Bio Factsheet

Number 83

Chromatography and its Uses in Biology

After studying this Factsheet the student should understand:

- **the principles and method of simple paper chromatography**
- **the range of chromatographic methods available**
- **the uses of chromatography in Biology**

Students may wish to use chromatography in laboratory and project work but should also be aware that examiners often set questions on the practical technique and applications of chromatography.

The principles and method of simple paper chromatography

Paper chromatography is the technique of **separation** and **identification** of chemical substances on sheets of filter paper by allowing a solvent to move along the paper. A drop of solution containing a mixture of the substances to be separated is placed on the filter paper near one end. The drop is allowed to dry leaving a 'spot' of the mixed substances. The position of the spot is called the **origin** and is noted. The end of the paper near the spot is immersed into a suitable solvent, without immersing the spot itself.

In **ascending** chromatography the solvent is in a pool at the bottom of a jar in which the paper is supported. The solvent is drawn up the paper by capillarity. In **descending** chromatography the solvent is in a trough from which the paper is hung and flows down the paper due to a combination of capillarity and gravity. In both cases, as the solvent flows over and past the spotted mixture it dissolves and carries along the substances from the spot. **Each substance generally moves at a different rate from the others**.

The solvent flow is allowed to move for a suitable time or for a reasonable length along the paper. The point where the solvent has reached is marked as the **solvent front**, and the paper is dried. The positions of the separated substances are noted and their distances from the origin measured. If the substances are colourless they are stained by applying appropriate chemicals to the paper. This technique is known as **one-way chromatography**. (Fig 1).

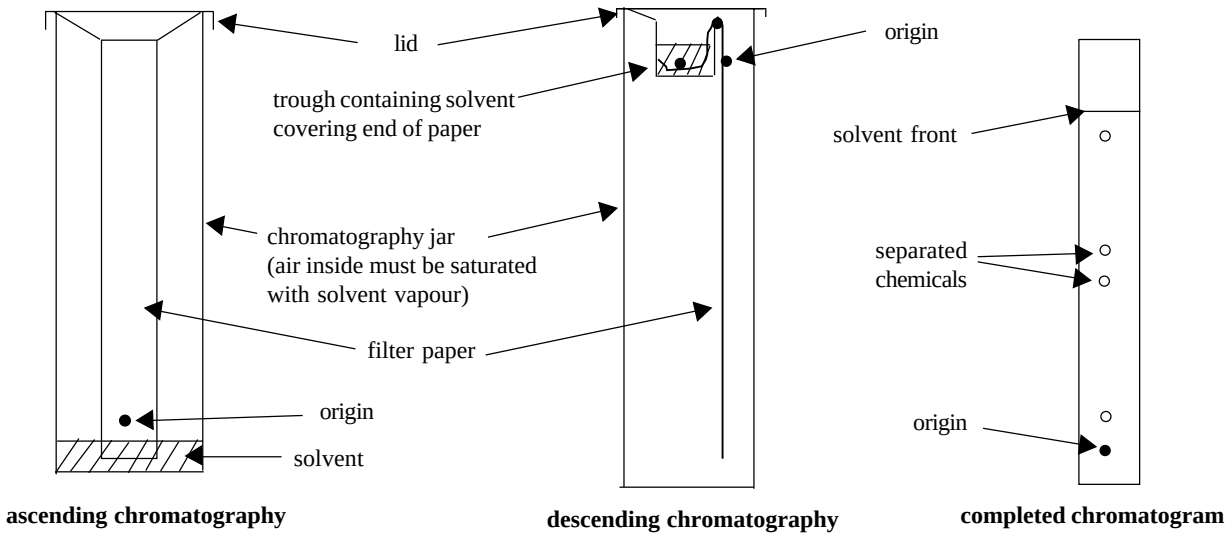
Two opposing types of force operate to enable the separation of the substances from the mixture.

- **Propelling** forces act to shift the substances from the origin and displace them along the solvent flow. Two propelling forces are involved. One is the **relative solubility** of the substance in the solvent – the more soluble a substance is in the solvent, the easier it is picked up by the solvent and moved along - more soluble substances will tend to outdistance the less soluble. The other propelling force is the actual **solvent flow** which 'washes' the substances along.
- **Retarding** forces act to impede the movement of the substances by holding them in the paper rather than allowing them into the solvent flow. Two main retarding forces exist. One is the degree to which the substance is **adsorbed** onto the cellulose fibres of the paper and the rate at which the substance is released from that adsorption as the solvent flows past. The other force relates to **partition** – the paper contains a water phase bound to the cellulose fibres and this water may dissolve and hold more of the substance than the actual separating solvent, thus inhibiting its movement into the solvent.

Other chromatography techniques

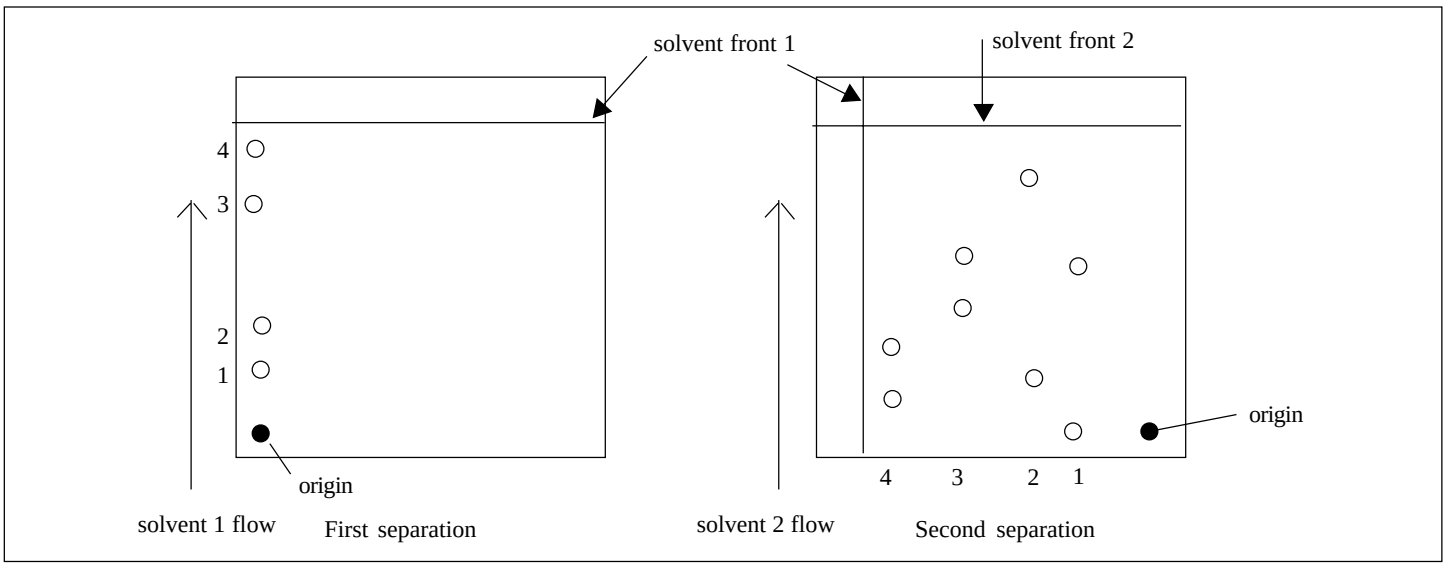
Better separation of substances may be achieved by a two way technique. In this case the origin is placed at the corner of a square piece of filter paper and solvent 1 is allowed to run through causing an initial separation. Solvent front 1 is marked and the paper dried. It is then turned through 90° and solvent 2 allowed to run, enabling a second separation. Solvent front 2 is marked and the paper dried.

Fig 1. One-way chromatography



A two-way chromatogram is illustrated in Fig 2.

Fig 2. Two-way chromatography



Chromatography can be carried out on media other than chromatography or filter paper. For example, in **thin layer chromatography (TLC)** the separation is done on media such as silica gel, cellulose powders, celite or alumina. The medium is laid, together with a binding substance, on sheets of glass and allowed to dry. A special spreader enables the medium to be spread at a precise thickness and density across the glass plate. The technique is then similar to paper chromatography but is always done in an ascending way.

TLC has several advantages over paper chromatography. For example:
- it will give better separation of lipid class compounds.
- it gives quicker separation so that running time is shorter
- resolution is better since the spots are more compact
- the chemicals can be easily recovered for analysis, by removing the media at the spots and washing the chemicals out. Even microgram quantities can be collected.
- strongly corrosive locating agents, such as sulphuric acid, can be sprayed onto silica gel and alumina plates without affecting the coating – paper would disintegrate.

Identification of separated compounds

Some compounds such as chlorophylls and carotenes may be recognised by their colour or by their fluorescence in ultra-violet light. Colourless compounds have to be stained by spraying the paper with suitable chemicals. For example, the stain ninhydrin colours amino acids, some in specific colours – the amino acid proline stains yellow and asparagine stains brown.

> ***Remember*** *– never spray locating agents in the open laboratory, but always in a fume cupboard and be careful to confine the droplets to the fume cupboard.*

Compounds may also be identified by their **R_f values** (relative flow value). The R_f value can be calculated by the formula:

$$R_f = \frac{\text{distance the substance has moved from the origin}}{\text{distance the solvent front has moved from the origin}}$$

R_f values are physical constants for specific substances in specific solvents on a particular chromatography medium. **Controls** can be performed by running chromatographs of the expected substances alongside the unknown mixture to see if the R_f values of the known and unknown compounds correspond.

Some practical tips when carrying out paper chromatography

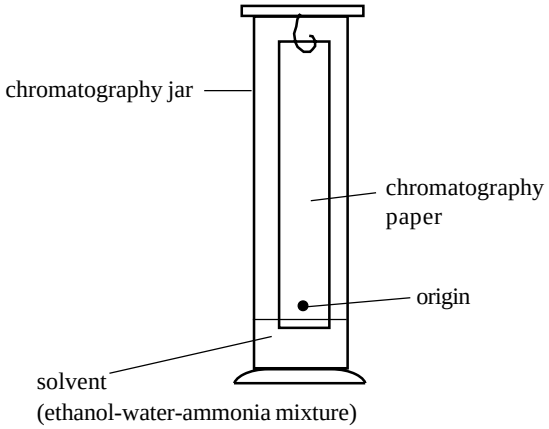
- do not handle the paper directly since sweat on the finger tips contains compounds such as amino acids which will contaminate the chromatogram.
- when placing the mixture at the origin use a fine capillary tube to dispense it and to stop it spreading. Immediately dry the spot using a hair dryer. Add five to ten spots to the origin in this way so that the mixture at the spot is concentrated.
- be careful that the surface of the solvent does not come above the origin, thus preventing separation on the paper.
- make sure that the paper hangs vertically in the solvent. If it does not then the solvent will rise up at an angle, making measurements difficult.
- make sure that the lid of the chromatography jar fits snugly – it is essential that the atmosphere in the jar is saturated with the solvent vapour, so that the solvent does not dry out when moving along the paper.
- many solvents are flammable so make sure that there are no naked flames or other ignition sources in the room. Preferably carry out the separation in the fume cupboard.
- mark the solvent front with a pencil mark which will not spread.
- dry the paper in a hot air oven at around 105°C.
- if a locating agent is used, spray it on the paper and allow it to dry in the fume cupboard.

Uses of chromatography in Biology

- to trace metabolic pathways. For example, in combination with radioactive tracing using carbon -14, two way chromatography was used to discover the reaction sequences of the Calvin pathway (light-independent stage of photosynthesis)
- to determine abnormal constituents in the urine of new born babies in order to discover possible genetic abnormalities, such as phenylketonuria (a symptom of various inherited abnormalities of phenylalanine and tyrosine metabolism).
- separation and identification of amino acids, sugars, lipids and electrolytes in various biological fluids, such as fruit juice, plant sap, urine and plasma.
- separation and identification of chlorophylls and anthocyanins from leaves and flowers, and the comparison of these from different plants to assess taxonomic (evolutionary) relationships.
- separation of pigments (pteridines) from insects such as Drosophila, and their comparison from different mutant strains of Drosophila.

Practice Questions

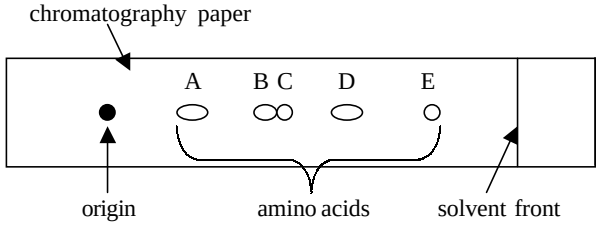
1. The diagram below shows a chromatography apparatus that could be used to identify the different amino acids in a sample of fruit juice. Since amino acids are invisible, a stain in spray form can be used to colour them.



(a) (i) Describe how the experiment would be set up to separate the amino acids in the fruit juice. 5

(ii) State **three** precautions which should be taken when setting up the apparatus. 3

(b) After a suitable time the chromatography paper was removed, the solvent front marked and the paper dried in a fume cupboard. It was then sprayed with ninhydrin and dried at 105°C to locate the amino acids. The R_f values of the amino acids were then measured. The diagram below shows the results that were obtained.



(i) What is meant by the term R_f value? 3

(ii) The table below gives the R_f values of some common amino acids.

Amino acid	Rf value
Cysteine	0.78
Valine	0.60
Proline	0.43
Arginine	0.20
Threonine	0.35
Lysine	0.14
Methionine	0.55
Phenylalanine	0.68
Serine	0.27
Tyrosine	0.45

Calculate the R_f values of amino acids A, D and E and suggest which they are. Show your working. 6

(iii) Suggest how amino acids B and C could be separated more clearly. 2

Total 19

Answers

1 (a) (i) lay chromatography paper flat on clean filter paper/paper;
use capillary tube to place small drops of fruit juice on origin;
dry each drop with hair dryer (to prevent spreading);
at least 5 drops to get a concentrated spot;
hang chromatography paper in jar so that solvent surface is over end of paper but below origin;
put lid on to make an airtight seal; max 5

(ii) do not touch chromatography paper with fingers since sweat contains amino acids;
atmosphere in jar must be saturated with the vapour of the solvent (so that the paper does not dry out);
make sure paper is hanging vertically so that solvent moves straight up/does not carry amino acids to edge of paper;
have no naked flames/ignition sources in the room/use a fume cupboard; max 3

(b) (i) distance moved by solute;
divided by the distance moved by the solvent front;
is a physical constant for each amino acid with a specific solvent; 3

(ii) A: $R_f = \frac{13}{66} = 0.20$; arginine; (measure to the centres of spots, allow ± 0.5 mm)

D: $R_f = \frac{31}{66} = 0.56$; methionine;

E: $R_f = \frac{52}{66} = 0.79$; cysteine;

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(iii) run another chromatogram at right angles to the first/two way chromatography;
using a different solvent; 2

Total 19

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