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DEPARTMENT OF BIOINFORMATICS

CERTIFICATE

This is to certify that Mr. **Shalmon N Anandas** of M.Sc. Part I Bioinformatics has satisfactorily completed the practical semester I course prescribed by the university of Mumbai during the academic year 2021-2022

TEACHER IN CHARGE **HEAD OF DEPARTMENT**

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Date: 27-10-2021

AIM: To separate plant pigments using paper chromatography.

INTRODUCTION:

Chromatography is a separation technique which was first invented by M. Tswett, a botanist in 1906 in Warsaw. In that year, he successfully performed the separation of chlorophyll, xanthophyll and several other coloured substances by percolating vegetable extracts through a column of calcium carbonate. the calcium carbonate column functioned as an adsorbent and the different substances got adsorbed to different extent and this gives rise to coloured bands at different positions on the column. Tswett termed this system of coloured bands as the chromatogram and the method as chromatography after the Greek words chroma and graphs meaning “colour” and “writing” respectively. It is a non-destructive procedure for resolving a multi-component mixture of trace, minor, or major constituents into its individual fractions. Different variations may be applied to solids, liquids, and gases.

Chromatography may be regarded as a method of separation in which separation of solutes occur between a stationary phase and a mobile phase. Essentially, the technique of chromatography is based on the differences in the rate at which the components of a mixture move through a porous medium (called stationary phase) under the influence of some solvent or gas (called moving phase). Paper chromatography is a type of a planar chromatography whereby chromatography procedures are run on a specialized paper. It is the simplest and most widely used of the chromatographic techniques because of its applicability to isolation, identification and quantitative determination of organic and inorganic compounds.

German scientist Christian Friedrich Schonbein (1865) first introduced it. In paper chromatography, the stationary phase is a special quality paper called chromatography paper. Mobile phase is a solvent or a mixture of solvents. A solution of the mixture is spotted on a line about 1 cm above from the bottom of the paper, called original line or base line and then suspended in a chromatography chamber containing suitable solvent. The solvent rises the paper by capillary action and flows over the spot. The paper selectively retains different components according to their differing partition in the two phases. The paper strip so developed is called Chromatogram. The spots of the separated coloured compounds are visible at different heights from the position of initial spot on the chromatogram. The distance travelled by the solvent from the original line is called solvent front. The relative adsorption of each component of the mixture is expressed in terms of its Retardation factor (Rf) (Retention factor). Photosynthetic plants convert light energy from the sun to chemical food energy. During photosynthesis, molecules referred to as pigments are used to capture light energy. Pigments are chemical compounds which reflect only certain wavelengths of visible light. Plant leaves contain four primary pigments: chlorophyll a (dark green), chlorophyll b (light green), xanthophylls (pale yellow) and carotenoids (dark yellow).

PRINCIPLE:

This technique is a type of partition Chromatography in which the substance is distributed between two liquids i.e., one is stationary liquid which is held in the fibres of the paper and called stationary phase and other is the moving liquid or developing solvent and called the moving phase. The components of the mixture to be separated migrate at different rates and appear as sports at different point on the paper.

In this technique a drop of the test solution is applied as a small spot on a filter paper and the post is dried. The paper is kept in close chamber and the edge of paper is dipped into a solvent i.e., the mobile phase. As the filter paper gets the liquid through its capillary axis and when it reaches the spot of the test solution, the various substances are moved by solvent system at various appends. When reached or travelled to a suitable length the paper is dried and spot are visualised by suitable reagents called visualising reagents. The movement of substance relative to the solvent is expressed in terms of RF values i.e., migration parameters. The Rf value is defined as the ratio of the distance moved by the solute (i.e., the dye or pigment under test) and the distance moved by the solvent (known as the Solvent front) along the paper, where both distances are measured from the common Origin or Application Baseline, that is the point where the sample is initially spotted on the paper.

$$Rf = \frac{\text{Distance travelled by the solute from the point of application}}{\text{Distance travelled by the solvent}}$$

Due the fact that the solvent front is always larger from the distance travelled by the solute, Rf values are always between 0 - one extreme where solute remains fixed at its origin and 1 – the other extreme where the solute is so soluble that it moves as far as the solvent. Rf values do not have units since it is a ration of distances.

REQUIREMENTS:

1. Sample: Given fresh vegetable leaves (Spinach leaves)

2. Chemicals:

Magnesium Carbonate – (Pinch)

Acetone – one drop

Petroleum Ether – (16 ml)

Diethyl ether – (4 ml)

3. Glassware:

Clean and dry glass pipette – 1 ml

Clean and dry glass pipette – 10 ml

Clean and dry glass beaker – 250 ml *4

Clean and dry development jar

4. Miscellaneous: Whatman Filter Paper (No.1) strip

Dropper

Marker

Labels

PREPARATION:

Preparation of the sample:

1. Crush the sample using mortar and pestle.

2. Add a pinch of magnesium carbonate to the mixture.

3. Add one drop of acetone to the mixture and mix thoroughly.

Preparation of the mobile phase:

1. Take a glass beaker and add 16 ml of petroleum ether.

2. Add 4 ml of diethyl ether in the beaker.

3. Add 1 drop of acetone to the mixture and mix thoroughly.

PROCEDURE:

1. Take a clean and dry long strip of Whatman Filter paper and draw a line with a pencil parallel to about 1 cm from one edge. This will indicate the bottom of the chromatogram.

2. Spot a point with the pencil on the middle of the line. Put a drop of the sample on the spot.

3. Repeat the process of putting and drying the drop of sample for 4 – 5 times.

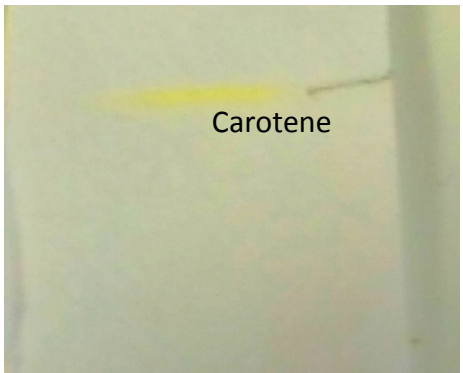
4. Fill the mobile phase in development jar about 2 cm and keep it closed for 15 minutes for saturation.

5. Keep the strip of the Whatman Filter paper inside the jar well straight and let the chromatogram develop.

6. Keep the whole apparatus as such for 5 – 10 minutes and see that mobile phase has risen nearly up to the top of the paper.

7. Measure the Solute and Solvent run and calculate the Rf values of the respective pigments and interpret your results

OBSERVATIONS:



Xanthophyll

Chlorophyll a

Chlorophyll b

Band Colour	Plant Pigment	Distance (cm)	Rf (use formula)
Dark Yellow	Carotene	6.5 cm	0.85
Pale Yellow	Xanthophyll	4.4 cm	0.55
Dark Green	Chlorophyll a	2.2 cm	0.27
Light Green	Chlorophyll b	0.9 cm	0.11

CALCULATIONS:

Distance from origin line:

a.) Chlorophyll a - 1.5 cm

b.) Chlorophyll b – 0.9 cm

c.) Xanthophyll – 2.5 cm

d.) Carotene - 6.6 cm

$$Rf = \frac{\text{Distance travelled by the solute from the point of application}}{\text{Distance travelled by the solvent}}$$

a.) For Chlorophyll a:

Rf = = 0.27

b.) For Chlorophyll b:

Rf = = 0.11

c.) For Xanthophyll:

Rf = = 0.55

d.) For Carotene:

Rf = = 0.86

RESULTS:

The plant pigments were separated successfully using paper chromatography. Pigments are separated on the paper and show up as four different coloured bands with different distance from the origin line. The topmost dark yellow band pigment in the separation on chromatogram represents carotene. The pale yellowish appearing below represents xanthophylls. The next dark green and light green band represents chlorophyll a and chlorophyll b respectively. The solvent run distance from the origin line was 8 cm.

CONCLUSION:

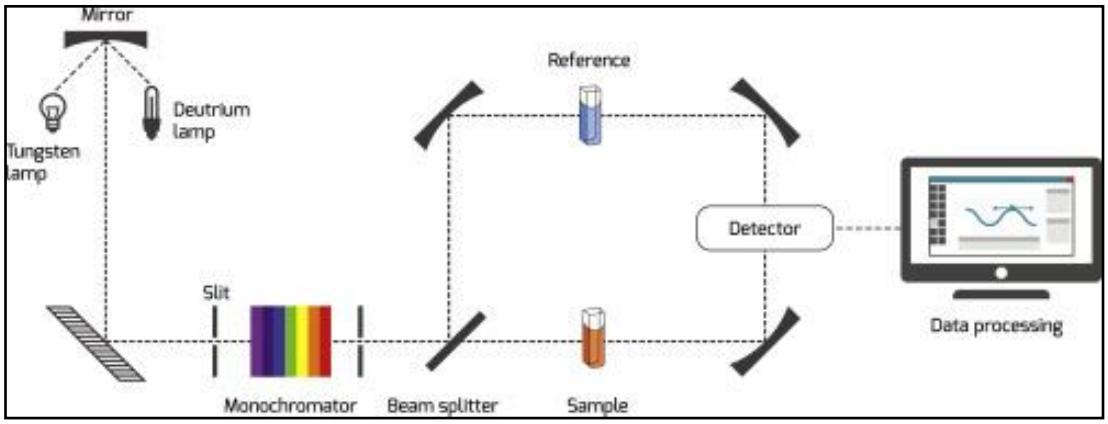
Separation of different plant pigments on strip is because paper chromatography separates compounds based on their different rates of migration on filter paper. The rate of migration depends upon the solvent which is flowing up and on the relative adsorption which holds the molecules tightly to the paper. It also shows that carotene has greater affinity towards mobile phase solvent and chlorophylls has greater affinity towards water immobilised on pores of paper, this gives insight of the polarity of the pigment.

UV Visible Spectrophotometer

AIM: To Determine Concentration of unknown Protein sample by Biuret Method Using UV Visible Spectrophotometer

PRINCIPLE:

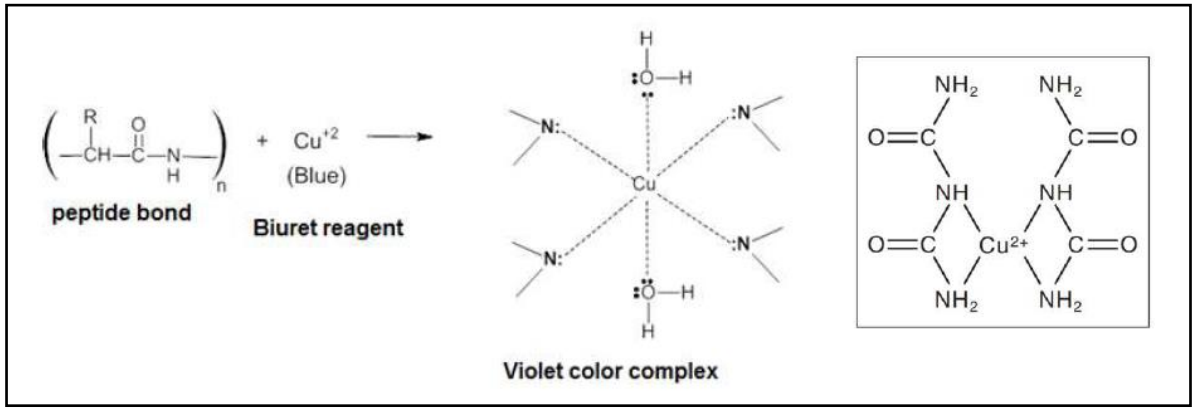
Spectrophotometers are used to measure the concentration of known substance in a solution. It is done by passing light through the substance and measuring light intensity as a function of wavelength. For measuring both ultraviolet and visible light, two lamps are usually required: 1) Tungsten filament lamp which produces wavelength in the visible region; 2) Hydrogen / Deuterium lamp which is suitable for ultraviolet region. There are two kinds of optical arrangements: 1) Single beam [A single beam spectrophotometer uses a reference standard to standardize or blank the instrument before taking measurements.] 2) Double beam [A double beam spectrophotometer splits the beam of light in two different paths; one passes through sample other passes through reference standard.]. UV Visible Spectrophotometer follows Beer-Lambert Law which states that there is a linear relationship between the concentration and the absorbance of solution, which enables the concentration of solution to be calculated by measuring the absorbance.



Construction of UV Vis Spectrophotometer

Biuret is a small compound that forms when urea is heated allowing two urea molecules to join. Urea molecules fused in this manner produce amide groups (-NH) at the center of the

molecule which bind to cupric ions at a basic pH. The copper complexes that result from this interaction produce a strong blue color that can be measured with a spectrophotometer. Proteins also contain amide groups. When an amino group and a carboxyl group join to form a peptide bond, the amino group (-NH2) becomes an amide group (-NH). At basic pH, the proteins forms complex with copper ions. Thus, in this assay you will combine protein samples with Biuret Reagent which contains copper ions in a basic solution. The copper ions will complex with the amide groups in the proteins to create a blue color that will be measured using a spectrophotometer. The intensity of the color formed is directly proportional to the number of peptide bonds participating in the reaction and thus in the amount of protein present. This relationship allows a standard curve to be created that is used to calculate the concentration of protein in an unknown sample.



Reaction of Biuret and copper complex

(Biuret reagent reacts with an alkaline solution of CuSO4 to form a violet chelate compound.)

MATERIAL & METHODOLOGY

Sr. No.	Material	Methodology
Chemical Required		
1.	Biuret reagent	Dissolve 0.3 g of CuSO4 and 0.9 g of Sodium Potassium Tartrate in 50 ml 0.2 M NaOH solution. Add 0.5 g of KI and make up the volume to 100 ml with 0.2 M NaOH.
2.	0.2 M NaOH	Dissolve 1.6 gm NaOH in 100 ml distilled water
3.	BSA stock (standard) solution (10 mg/ml)	Dissolve 100 mg of BSA in 10 ml distilled water.
4.	Distilled water.	
Instruments Required		
1.	UV Vis Spectrophotometer	
2.	Weighing Balance	
Glassware & Miscellaneous		
1.	Measuring cylinder	100 ml
2.	Volumetric Flask	2 x 100 ml
3.	Beaker	3 x 250
4.	Test tubes Stoppered	1
5.	Test tubes	7
6.	Pipette	2 x 1ml 1 x 10ml
7.	Bubbler	2 small 1 big
8.	Tissue paper	1 roll
9.	Stirrer	1
10.	Test tube stand	1
11.	Cuvette	2

PROCEDURE:

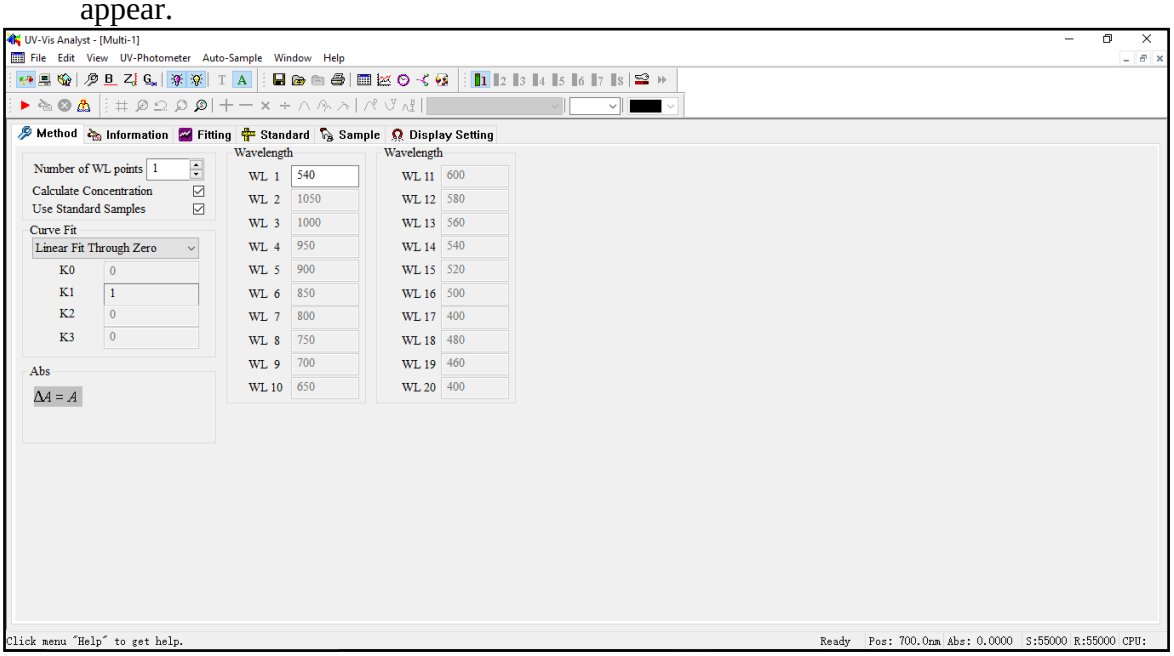
A) Preparation of Standard Dilutions and unknown protein for Biuret test

1. A stock solution of BSA protein was prepared (10mg/ml) and kept in a labelled beaker.
2. 150 ml of Biuret reagent was made by proper measuring cylinder.
3. In the test tube, dilution of proteins was made with different volume from the stock by mixing appropriate amount of BSA stock and water as mentioned in the Table 2.1.
4. 4 ml of Biuret reagent is added to all the test tube.
5. In another test tube, 1 ml of unknown sample is taken and 4 ml of Biuret reagent is added to it.
6. Incubate all the test tubes for 30 minutes at room temperature.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis using UV Vis Spectrophotometer.

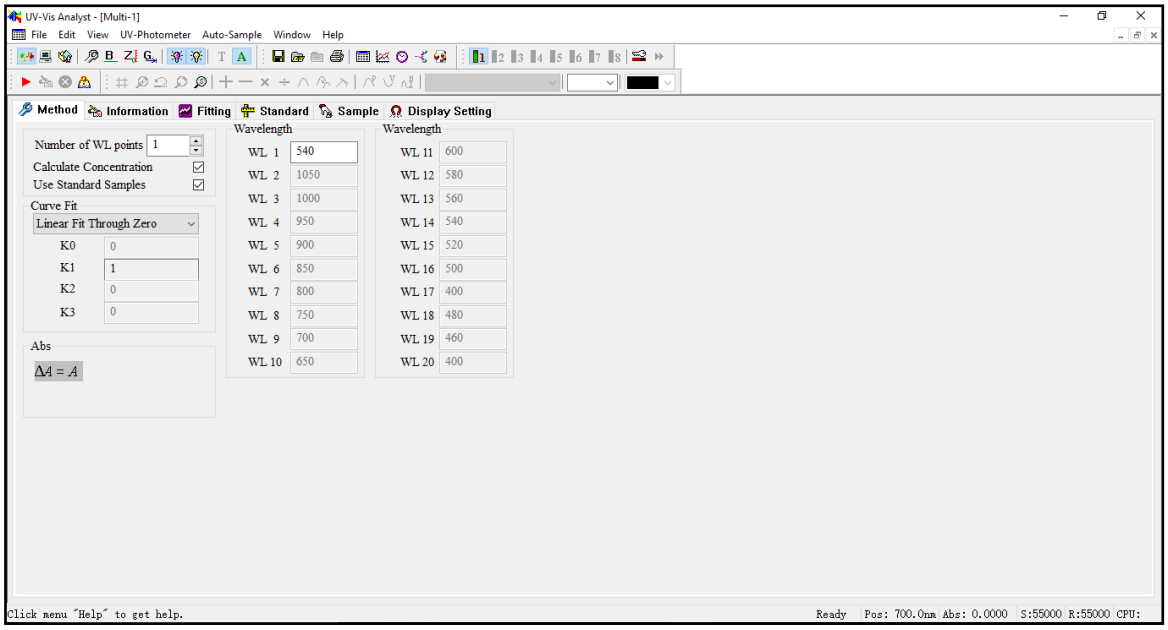
BSA Concentration (mg/ml)	BSA stock (ml)	Distilled Water (ml)	Biuret reagent (ml)		OD values (Absorbance at 540nm)
0	0.0	1.0	4	Incubate for 30 minutes at Room Temperature	
2	0.2	0.8	4		
4	0.4	0.6	4		
6	0.6	0.4	4		
8	0.8	0.2	4		
10	1.0	0.0	4		
Unknown (Test solution)	1 ml (sample)	_	4		

B) Procedure for using UV Vis Spectrophotometer for taking absorbance.

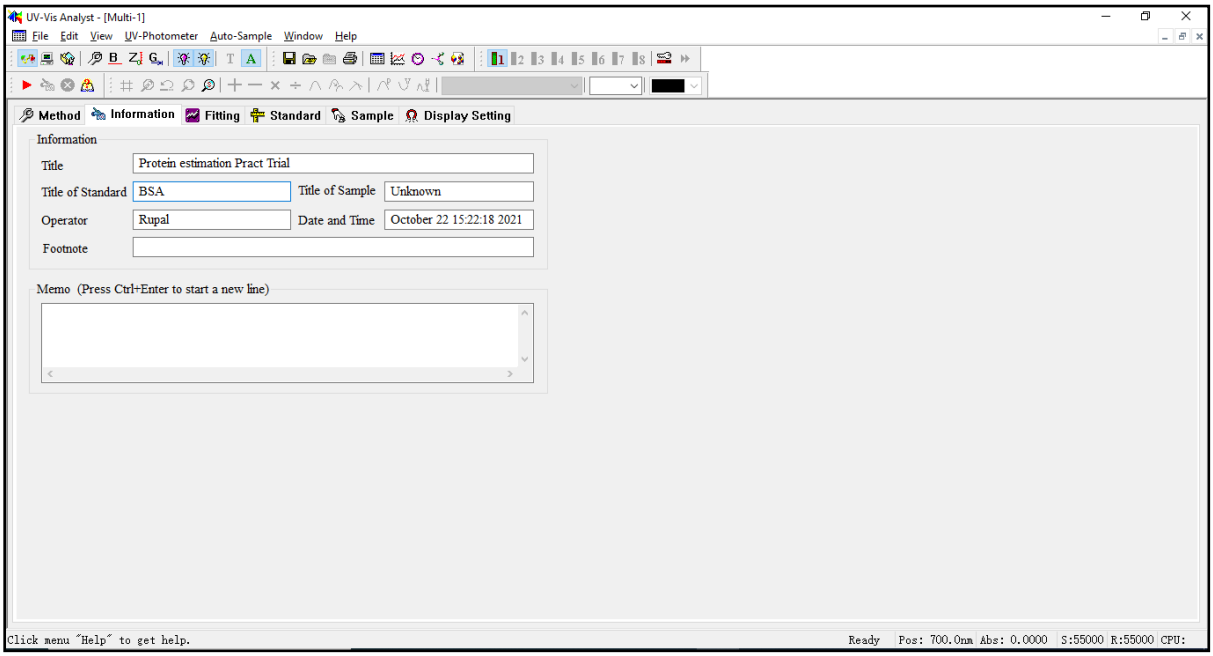
1. Open UV Analyst Software
2. Put Blank cuvette and click on  for Auto zero Calibration.
3. Click Fixed Point Measurement  on the toolbar. The Following form will appear.



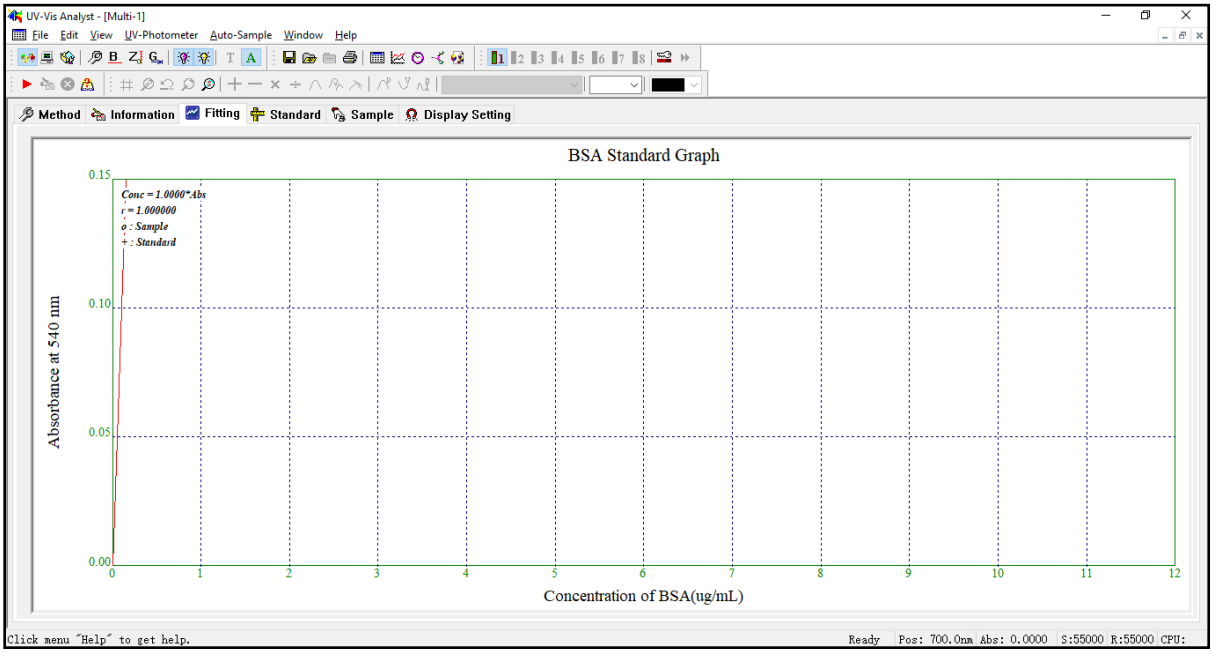
4. Click the **Method tab**.
5. Type the number of wavelength points (1) in the Number of **WL Points** box, or click the up/down arrows next to the box set the wavelength points. Tick the two boxes **Calculate Concentration** and **Use Standard Samples**. Choose the curve fit (Linear passing through origin). Write the wavelength required in “WL 1” box.



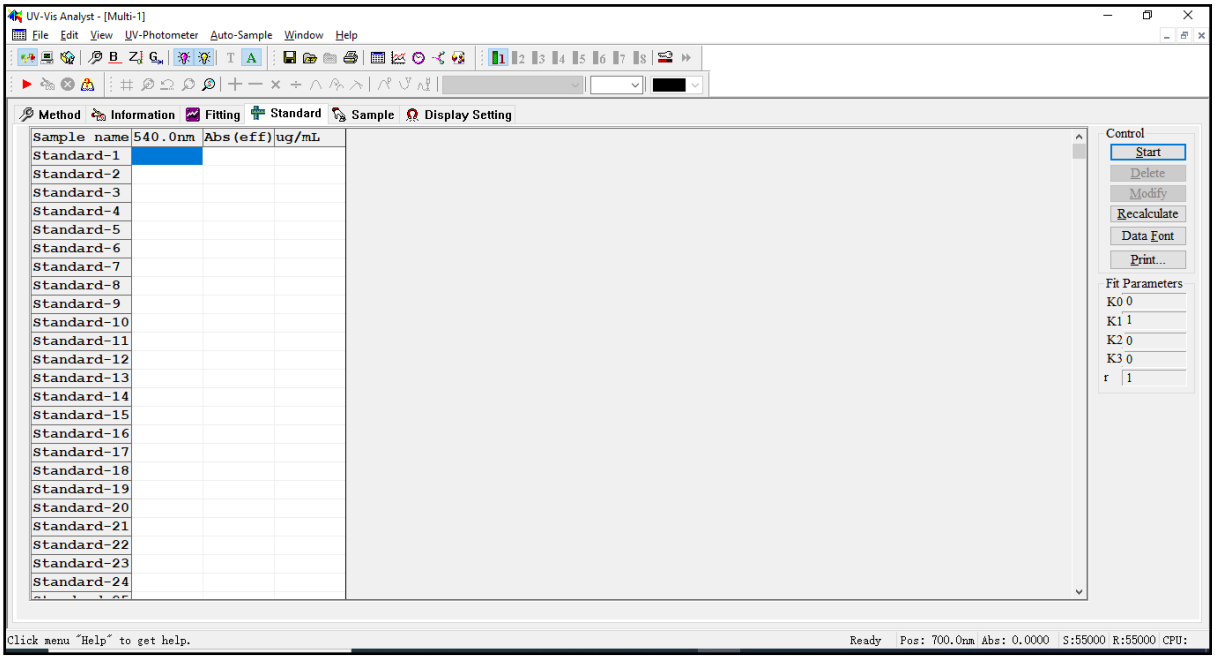
6. Click the **Information tab**. Enter Title of practical, title of standard & sample and Name of Operator.

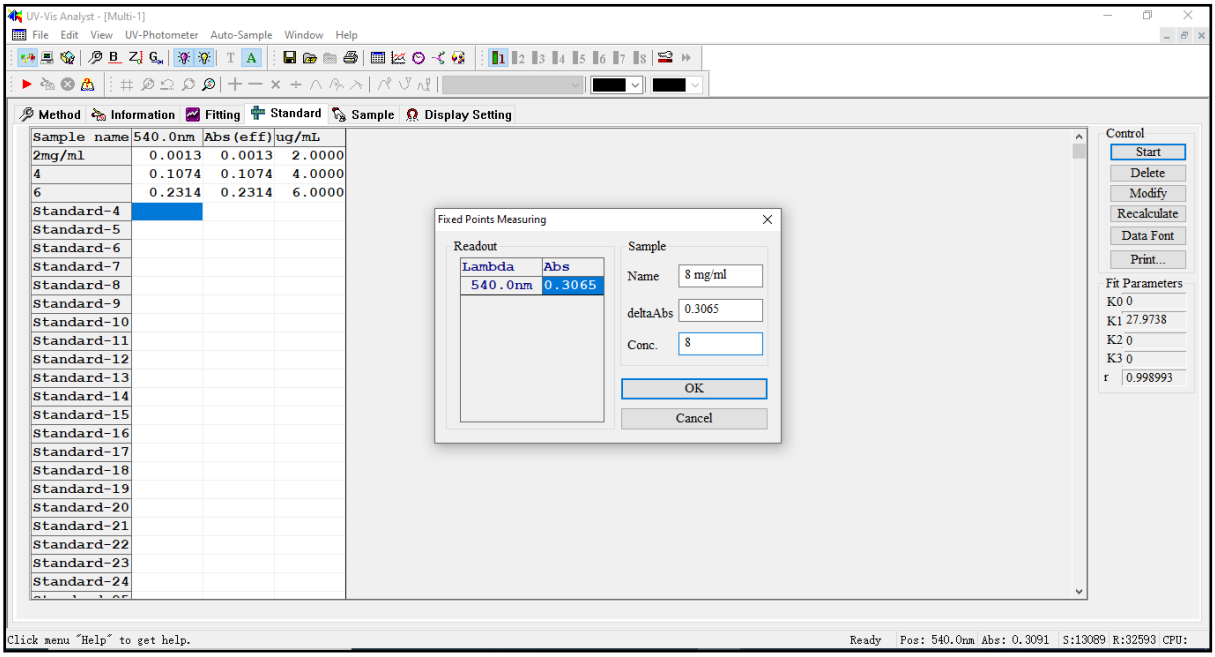


7. Click the **Fitting tab.** This tab will show empty graph until absorbance for standard and unknown are calculated.

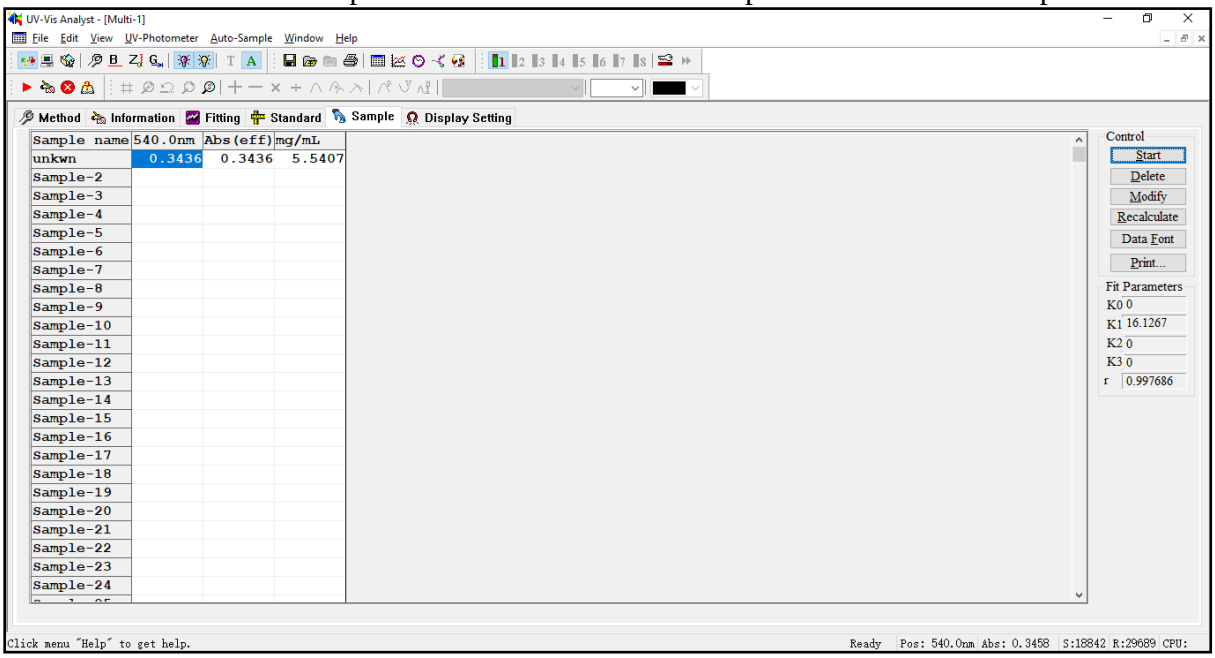


8. Click the **Standard tab.** Put the first solution of standard dilution in UV Vis Spectrophotometer and click on start tab to take the absorbance. After absorbance is taken Give name to the dilution & enter concerntration of dilution and click on OK to save the absorbance taken. The photometric data for standards will be listed in the Standard table. Perform the same step for all standard dilutions made.

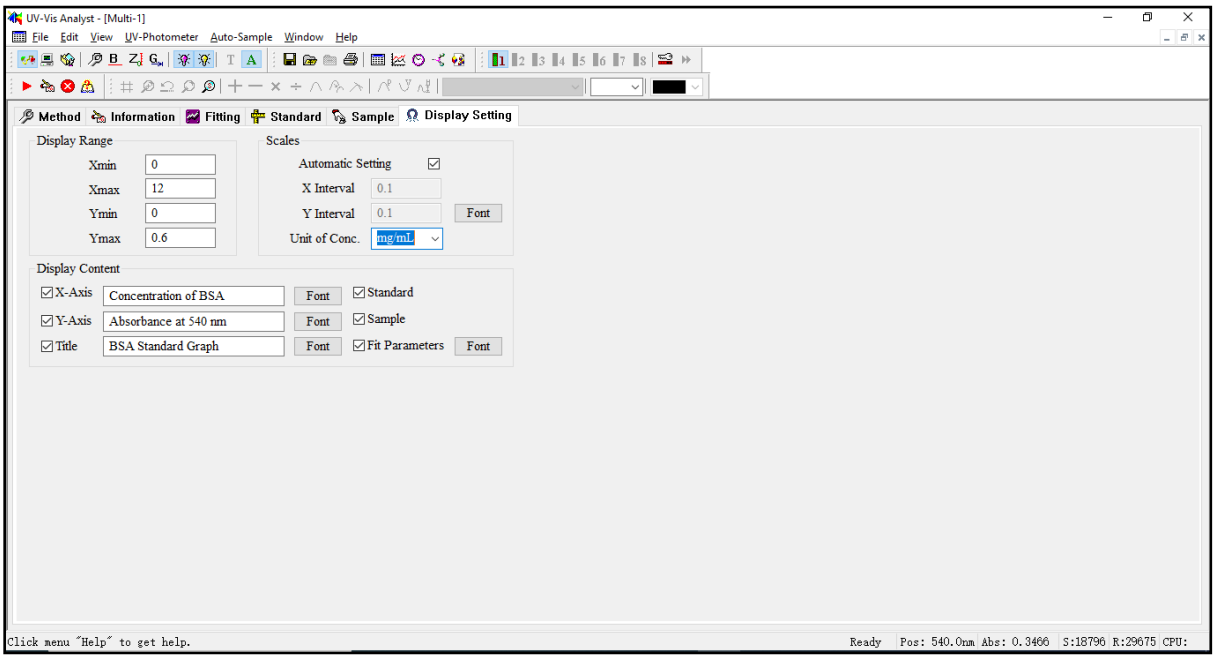




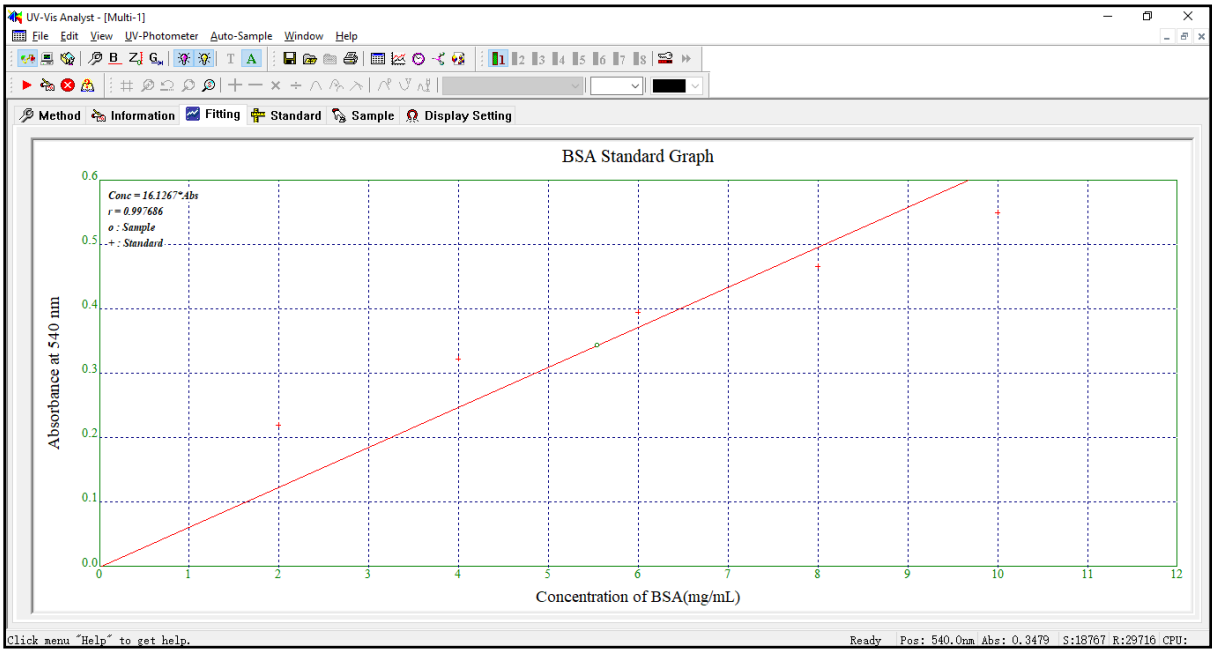
9. Click the **Sample tab**. Put the first solution of Unknown sample in UV Vis Spectrophotometer and click on start tab to take the absorbance. After absorbance is taken; Give name to the dilution. The concerntration of unknown is calculated by the software and value is displayed below the absorbance. Click on OK to save the absorbance taken and concerntration calculated. The photometric data for sample will be listed in the Sample table. Perform the same step for all Unknown samples.



10. Click the **Display Setting tab.** Adjust the range of X-axis and Y-axis values required. Choose the unit of concerntration. Give Title of the graph, X-axis and Y-axis.



11. Click the **Fitting tab.** After changing the display setting and taking absorbance for Standard & Unknown, click again on the fitting tab to view the standard graph generated.



12. Click on Print and the result in .pdf format.

Result:

The given unknown sample contains 5.5407 mg protein/ml.

Conclusion:

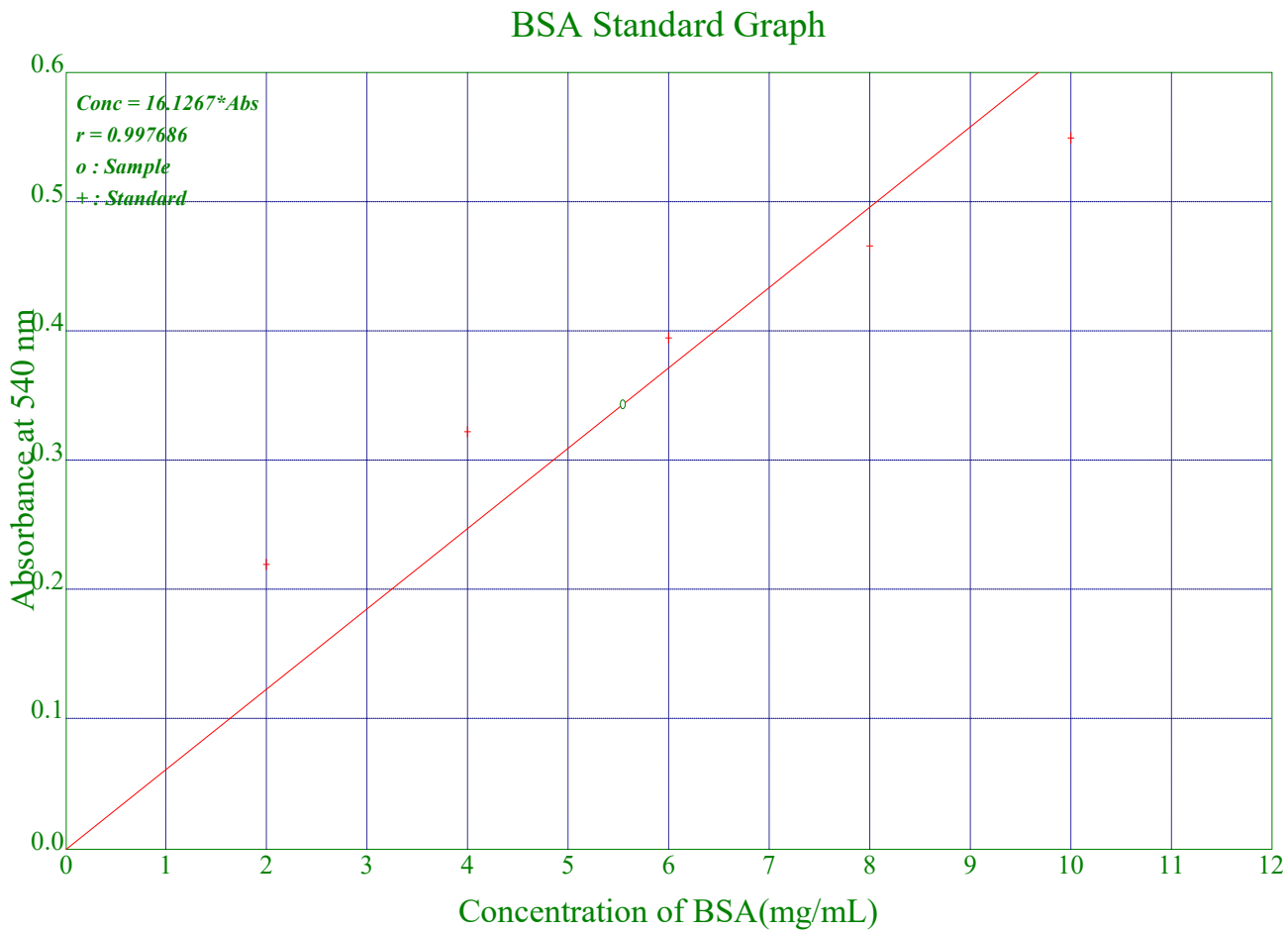
Determination of Concentration of unknown Protein sample was done by Biuret Method Using UV Visible Spectrophotometer.

Protein estimation Pract Trial

Operator:Rupal

Date & Time:October 22 15:22:18 2021

Memo:



BSA

Sample name	540.0nm	Abs(eff)	mg/mL
2mg/ml	0.2192	0.2192	2.0000
4mg/ml	0.3218	0.3218	4.0000
6mg/ml	0.3946	0.3946	6.0000
8mg/ml	0.4655	0.4655	8.0000
10mg/ml	0.5485	0.5485	10.0000

Unknown

Sample name	540.0nm	Abs(eff)	mg/mL
unkwn	0.3436	0.3436	5.5407

THIN LAYER CHROMATOGRAPHY

AIM: To separate and identify major components from given turmeric sample by performing Thin Layer Chromatography (TLC).

REQUIREMENTS:

1. Chemicals: Chloroform & Methanol.
2. Glasswares: Pipettes, Twin trough chamber, Capillaries, Stoppered test tubes.
3. Miscellaneous: TLC Silica Gel 60 F254 Plate, Forcep, Pencil, Scale, Vortex instrument, Filter paper, eppendorf tubes.
4. Sample & Standard: Turmeric sample and Curcuminoids standard.

THEORY:

-Curcuminoids are natural polyphenol compounds derived from turmeric (*Curcuma longa*), which is a member of the ginger family.

-Curcuminoids is a family of active compounds within turmeric which includes Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin. Among them, curcumin with bright yellow colour is the principal component.

Properties and uses of curcuminoids:

1. Turmeric is one of the most popular medicinal herb, with a wide range of pharmacological activities.
2. **Antioxidant activity:** Turmeric dramatically increases the antioxidant capacity of the body.

   a. Oxidative damage is believed to be one of the mechanisms behind aging and many diseases.
   b. It involves free radicals, highly reactive molecules with unpaired electrons.

c. Free radicals tend to react with important organic substances, such as fatty acids, proteins or DNA.
d. The main reason antioxidants are so beneficial is that they protect body from free radicals.
e. Curcumin is a potent antioxidant that can neutralize free radicals due to its chemical structure.
f. In addition, curcumin boosts the activity of body's own antioxidant enzymes.
g. In this way, curcumin delivers protection against free radicals. It blocks them directly, then stimulates body's own antioxidant defences.

3. **Anti-Inflammatory activity:**

a. Curcumin is a potential anti-inflammatory agent. It is so powerful that it matches the effectiveness of some anti-inflammatory drugs, without the side effects.
b. It blocks NF-kB, a molecule that travels into the nuclei of cells and turn on genes related to inflammation. NF-kB is believed to play a major role in many chronic diseases. Also curcumin suppress inflammation through many different mechanisms.

4. **Improve brain function:**

a) Curcumin boosts levels of Brain Derived Neurotrophic Factors (BDNF), which increases the growth of new neurons and fights various degenerative processes in brain.
b) By doing this, it may be effective in delaying or even reversing many brain diseases.

5. **Lowers risk of heart disease:** Curcumin has beneficial effect on several factors known to play a role in heart disease. It improves the function of the endothelium and is a potent anti-inflammatory agent and antioxidant.
6. Several studies have shown that turmeric can prevent and even helps in **treatment of cancer**.
7. Curcumin can cross the blood-brain barrier and has been shown various improvements in the **pathological process of Alzheimer's disease**.
8. **Arthritis** is a common disorder characterized by joint inflammation. Many studies have show that curcumin can help treat symptoms of

arthritis and in some cases is more effective than anti-inflammatory drugs.

9. Studies have shown that curcumin has incredible benefits against **depression**.
10. Due to antioxidant activity of curcumin it is predicted that it may help **delay ageing** and can help in alleviating symptoms of age-related chronic diseases.

PRINCIPLE:

It is based on the principle of adsorption and relative affinity of components towards the stationary and the mobile phase. The components of sample under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the components with higher affinity towards stationary phase travel slowly while the other components travel faster. Affinity of component is defined by its polarity. Since silica is polar in nature, polar components from sample interact more with stationary phase (silica) and thus travel slowly while non-polar components interact less with stationary phase and thus travel faster. Therefore, the separation of components from the sample mixture is achieved. Once separation occurs, the individual components are visualized as spots/bands on the plate.

PROCEDURE:

1. Preparation of mobile phase (solvent): Take 19ml of chloroform and 1ml of methanol in stoppered test tube with the help of pipettes. Vortex or shake test tube for mixing of solvents.
2. Preparation of sample and standard solution: Weigh 0.5g of sample and standard in different eppendorf tube and add 5ml of methanol in each tube. Sonicate for 05 minutes.
3. Saturation of twin trough chamber: Place filter paper inside chamber and pour prepared mobile phase on top of filter paper. Immediately close the chamber with lid. Leave chamber undisturbed for 15-20minutes.
4. Spotting of sample and standard: Before spotting, mark application point (1cm above from bottom) and solvent front (7cm above from application

point) by pencil. Finely spot sample and standard spot (at some space in between) using pointed capillary.

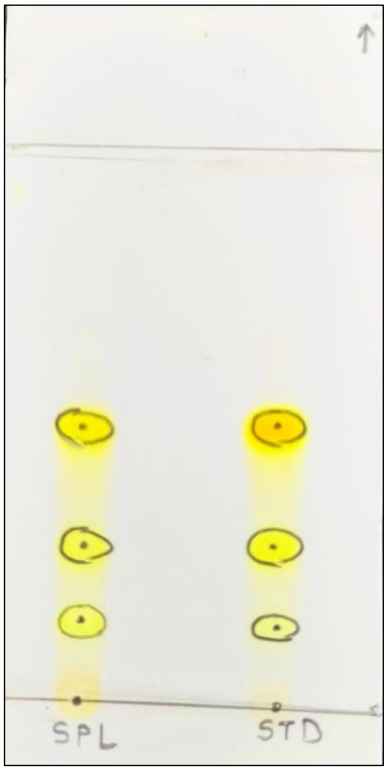
5. Plate run: Place plate in chamber carefully (facing filter paper) with the help of forcep and allow it to run till solvent front. Remove plate with forcep after complete run.

6. Encircle three spots for both sample and standard using pencil. Measure the distance of all three spots from center to application point for both sample and standard.

7. Calculate Retension factor (Rf) by using the formula:

$$Rf = \frac{Distance\ travelled\ by\ the\ solute\ from\ the\ point\ of\ application}{\text{distance } travelled\ by\ the\ solvent}$$

FIGURE: Separation of turmeric components on TLC plate



OBSERVATION TABLE:

Sample	Distance travelled by solute (in cm)	Distance travelled by solvent (in cm)	Rf value
Spot 1 (BDMC)	1.2	7.0	0.17
Spot 2 (DMC)	2.4	7.0	0.34
Spot 3 (Curcumin)	3.8	7.0	0.54

Standard	Distance travelled by solute (in cm)	Distance travelled by solvent (in cm)	Rf value
Spot 1 (BDMC)	1.4	7.0	0.20
Spot 2 (DMC)	2.5	7.0	0.35
Spot 3 (Curcumin)	3.8	7.0	0.54

Where, BDMC: Bisdemethoxycurcumin & DMC: Demethoxycurcumin.

CALCULATIONS:

- **Sample:**

1. $\mathrm{Rf} = \frac{1.2}{7.0} = 0.17$

2. $\mathrm{Rf} = \frac{2.4}{7.0} = 0.34$

3. $\mathrm{Rf} = \frac{3.8}{7.0} = 0.54$

- **Standard:**

4. $\mathrm{Rf} = \frac{1.4}{7.0} = 0.20$

5. $\mathrm{Rf} = \frac{2.5}{7.0} = 0.35$

6. $\mathrm{Rf} = \frac{3.8}{7.0} = 0.54$

RESULT:

TLC technique was performed with turmeric as sample, curcuminoids as standard and by using mobile phase composition of chloroform:methanol (19:1) (v/v).Three separate spots were obtained for three components of turmeric namely, curcumin, demethoxycurcumin and bisdemethoxycurcumin at a distance of 1.2cm. 2.4cm and 3.8cm respectively.

CONCLUSION:

Sample was run on TLC plate and three different spots of Curcumin, DMC, BDMC were observed. Rf values for these spots were calculated and found to be similar to that of Rf values of standard for respective spots confirming identity of each component. By observation, curcumin was found to be the major component followed by DMC and BDMC.