Experiment 3

- Separation of sugars by paper chromatography
- TLC of herbal extract
- Distillation of volatile oils and detection of phytoconstituents by TLC

PAPER CHROMATOGRAPHY

INTRODUCTION

Chromatography is a separation technique, in which the separation of a mixture of components into the individuals using stationary phase and mobile phase. The sugars are separated using paper chromatography. Paper chromatography is partition chromatography, in which the mobile phase and stationary phase are liquid. The mixture of compounds is separated based on their partition coefficient. The components have more soluble in the stationary phase travel slower and components, which are more soluble in the mobile phase, travel faster. Thus, compounds are separated because of their differences in partition coefficient. No two components have the same partition coefficient for the combination of stationary phase, mobile phase, and other conditions. In paper chromatography, the cellulose layer on the filter paper contains moisture, which acts as a stationary phase. The organic solvents or buffers are utilized as mobile phases.

Preparation of Solution

Standard solutions: Prepare the aqueous solution of any three of the following by dissolving in water at a concentration of 20–50 μg/ml of each sugar. The sugars are: D-glucose, D-fructose, D-xylose, L-rhamnose, D-galactose, Lactose, maltose, sucrose, D-mannose.

Preparation of mixture solution of sugars: Add a few drops of each sample sugar solution in a dry test tube.

Sample solution: The plant material or fruit juice is ground and extracted with a suitable solvent to concentrate the sugars. These sugars are diluted or concentrated or applied directly.

Procedure

- Prepare the mobile phase selected for the analysis and transfer in the developing chamber, cover the led and allow the chamber to be saturated with the mobile phase.

- Cut the sheet of Whatman 1 chromatography paper in the proper size, remark the solute application line about 1.5 cm from the lower edge of the paper with the help of a pencil.
- Mark the line at equal distance on the solute application line based on the number of standards and samples.
- Label the paper at the top with the name of each of the sugars and label the last unknown.
- Use a fine capillary to place the drops of the solutions of the sugars, glucose, fructose, maltose, lactose, and the mixture.
- After spotting, dry the paper with a hot air dryer for one minute, repeat this step.
- Place the spotted paper in the chromatographic tank and make the development by using the ascending technique.
- Close the tank with a lid, allow the solvent to flow for about 30–45 minutes.
- Remove the paper and immediately mark the position of the solvent front with a pencil.
- After the chromatogram has dried, spray the paper with the detecting reagent.
- Circle the position of each spot with a pencil.
- Calculate the Rf value for each spot and also for the spots the mixture contained.

Chromatographic Conditions

Mobile Phase Solvents

- *n*-butanol: acetic acid: water (4:1:5 v/v)
- Isopropanol: pyridine: water: acetic acid (8:8:4:1 v/v)

Spray Reagent

Resorcinol reagent: 1% ethanolic solution of resorcinol and 0.2N HCl (1:1 v/v) visualize spots by heating at 90°C.

Formulae

R_f value = Distance traveled by solute/distance traveled by the solvent

Sugar	**R_f values**	
	Mobile phase: *n*-butanol: acetic acid: water (4:1:5 v/v)	**Mobile phase** Isopropanol: pyridine: water: acetic acid (8:8:4:1 v/v)
Glucose	0.64	0.18
Galactose	0.62	0.16
Fructose	0.68	0.25
Ribose	0.76	0.31
Lactose	0.46	0.09
Maltose	0.50	0.11
Sucrose	0.62	0.14

THIN LAYER CHROMATOGRAPHY

INTRODUCTION

Thin layer chromatography (TLC) is a method for identifying substances and testing the purity of compounds. TLC is a useful technique because it is relatively quick and requires small quantities of material. Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.

Principle

- TLC has been included under both adsorption and partition.
- Separation may result due to adsorption or partition or by both phenomena depending upon the nature of adsorbents used on plates and solvent system used for development.
- Thin-layer chromatography (TLC) is a chromatography technique used to separate mixtures.
- Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose (blotter paper).
- This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Procedure

- Prepare the TLC plate and keep it for saturation.
- Make the extract of the powdered drug and spot it on the saturated TLC plate.
- Make the solvent system and let it saturate for half an hour. The run the solvent system till ¾ that of the plate.
- Dry the plate on the hot plate and then spray it with the spraying agent.
- Let it get air-dried and mark the spot and trace the spot on the tracing paper.

R_f value = Distance traveled by solute /distance traveled by the solvent

Cautions

Do not misuse the chemicals and keep the plate properly in the solvent chamber.

Thin-layer chromatography can be used to:

- Monitor the progress of a reaction
- Identify compounds present in a given substance
- Determine the purity of a substance

DISTILLATION OF VOLATILE OILS AND DETECTION OF PHYTOCONSTITUENTS BY TLC

INTRODUCTION

Generally, volatile oils are extracted from the plant material by four methods, i.e. distillation, steam distillation, expression, and enfleurage. Most oils are distilled in a

single process within 2–5 hours, while a few of them require a second step to purify through fractional distillation. Solvent extraction 'is one of the most promising methods of essential oil extraction but recently newer techniques like microwave-assisted or supercritical fluid extraction (SCF) became more popular. Microwave extraction is a mild and controllable processing tool that allows oil extraction with or without solvent or even dry extraction within a very short time. The use of SCF inessential oil extraction has gained much more attention recently due to the high selectivity in separation, nontoxic residue, and thermal degradation which produces the highest quality essential oil than conventional distillation methods. Many factors are responsible for the quality of distilled essential oils like time, temperature, pressure, and distillation equipment. Essential oils are composed of fairly delicate components which can be altered or destroyed or their activity lost by improper methods of extractions.

Method for Extraction Volatile Oil

- Select the appropriate clevenger apparatus for volatile oils having density either higher than or lower than water.
- Take the accurately weighed quantity of fresh or dried plant material consisting of the flowers, leaves, wood, bark, roots, seeds, or peel (e. g. eucalyptus leaves, lemongrass leaves, clove buds, etc).
- Reduce the material to coarse-sized particles.
- Place the material in the RBF of the Clevenger apparatus.
- Add a sufficient quantity of water. Attach the assembly assign the figure.

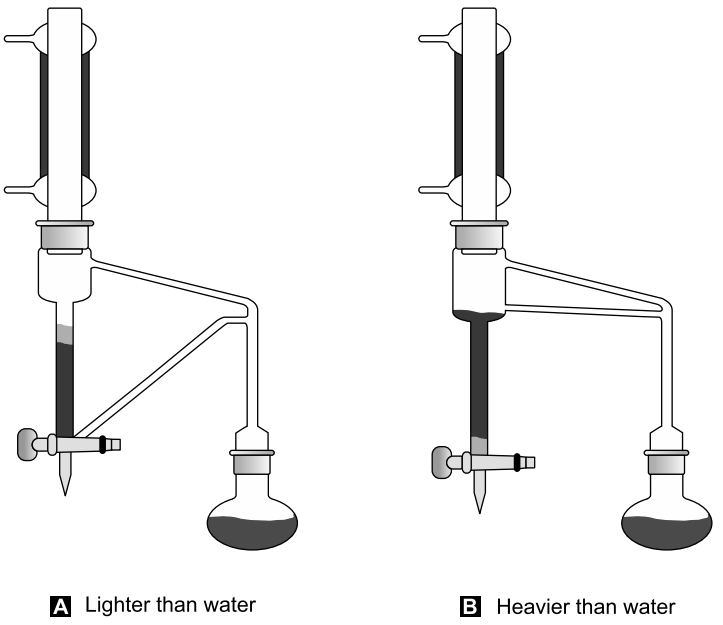

Fig. 3.1: Clevenger apparatus for volatile oil determination

- Heat the mixture for 1 hr. vapors of both water and volatile oil will pass towards the condenser and then the collector tube.
- Allow the mixture to cool and separate.
- Read the volume of oil directly from the graduated tube.

Calculation

- Weight of plant taken (gm) (a)
- Weight of volatile oil obtained (ml) (b)
- % yield = (b×100)/a

Detection of Phytoconstituents by TLC

1. Prepare a suspension of the silica gel G in distilled water; prepare the plate either of spreading, pouring, dipping method. Allow the coated plates to dry in the air, heat at 100° to 105° for at least 1 hour, and allow cooling
2. Allow the saturate the developing chamber with the vapors of solvents of mobile phase by keeping this chamber aside for 30 min
3. Apply the spot on the activated silica gel plate using capillaries and mark it with a pencil
4. Place this plate into the developing chamber and put the lid on it
5. Allow to run the mobile phase on the silica gel plate (stationary phase) to its 80% the height
6. Mark the level of solvent front immediately after removing the plate from the developing chamber
7. Dry the plate to room temperature and observe the plate either in the iodine chamber of the UV chamber at 254 nm or 366 nm.

R_f Value

Measure and record the distance of each spot from the point of its application and calculate the Rf. value by dividing the distance traveled by the spots by the distance traveled by the front of the mobile phase.

Eugenol

Experimental condition for TLC

- **Stationary phase:** Silica gel GF254
- **Mobile phase:** Toluene (100)
- **Visualizing agent:** Vanillin sulphuric acid
- *Phytoconstituents identification*
  - Eugenol—Rf. 0.4; orange-brown
  - Aceteugenol—pinkish-yellow below eugenol.

Thymol

- Stationary phase: Silica gel GF254
- Mobile phase: Toluene: ethyl acetate (3:1)
- Visualizing agent: Anisaldehyde sulphuric acid

Phytoconstituents identification

- Thymol–R_f. 0.72; pink

Experiment 4

Analysis of crude drugs by chemical tests:

- Asafoetida
- Benzoin
- Colophony
- Aloes
- Myrrh

ASAFOETIDA

Biological source: It is the oleo gum resin obtained from roots and rhizomes of *Ferula foetida*.

Fam: Umbelliferae

Physical Characteristics

- *Appearance:* Crystalline
- *Color:* Yellowish-brown
- *Odor:* Intense penetrating and alliaceous odor
- *Shape:* Tear and masses
- *Taste:* Bitter acrid and alliaceous taste
- *Solubility:* Partially soluble in alcohol

Chemical Constituents

- The main constituents are volatile oil (10–17%), resin (40–65%) and gum (1.5–10%).
- The resin consists of resene (asaresene A) and volatile oil.
- It also contains 1.5 percent of free ferulic acid and 16 percent of unstable ester of ferulic acid with asaresinol.
- The volatile oil contains pinene and various disulfides (C7H14S2, C11H20S2, C10H16S2).
- Ferulic acid yields umbellic acid, when it is treated with hydrochloric acid, loses water molecules and forms umbelliferone.
- Free umbelliferone is absent in asafoetida.

Chemical Test (Table 4.1)

Table 4.1: Chemical test for asafoetida

Sr. No.	*Test*	*Observation*	*Inference*
1.	Triturate with water	Yellowish orange Emulsion	Asafoetida present
2.	Treat the fractured surface with 50% HNO_3	Green color	Asafoetida present
3.	Treat the fractured surface with conc.H_2SO_4	Red or reddish-brown color	Asafoetida present
4.	Combined umbelliferone test: Triturate 0.5 g of drug + sand + 5 ml HCl, add 2 ml water, filter, filtrate + equal volume of NH_3	Blue fluorescence (umbelliferone)	Asafoetida present
5.	Alcoholic extract + phloroglucinol and conc HCL	Pink color	Asafoetida present
6.	On burning	Yellow flame	Asafoetida present

Uses

- Carminatives
- Antispasmodic
- It is a powerful nervine tonic
- Used in the treatment of hysteria, bowel stimulant, expel flatulence, relieves constipation and flavoring agent.

BENZOIN

Biological source: It is a balsamic resin obtained from the incision on the stem of *Styrax benzoin.*

Fam: Styraceae

Physical Characteristics

- *Appearance:* Crystalline
- *Color:* Greyish brown
- *Odor:* Aromatic and characteristics, balsamic
- *Shape:* Tear
- *Taste:* Sweet and slightly acrid

Chemical Constituents

- It should contain not less than 25% of total balsamic acid concerning the dry alcohol-soluble matter.
- Sumatra benzoin contains free balsamic acid 25% (benzoic acid and cinnamic acid (20%)) and ester derived from them.
- Triterpenoids acid such as sumaresinolic acid and sia recinolic acid are also present.
- The major constituents of Siam benzoin (less amount of cinnamic acid) is an ester coniferyl benzoate (about 76%)
- The drug also contains styrol, vanillin, and phenyl propyl cinnamate.

Chemical Test (Table 4.2)

Table 4.2: Chemical test for benzoin

Sr. No.	*Test*	*Observation*	*Inference*
1.	Heat 0.5 g of benzoin in a dry test tube	Evolve white fumes which condense to form white crystalline sublimate in the upper part of the tube.	Benzoin present
2.	Alcoholic solution of sample + water	The white milky solution formed	Benzoin present
3.	Alcoholic solution of a sample of litmus treat with litmus paper	Acidic to litmus paper.	Benzoin present
4.	Heat 0.5 g with 10 ml solution of potassium permanganate + 1 drop of conc. Sulphuric acid	The bitter almond smell of benzaldehyde	Sumatra benzoin
5.	2.5 g drug + 10 ml ether shake and warm. pour 2 to 3 ml of this extract in a porcelain dish containing 2 to 3 drops conc. sulphuric acid	The deep reddish-brown color is produced in a porcelain dish. Purple-red color	Sumatra Siam benzoin
6.	Triturate drug with alcohol, filter, filtrate + ferric chloride solution (alcoholic)	Bright green color	Siyam benzoin
7.	Heat a small quantity of benzoin in the test tube cover the opening of the test tube with a glass slide cool the content and examine the slide under a microscope	Crystals of cinnamic acid are observed	Benzoin conformed

Uses

- Antiseptic
- Expectorant
- Carminative
- Stimulant
- Diuretics

COLOPHONY

Biological source: Colophony is a solid residue left after distilling off the volatile oil from the oleoresin obtained from *Pinus palustris* (longleaf pine) and other species of *Pinus* such as *P. pinaster, P. halepensis, P. massoniana, P. tabuliformis, P. carribacea* var., belonging to family: Pinaceae.

Organoleptic Characters

- *Color:* Amber colored or sometimes, yellowish to yellowish-brown.
- *Odor:* Faint.
- *Taste:* Angular, translucent masses.
- *Size:* Varies in size.
- *Appearance:* Glossy appearance with brittle nature.

It has a solubility in alcohol, chloroform, ether, fixed oil, essential oils, light petroleum, and glacial acetic acid but is insoluble in water.

Chemical constituents: It mainly contains unsaturated resin acids principally

- Abietic acid (nearly 90 percent).
- Esters of oleic acids.
- Volatile oil (0.5 percent).
- Resines (5 to 6 percent).
- Sipinic acid.
- Pimaric acid, etc.

Chemical Tests (Table 4.3)

Table 4.3: Chemical test for colophony

Sr. No.	*Test*	*Observation*	*Inference*
1.	To a solution of powdered resin (0.1 g) in acetic acid (10 ml) one drop of conc. sulphuric acid is added in a dry test tube	A purple color, readily changing to violet, is formed	Colophony present
2.	To a petroleum ether solution of powdered colophony twice its volume of dilute solution of copper acetate is shaken	The color of the petroleum ether layer changes to emerald-green due to formation of copper salt of abietic acid	Colophony present
3.	To alcoholic solution of colophony sufficient water is added	It becomes milky white due to precipitation of chemical compounds	Colophony present
4.	Alcoholic solution of colophony	Turns blue litmus to red due to the presence of diterpenic acids	Colophony present

Uses

- Stiffening agent in ointments.
- Adhesives, plasters and cerates.
- Diuretic in veterinary medicine.
- Commercially it is used to manufacture varnishes, printing inks, cement, soap, sealing wax, wood polishes, floor coverings, paper, plastics, fireworks, tree wax, rosin oil, and waterproofing cardboard.
- The abietic acid has antimicrobial, antiulcer, and cardiovascular activity.

ALOES

Biological source: Aloe is the dried juice collected by incision, from the bases of the leaves of various species of Aloe.

- *Aloe barbadensis:* Miller (or **Curacao Aloe**);
- *Aloe ferox:* Miller (or **Cape Aloe**);
- *Aloe perryi:* Baker (or **Socotrine Aloe**);
- *Aloe africana:* Miller and A*loe spicata* Baker (or **Cape Aloe**).

All these species belong to the family: *Liliaceae.*

Four important varieties

1. Curacao or barbados aloe
2. Socotrine aloe
3. Zanzibar aloe
4. Cape aloe

Organoleptic Characters

Color: Bright yellow-ish or rich reddish-brown to black. Sometimes, it is vitreous and small fragments are then of a deep garnet-red color and transparent.

- *Taste:* Aloes possesses the nauseous and bitter taste that is characteristic of all aloes
- *Odor:* Disagreeable, penetrating odor.

It is almost entirely soluble in 60% alcohol and contains not more than 30% of substances insoluble in water and 12% of moisture.

Chemical Constituents

- The most important constituents of aloes are the three isomers of aloins, barbaloin, β-barbaloin and isobarbaloin
- Other constituents are amorphous aloin, resin, emodin and aloe-emodin.
- Barbaloin is present in all varieties; it is slightly yellow-colored, bitter, water-soluble, crystalline glycoside.
- Isobarbaloin is a crystalline substance, present in curacao aloe and trace amount in cape aloe and absent in socotrine and zanzibar aloe.
- The chief constituents of socotrine and zanzibar aloe are barbaloin and β-barbaloin.

Chemical Tests (Table 4.4)

Boil 1 gm of drug with 100 ml water, allow it to cool; add 1 gm kieselguhr, stir it well and filter through filter paper.

Table 4.4: Chemical test for aloe

Sr. No.	*Test*	*Observation*	*Inference*
1.	*Borax Test:* Take 10 ml of aloe solution and to it add 0.5 gm of borax and heat	A green-colored fluorescence is produced	Presence of aloe-emodin anthranol
2.	*Modified anthraquinone Test:* To 0.1 gm of the drug, 5 ml of 5% solution of ferric chloride is added followed by the addition of 5 ml dilute hydrochloric acid. The mixture is heated on water bath for 5–6 min and cooled. An organic solvent (benzene or chloroform) is added and shaken. Separate the organic solvent layer and add an equal volume of dilute ammonia	The ammoniacal layer produces a pinkish red color	Aloe confirmed
3.	*Bromine test:* To 5 ml of aloe solution, add an equal volume of bromine solution	A bulky yellow precipitate is formed due to the presence of tetrabromaloin	Aloe confirmed
4.	*Nitrous acid test:* To 5 ml of aloe solution, add a little sodium nitrite and a few drops of dilute acetic acid	It produces pink or purplish color	Zanzibar and Socotrine aloes give negative test.

Contd.

Table 4.4: Chemical test for aloe

Sr. No.	*Test*	*Observation*	*Inference*
5.	*Nitric acid test:* 2 ml of concentrated nitric acid is added to 5 ml of aloe solution	Curacao aloe gives deep reddish-brown color, Socotrine aloe gives pale yellowish-brown color, Zanzibar aloe gives yellowish-brown color and cape aloe first produces brown color which on standing changes to green	Aloe confirmed
6.	*Cupraloin test:* 1 ml of the aloe solution is diluted to 5 ml with water and to it, 1 drop of copper sulfate solution is added. Bright yellow color is produced which on the addition of 10 drops of a saturated solution of sodium chloride changes to purple and the color persist if 15–20 drops of 90% alcohol is added	This test is positive for Curocao aloe, faint for Cape aloe, and negative for Zanzibar and Socotrine aloes	Aloe confirmed

Uses

- Stimulating purgatives,
- Abortifacient.
- Emmenagogue,
- Emollient,
- Stimulant, stomachic, tonic, and vulnerary.
- Antibacterial activity.

MYRRH

Biological Source

Myrrh is an oleo-gum-resin obtained from the stem of *Commiphora molmol* Eng. or *C. abyssinica* or other species of Commiphora, belonging to the family: Burseraceae.

Organoleptic Properties

Color: Reddish-brown.

- *Odor:* Aromatic
- *Taste:* Aromatic, bitter, and acrid.

Chemical Constituents

- Myrrh contains resin (25–40%), gum (57–61%), and volatile oil (7–17%).
- A large portion of the resin is ether-soluble containing α-, β-, and γ-commiphoric acids, resenes, the esters of another resin acid and two phenolic compounds.

- The volatile oil is a mixture of cuminic aldehyde, eugenol, cresol, pinene, limonene, dipentene, and two sesquiterpenes.
- The disagreeable odor of the oil is due to mainly the disulfide.
- The gum contains proteins (18%) and carbohydrates (64%) which is a mixture of galactose, arabinose, glucuronic acid, and an oxidase enzyme.

Chemical Tests (Table 4.5)

Table 4.5: Chemical test for myrrh

Sr. No.	*Test*	*Observation*	*Inference*
1.	When myrrh is triturated with water	A yellowish-brown emulsion is obtained	Myrrh present
2.	An ethereal solution of the drug is treated with bromine vapors When moistened with nitric acid	Yellow color Purple color	Myrrh present

Uses

- Carminative
- Antiseptic.
- Stimulant.
- Used in mouth wash and toothpaste.