

Aim of the experiment

Systematic extraction, isolation, purification and identification of **Basic poison** (Caffeine and Lorazepam) by various analytical techniques.

Requirements

- a) **Test sample:** Food curry (divided in two parts) spiked with caffeine and lorazepam.
- b) **Materials:** Beaker(s), capillary tube, filter paper sheet, separating funnel, measuring cylinder, glass rod, conical flask, tripod, TLC plate, TLC chamber, china dish.
- c) **Instrument:** Hot plate, UV chamber.
- d) **Chemicals:** Silica gel-G, chloroform, ammonia, acetone, potassium iodide, hydrochloric acid, ammonium sulphate, methanol, tannic acid, iodide, sodium hydroxide, dioxane, benzene, and ammonium hydroxide.

Theory and Principle

- a. Thin layer chromatography, or TLC, is a method for analysing mixtures by separating the compounds in the mixture. 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 owing to affinity with stationary phase, separation is achieved. The R_f value is used to quantify the movement of the materials along the plate. R_f is equal to the distance travelled by the substance divided by the distance travelled by the solvent. Its value is always between zero and one. The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions such as solvent system; adsorbent; thickness of the adsorbent; amount of material spotted and temperature are also constant.
- b. Beer- Lambert's law: According to this law, when a monochromatic light passes through a transparent solution, the rate of decrease in the intensity of transmitted light w.r.t. thickness and concentration of solution is directly proportional to the frequency of incident light.

$$A = \log I_0/I_t = Ecl$$

Where, c = concentration of solution; l = thickness of solution, E = constant; A = Absorbance

Procedure

1. **Extraction, isolation and purification of caffeine and lorazepam from sample:**

- a) Add 50 ml of hydrochloric acid (for digestion of sample) to the sample matrix in beaker along with equal amount of water.
- b) Then keep the beaker(s) on hot plate to heat for 20-30 minutes (or till digestion is completed).
- c) During heating hydrochloric acid can be added if loss of volume is observed.
- d) Allow the beaker to cool. Further, digested sample to be filtered.
- e) Measure the pH and if acidic, add ammonia to maintain the pH.
- f) Extract the filtrate twice extracted using chloroform as a solvent in a separating funnel.
- g) Keep the separating funnel in stand on tripod for five- ten times. chloroform to be collected and discard the aqueous layer.
- h) Use ammonium sulfate for purification.
- i) Evaporate the extract on hot plate to obtain a dry residue then reconstitute using methanol for TLC and UV analysis.

2. Colour Test

Sl. No	Test for Caffeine	Observation	Inference
1.	10 mg of extracted sample to be taken in china dish + 1 ml of hydrochloric acid + 0.1 gm of potassium perchlorate. Evaporate to dryness on water bath. a. Exposure the residue to dil. Ammonium solution. b. Saturated solution of caffeine + tannic acid c. 5 ml of saturated solution + 1.5 ml of 0.5 gm of iodine + few drops of dilute hydrochloric acid	Purple color will appear. Disappear on addition of any alkali. White precipitate forms. Clear solution. Brown precipitate will appear which is soluble in sodium hydroxide	Caffeine may be present.

3. Thin layer Chromatography: -

Stationary Phase: Silica Gel-G

Mobile phase for caffeine: Acetone: Water: Ammonia (90:7:3)

Mobile phase for Lorazepam: Dioxane: Benzene: Ammonium Hydroxide (20 : 75 : 5)

Preparation and Activation of TLC plates

- a. The glass TLC plates should be cleaned with water and then air-dried.
- b. The silica gel slurry is prepared by mixing a sufficient amount of silica gel-G with distilled water in the ratio of 2:1 while stirring it slowly with a glass rod, till the desired consistency (with homogenous nature) is achieved.
- c. Pour the slurry onto the glass slide from one side and spread the slurry evenly to obtain uniform thickness. Let the plates air dry.
- d. Before an hour or two of experiment TLC plates keep the plates for activation by placing in a thermostatically controlled oven at 110°C for a duration of 60 minutes.
- e. TLC plate is observed for non-uniformity or if any cracks develop owing to over activation, such plates must be discarded.

Preparation of mobile phase

In TLC tank / TLC development chamber, 100 ml solvent system to be prepared by mixing Acetone: Water: Ammonia in 90:7:3 ratio for caffeine and Dioxane : Benzene : Ammonium Hydroxide in a ratio of 20 : 75 : 5 for lorazepam .

Chamber should be covered with a lid and stand for at-least 30 minutes for the saturation of chamber such that equilibrium is obtained before placing the TLC plate for development.

Spotting of sample

- a. Spotting is done using a capillary tube immersed into the sample solution such that liquid is drawn into the capillary.
- b. Very gently the end of the capillary tube is pressed on the prepared TLC plate at a distance of 2 cm from the base. Spotting was done carefully to avoid tailing of sample as well as contaminating the adjacent sample. The spot to be air-dry and repeated spotting directly over the original spot, if needed.
- c. Two spots to be done; one the extracted sample and other, standard in methanol.

TLC plate development

- a. Before placing the TLC plate in the developing chamber, double check was done such that the solvent level is below the sample spot. The sides of the plate did not touch the jar.

- b. Place the plate vertically in the developing jar with the top end leaning against the jar.
- c. The jar is sealed and allow the solvent to run to rise until 2-3 cm from the top of the plate.
- d. Take out the plate from the tank and mark immediately for the solvent front with a pencil. Allow the plate to dry.

Visualization of sample

The plate is sprayed with potassium iodide for caffeine and dragendroffs reagent for lorazepam to view the spots.

4. UV-Visible spectroscopy: -

- a) Instrument is switched on and allow it to stand for 20 min for warm-up/ check parameters.
- b) Cuvettes should be rinsed with methanol and the baseline run is produced using methanol in both reference and sample cell covered with lid.
- c) The extracted sample and standard reconstituted in methanol are analyzed in spectra mode.
- d) Cuvettes were rinsed using methanol, further with distilled water and placed in case. Shut down the instrument to printing of spectra.

Observation and Calculation

1. Thin Layer Chromatography

Rf value of Caffeine (extracted sample):

Distance travelled by solute (x) =

Distance travelled by solvent (y)=

$Rf\ value = x / y =$

Rf value of Lorazepam standard):

Distance travelled by solute =

Distance travelled by solvent =

$Rf\ value = x / y =$

2. UV-Visible spectroscopy

The spectra show peaks at _____, which are prominent to Lorazepam

The spectra show peak at _____, which is prominent for caffeine.

Conclusion: Thus, on the basis of observations from color test, TLC and UV analysis, it can be concluded that the test sample is _____ belonging to CNS stimulant and CNS depressant (benzodiazepine) class of basic poison.