Chromatography
An Educational Tool

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The technique of chromatography is studied to bring forth the underlying concepts such as adsorption, partition, polarity/non-polarity of a compound. The materials required are very simple such as chalk, starch, ink, etc. Once the concepts are understood, the technique can be easily extrapolated to analyze complex biological samples as well.

Introduction

Chromatography is an important separation technique that is usually included in the biology curriculum at the university level. Many a time, the experiments involving chromatography are performed without much emphasis on the underlying concepts. During the Indian National Biology Olympiads (INBO-1999, 2000), this technique was examined in detail and several biochemical concepts were brought forth. Students themselves were asked to explore the principles of separation. For this, the experiments devised were simple, not requiring any special apparatus or chemicals and were highly flexible.

A few experiments described herewith can be performed even at the school level. These experiments apart from stirring interest among students, clarify several concepts such as adsorption (*Box* 1), partitioning of solutes, polar/non-polar nature of solvents and solutes.

Adsorption Chromatography

The technique of chromatography was introduced as early as 1906 by a Russian scientist Michael Tswett. He used a column of calcium carbonate ($CaCO_3$) for separation of leaf pigments. This technique was called as 'adsorption chromatography' (see *Box* 1).

Experiment 1 (on adsorption chromatography): Take a white chalk. Engrave a circular ridge on it about 1 cm from the end.

Box 1. Adsorption

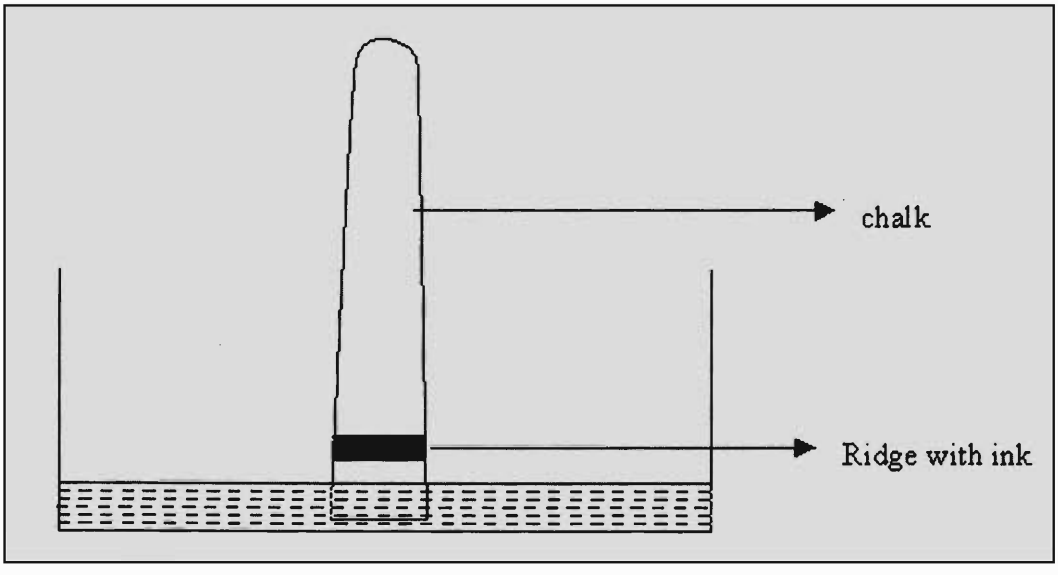
Adsorption differs markedly from the frequently used term absorption. In absorption, one substance (A) penetrates into the another substance (B) and occupies the intermolecular spaces of B. On the other hand, adsorption is a surface phenomenon in which atoms, molecules or ions of A are attracted at the surface of B. Separation in this technique is the net result of adsorption and desorption. During the course of time, A gets concentrated on B. A is called *adsorbate* and B is called *adsorbent*. A variety of inert materials can be used as adsorbents. e.g. charcoal, calcium carbonate, alumina.

(*Figure* 1). In the ridge add 4-5 drops of microtip black ink (of trademark PIK^R) in a circular fashion. Allow the ink to dry for a minute. In a small container (plate or beaker) pour a few milliliters of tap water and vertically place the chalk inside. Ensure that the ink mark is above the water level. Let the water run through the chalk column and reach the top. Observe various colored bands (*Figure* 2).

The following concepts can be discussed now.

1. Ink is composed of many different color components.
2. These ink components differ from each other in their adsorptive properties.
3. These components also show differential polarity.

Figure 1. Chalk with ridge.



The above experiment can be further modified in various ways:

1. Using other polar/non-polar solvents like ethanol, acetone, and ether (*Box* 2).
2. Using inks from different manufacturers.
3. Using some other inert adsorbent material. Eg. starch.

After doing these simple experiments and collecting the data, the following questions can be asked:

1. Why does one get good separation with water, alcohol, etc. and poor separation with ether?
2. What information do we get about the nature of the ink components in terms of polarity/non-polarity?
3. What kind of separation do we get from red or blue ink? Why?

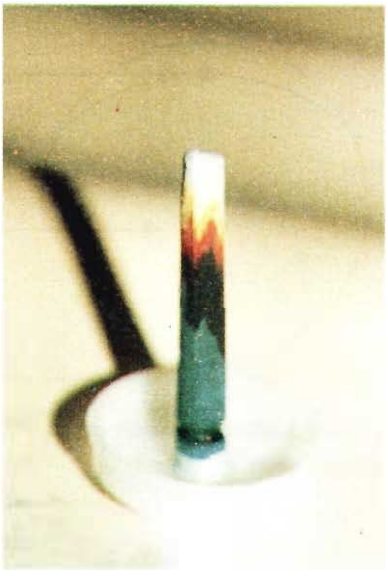


Figure 2. Colour bands formed on chalk.

Adsorption chromatography can also be performed using another easily available adsorbent material namely starch used at homes for clothes (e.g. Revive® starch).

Experiment 2. **Take 3 teaspoonsful of starch powder in a dry flat plate or a glass petri-dish. Tap or shake gently to spread it in the**

Box 2.

Polar Molecules: In a molecule, when one atom is more electronegative than another, a partial charge separation occurs. Such bonds with partial positive and negative centers are called polar covalent bonds. They make the entire molecule polar in nature depending on the shape of the molecule and orientation of the polar bonds. e.g. H_2O, HF, HCl, NH_3, H_2S, etc.

Non-polar Compounds: In a molecule, when both the atoms are same (e.g. H_2, O_2, N_2) or both the atoms have similar electronegativities (e.g. C-H bond), the bonding electrons are shared equally between the two atoms. Such bonds are called non-polar bonds. They impart non-polarity to the entire molecule. e.g. Benzene, alkanes, lipids, fatty acids.

Like Dissolves Like: 'Like dissolves like' is an extremely useful thumbrule. Highly polar compounds dissolve in highly polar solvents while non-polar or weakly polar compounds dissolve in non-polar or weakly polar solvents.

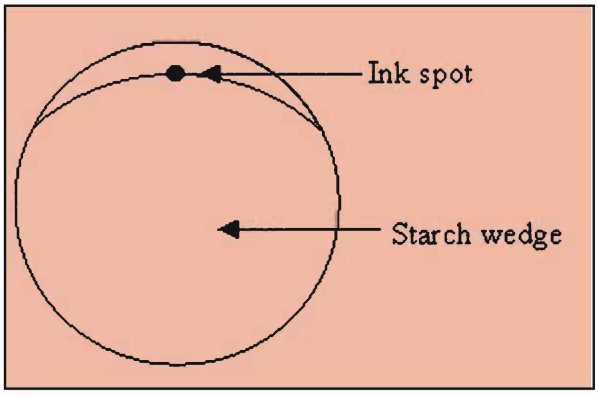


Figure 3. Starch powder on a petri dish.

plate in such a way that the thickness gradually decreases from edge to centre of the plate. It forms a wedge (see *Figure* 3). Add 1-2 drops of ink used in the previous experiment as shown in the figure. Allow it to dry for 1-2 minutes. In a glass dropper/capillary/pipette, take a few milliliters of a solvent such as alcohol. Add a drop of this solvent on the ink spot. Slowly go on adding the solvent dropwise till the ink separates into its colored components. You can slightly tilt the plate while adding the solvent. This procedure is also called as 'wedge-shaped chromatography'.

As mentioned in the earlier experiment, this experiment can also be modified by changing the solvents as well as ink samples (*Table* 1).

The following questions can now be asked:

1. Why does the separation pattern differ with chalk and starch with the common solvent (water)? (*Box* 3)
2. Why does the order of separation of different color components change by changing the adsorbent?
3. In order to get good separation with ether, which solvent would one add (mix) to it?

Adsorbent: $CaCO_3$	Adsorbent: Starch	Solvent used
+	±	Water
+	+	Ethanol
–	–	Ether

\+ : Good separation
– : No separation
± : No separation or delayed separation

Table 1. Separation of black ink using different adsorbents and solvents.

Box 3. Some Properties of Adsorbents which Affect Separation

Starch is a naturally occurring polysaccharide (polymer of glucose). It exists in two forms: amylose (water-soluble) and amylopectin (insoluble in water). Owing to numerous hydroxyl groups present in its structure, it is a polar molecule, which can absorb large quantities of water. This slows down the separation when water is used as a solvent. Chalk on the other hand, is mainly calcium carbonate, a salt relatively insoluble in water. (solubility: 0.0013g/100g water). This physical property allows water to percolate readily through the column. Hence separation with water as a solvent is fast.

Partition Chromatography

Partition chromatography involves an important concept of partitioning (solubilizing) of different solutes in different solvent systems.

Partitioning can be observed using various indicators commonly used in the chemistry laboratory such as methyl orange, thymol blue, methyl red, etc. Sudan red is another such important staining solution usually available in biology laboratory. It is used to stain lipoid structures of cell.

Experiment 3. In a glass test tube, add 3 mL of water and 3 mL of diethyl ether. This makes a two-phase solvent system. Add 1-2 drops of any one of the above indicators and observe the solubility of the indicators in the two phases.

The following questions can be raised:

1. In which solvent phase does each of the above indicators dissolve? Why?
2. Try to hypothesize their structural features. (Presence of charged groups, uncharged polar groups or long alkyl chains, etc.)

Paper Chromatography

At this stage, paper chromatography can be introduced, as it is a type of partition chromatography. Two solvent systems are involved in paper chromatography (*Box* 4). One solvent is water bound to cellulose fibers of paper. This water is also called as

stationary phase. The second solvent is the solvent in which the paper is dipped. It is also called as a mobile solvent. The choice of mobile solvent depends on the type of solutes (polar/non-polar) to be separated.

Experiment 4. You are provided with a mixture of thymol blue and Sudan red. You have to suggest an appropriate solvent/ solvent system as a mobile phase and perform a paper chromatography to separate them.

Students find this task very interesting as they develop their own method of separation based on the findings of earlier experiments of partitioning. They come up with various answers, which can be discussed. The following questions can be raised:

1. Is water/ether the only appropriate solvent or both?
2. Can any other polar/non-polar solvent be used? How will the separation differ?

The technique of chromatography can be now applied for separation of various biomolecules such as proteins, sugars, lipids, etc. Various plant pigments can be separated using chromatography. In fact, paper chromatography is routinely

Box 4.

Chromatography Paper: Paper is made up of cellulose molecules. Cellulose molecule is a polymer of glucose in which, the glucose molecules are linked to each other by β-1,4 linkages. Cellulose molecules are present in extended or linear conformation. Thus the structure of paper gives rise to a system of interconnected capillaries of varying size and shape. The rate of penetration of any liquid into the paper is governed by physical and geometrical properties of the capillary channels as well as on the physical properties of penetrating liquid. An unprinted portion of a newspaper, ordinary filter paper, all can act as chromatography papers. However, the non-uniform physical character and impurities in the paper hinder a good separation. There are specially manufactured papers for chromatography which have low organic and inorganic impurities and uniform physical characteristics. e.g. Whatman paper.

You can try out different papers and make your observations.

Cellulose Bound Water: There are multi-layers of loosely bound water around the fibers of cellulose, which take part in partitioning. Thus paper bound water becomes the stationary phase in paper chromatography.

performed in most of the colleges to separate pigments of green leaves. Students are aware that these leaves contain chlorophyll pigments. These pigments perform the function of photosynthesis and also impart green colour to leaves. Now, instead of green leaves, red/brown/violet coloured leaves can be shown to the students (e.g. coleus, setcretia/tradescantia). Following questions can then be raised:

1. Why do these leaves not look green?
2. If they contain any other pigment, does it also perform the function of photosynthesis?
3. Is chlorophyll mandatory for the function of photosynthesis?
4. How can these pigments be separated?

Experiment 5. Prepare a two phase solvent system in a test tube by adding 3 mL of water and 3 mL of diethyl ether. Macerate the brown/violet leaf in water. Add 3-4 drops of it to the test tube. One sees a green ether layer and violet-brown water layer. In a similar way, flower petals of any color (red, blue, yellow, etc.) can be macerated in water. A few drops of these is added to ether-water solvent system. It immediately colours the aqueous (water) phase, ether remains colourless.

Several points can be discussed now.

1. Presence of chlorophyll in non-green plants.
2. Presence of water-soluble pigments in non-green leaves as well as in flower petals.
3. Polar nature of these pigments – namely anthocyanins (red, blue) and flavanols (yellow).
4. Non-polar nature of chlorophylls.

Let's try to separate the pigments of non-green leaves effectively using two-dimensional chromatography.

Experiment 6. Cut a chromatography paper 8 cm × 8 cm. Prepare water extract of a non-green leaf as before using minimum quantity of water. Spot about 10 microliters (≈ 2 drops) at one corner of the paper leaving about 1cm distance from each edge

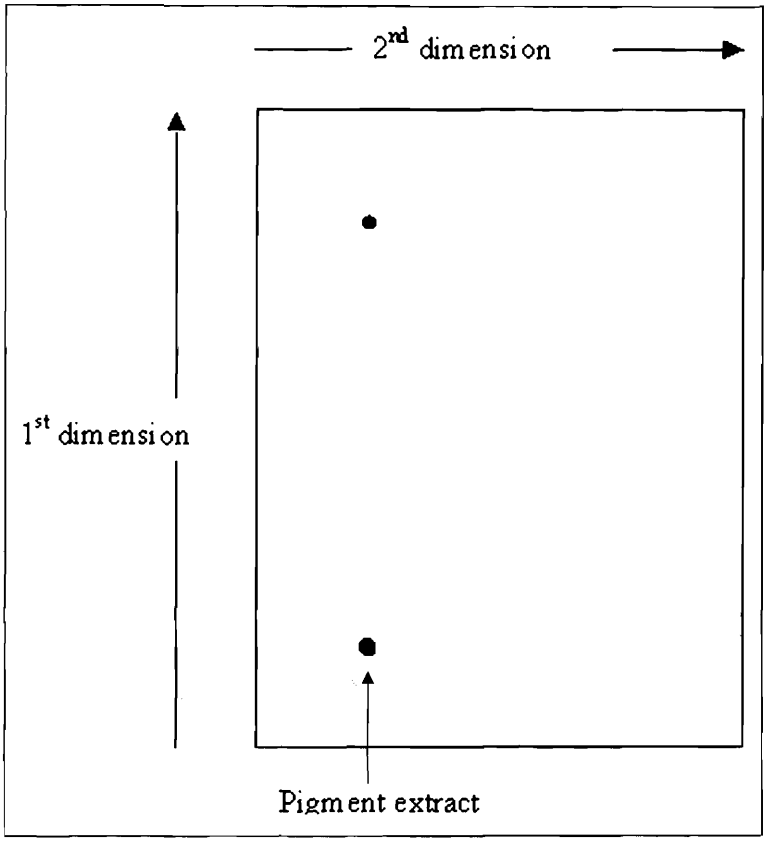


Figure 4. Two-dimensional chromatography paper.

(*Figure* 4). To a glass beaker (capacity 1 L), add 10 ml of diethyl ether and cover the beaker tightly with aluminium foil. After the spot has dried, place the paper in the beaker. Immediately cover. Allow the solvent to run. Remove the paper when solvent reaches to a height of about 1 cm below the top edge. At this stage, you have already got anthocyanins separated from other non-polar pigments. Allow the paper to dry. Now perform the chromatography in the second dimension using the following solvent system:

Petroleum ether (40-600) : 90% acetone :: 100 :12.

The second solvent effectively separates carotenes, xanthophylls (both yellow); chlorophyll a and chlorophyll b (*Figure* 5).

One can replace the solvent of first dimension chromatography by petroleum ether. Does it give separation similar to diethyl

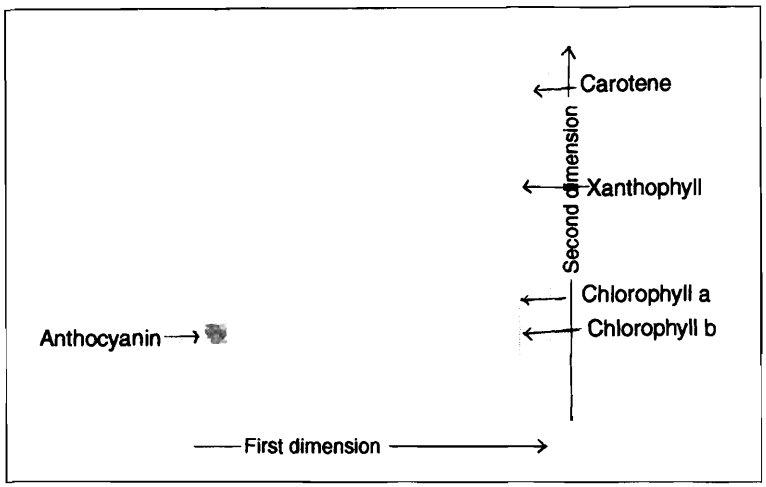


Figure 5. Scanned two-dimensional chromatogram for separation of plant pigments.

ether? What can be deduced about the polarity or non-polarity of the two solvents?

Thus, beginning with the simple experiment of chalk chromatography one can perform various investigations. These experiments help strengthen several concepts of adsorption, partition, solubility of solutes in polar/non-polar solvents, etc. This way, students also learn scientific methods: they hypothesize, perform experiments, learn to verify their hypothesis and modify the experiments, which further support or disqualify the hypothesis.

Acknowledgement

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Suggested Reading

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[3] N P O Green, G W Stout and D J Taylor, *Biological Science*, Cambridge University Press, 1996.