justifiable claims on labels. In fact, it is predicted that appropriate regulations will rejuvenate the market in response to growing concerns about the regulatory environment for herbal remedies.

Trends and Developments

The rationalization of the new multidrug and multi-target concept of therapy in classical medicine is likely to have great implications on the future basic research in phytomedicine and evidence-based phytotherapy. It requires concerted cooperation between:

- Phytochemists,
- Molecular biologists,
- Pharmacologists,
- Clinicians,

With the aim of using modern hightech methods for standardization of phytopreparations, of integrating new molecular biological assays into the screening of plant extracts and plant constituents, and of increasing studies on the efficacy proof of phytopreparations using controlled clinical trials. This should be paralleled or followed by pharmacokinetic and bioavailability studies.

One major concern will be the investigation of the multivalent and multi-target actions of plant constituents and standardized extracts, with the aim of rationalizing the therapeutic superiority of many plant extracts over single isolated constituents.

Increased effort in three major research areas will be crucial:

(1) Efforts to develop suitable standardization methods for phytopreparations

(2) The integration of molecular biological assays into the screening of plant extracts, single isolated compounds thereof and phytopreparations; and

(3) The performance of further placebo-controlled, mono- or double- blind, clinical trials, paralleled or followed by pharmacokinetic and bioavailability studies.

Herbs are still marketed without sufficient research but evidence must always be shown to consumers to support claims of products. More clinical studies are needed and doctors, along with other professionals, should work towards on untangling this herbal maze. Standards should be developed for each natural health product and the same regulatory standards that apply to manufactured pharmaceuticals should apply equally to herbal products as well. Unlike conventional drugs, herbal products are not regulated for purity and potency and this could cause adverse effects and drug interactions. Herbal manufacturing processes should be refined in order to improve the purity, safety and quality of products and the herbal industry needs to follow strict guidelines, for herbal products are now classified as medicines. Manufacturers and producers tend to resist these laws because such laws will increase cost, which will have to be passed on to consumers, and thus the appeal or herbal drugs might then be lost. The media help significantly to provide information about natural health products to consumers. One of the biggest problems with many mass media stories today is scientific inconsistency. With proper enforcement of regulations, more products that are legitimate will come to the market and the consumer will see justifiable claims on labels and these regulations will rejuvenate the market. Herbal medicines still have value because they have a long history.

Finally, it is sometimes asked whether natural health food stores require legislation?. The answer should be yes.

Promoting herbal products for medical conditions should be regulated in a similar fashion to shops that dispense pharmaceutical products.

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