

# Chromatography

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## Introduction

Chromatography in all its variations is one of the most widely used and most potent of all the laboratory purification methods in the chemist's armamentarium. First demonstrated by Michael Tswett, a Russian botanist who reported the separation of plant pigments (coloring agents) by this method in 1903, chromatography has since been applied to every conceivable type of compound in a wide variety of uses. The name chromatography stems from the early application to mixtures of colored compounds; the separation amounted to graphing the color (Greek: chroma = color).

If a mob of 1000 people of similar stamina, consisting of 500 weighing 50 kg and 500 weighing 90 kg randomly mixed together, set out to run through a very closely planted thick forest, those awaiting their arrival on the other side of the forest would note that the first 500 to arrive all weighed 50 kg and the second 500 arriving some time later all weighed 90 kg. The thin people had run through the spaces between the trees easily: the wider people had to struggle.

Chromatography involves an analogous principle. A mixture of two or more compounds is placed on a section of stationary material over which a fluid is slowly passed. The various compounds will be attracted to (adsorbed on or dissolved in) the stationary material, but will also have a competing tendency to dissolve in the fluid and pass slowly through the stationary material and out. This competition will cause some compounds of mixture to move through relatively quickly (they will be very soluble in the fluid and only weakly absorbed – the thin people) and some to move through very slowly (much less soluble in the fluid, strongly absorbed – the wider people). By collecting and analyzing the fluids leaving the stationary material in small batches, one can separate compounds that can never be purified of each other by other by any other technique. Alternatively, for analytical purposes, one could stop the fluid flow at some point before the compounds leave the stationary material and inspect their relative positions to see how many compounds are present and what their chromatographic properties are. Both approaches have been widely used,

Numerous forms of chromatography have been developed:

a) **Column chromatography**--The stationary material is often crushed mineral (silica gel, for instance) and the moving phase a solvent (acetone, benzene, etc.). The stationary material is packed in a vertical glass tube (column) and a sample to be separated is placed at the top of a column. Then solvent is added to the top of the column and passed through the sample and column of stationary material. The emerging liquid from the bottom of the column is collected in portions to isolate the individual compounds composing the sample. The method is useful as an analytical (identification) and preparative technique (10 mg – 1 kg of sample). A variation, high speed liquid chromatography (HSLC), has recently been developed where the above process is automated. The solvent is rapidly forced through the column under high

pressure, the emerging liquid is analyzed continuously, and its content is indicated in a strip of chart paper. This method is used both for analytical (1 ug-100 mg) and preparative work.

b) **Gas chromatography**- (variously known as GC, GLC – gas liquid chromatography or VPC – vapor phase chromatography) – The stationary material is an oily liquid coated thinly on a crushed mineral and the moving liquid is a gas (He, N<sub>2</sub>). The sample is injected into the column at an elevated temperature (50<sup>0</sup> – 250<sup>0</sup>C) and the compounds in the gas emerging from the end of the column are continuously analyzed and record on a chart as in HSLC above. This method is used predominantly for analytical work and is capable of difficult separations (e.g. mixtures of structural alkali isomers).

c) **Thin layer chromatography** – (TLC) – both stationary and moving materials are as in column chromatography. However, the adsorbent is coated on a glass plate in a thin layer, the mixture is deposited neat one edge of the layer as a spot or a streak and the plate then dipped in a shallow bath of a solvent. The solvent rises up the layer through capillary action (this is called developing the plate). When it reaches to the top, the positions of the spots of the various compounds in the mixture are observed, or, in the preparative mode, the adsorbent holding the spot of each is scraped off separately for recovery of the substance. This technique is useful for analytical or preparative (up to 1 g) applications.

d) **Paper chromatography** – This is a variation of (TLC) where the stationary substance is a strip of paper. It is useful for analytical purposes only, in situations where the compounds to be identified are suitably adsorbed not on silica gel but on paper.

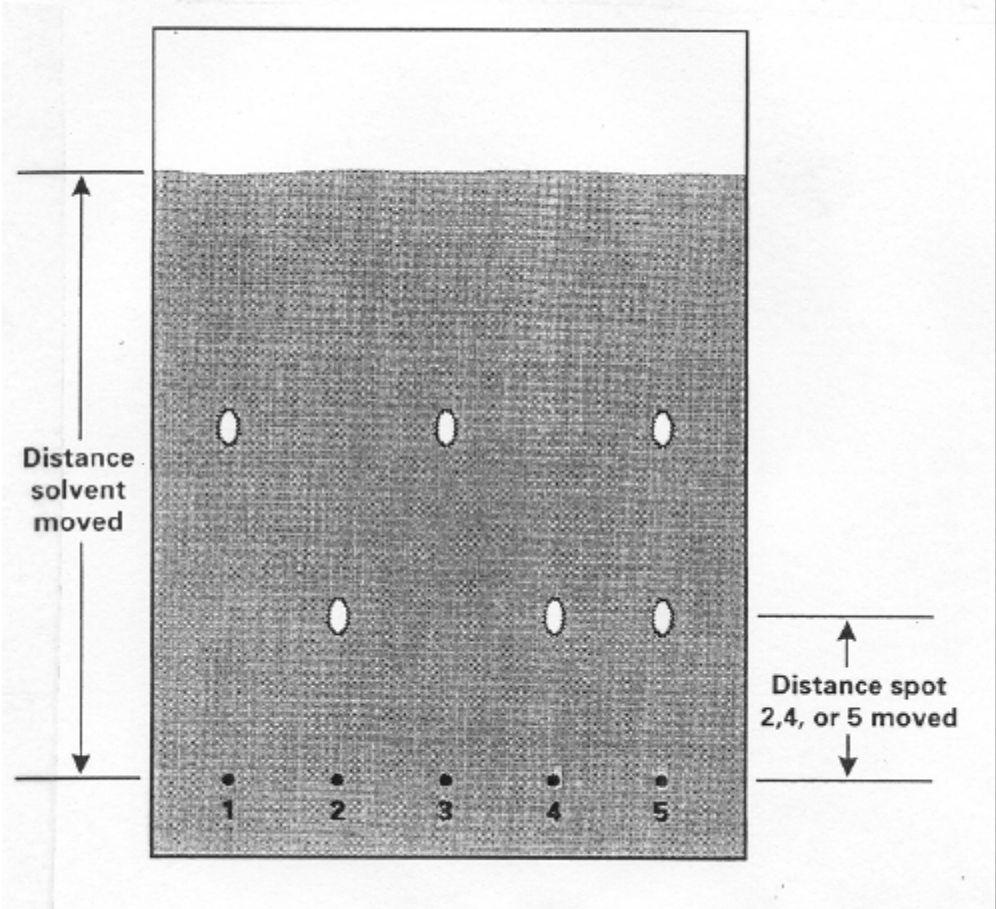
In this experiment paper chromatography will be applied to different samples and the components of the mixture characterized. The experimenter controls three variables in paper chromatography: solvent, paper, and distance solvent moves. The latter is difficult to repeat precisely and to compare experiments the ratio called the representative fraction, R<sub>f</sub>, is calculated. The representative fraction, R<sub>f</sub>, is defined by the equation:

$$R_f = \text{Distance from center of spot to starting point} / \text{Distance from solvent front to starting point.}$$

Figure 1 is a sample chromatogram and shows how the quantities in the equation are determined from the experiment. The R<sub>f</sub> is independent of the distance the solvent was allowed to move and can be easily repeated.

Figure 1 also shows how paper chromatography can be used to determine whether a sample is a mixture and to identify the components of a sample. For example, the chromatogram in Figure supports the idea that spot 2 and spot 4 are identical because both have the same R<sub>f</sub>. For spot 5, we see the result of chromatographing a mixture of compounds. This mixture is clearly separated into two components and the identify of each component established. Sometimes when a mixture consisting of two compounds of similar R<sub>f</sub> is chromatographed the components of the mixture will not be clearly separated or “resolved”. The resolution of a chromatograph can be changed by using a different solvent, using a different stationary phase or by letting the solvent move farther along the chromatogram.

Figure 1—Sample Chromatogram



## Procedure

### A. Procedure for Amino Acid mixture

The following procedures can be used to identify an unknown amino acid, separate plant pigments, and separate and identify the amino acids present in orange juice and lemon juice.

1. Preparation of development tank
2. Preparation of the paper
3. Development of chromatogram
4. Location of the amino acids after development of the chromatogram

#### Preparation of the developing tank

1. Pour enough “Eluting solution” into an 800 or 1000 ml empty beaker until the bottom of the beaker is completely covered with liquid. Cover the beaker with a piece of aluminum foil. Note, the eluting solution is a 75:25:30 mixture of methyl ethyl ketone, propionic acid, and water.

#### Preparation of paper

2. Wear gloves when handling the paper as to avoid depositing amino acids from your fingers.
- 3.. With a lead pencil, draw a line, 1.5cm from a parallel to one of the narrow edges of the 12cm x 14 cm filter paper sheet provided.
4. Along this line, mark off tick marks 1 cm apart. Number the tick marks.
5. Distributed around the laboratory you will find watch glasses with a few milliliters of 5% amino acid solution in them. Using a glass capillary tube, draw up a small volume of amino acid solution into the capillary and then deposit the amino acid onto the paper by touching the capillary to one of the tick marks. Record the tick mark number and sample identity in your data record.. Do not apply samples to the tick marks in the center of the paper as the paper will be folded for developing the chromatogram.
6. Allow the spots to dry before developing the chromatogram..

#### Development of the Chromatogram

7. When the spots on the paper are dry, fold (wear gloves) the paper at the center and so the spots are on one edge.
8. Uncover the development tank and stand the folded chromatogram with the spots at the bottom in the eluting solution. Recover the development tank.
9. Let the solvent run up the chromatogram until the solvent front is about 1 cm from the top of the chromatogram. At this point remove (wear gloves) the chromatogram from the development tank and mark with a pencil the location of the solvent front (See Figure 1). Hang the chromatogram to dry in the hood.

Location of the amino acids after development of the chromatogram

10. Clip a test-tube holder (large clothes pin) to the bottom of the dry chromatogram and then spray the chromatogram with 1% ninhydrin solution. **DO THIS IN THE HOOD TO AVOID INHALING THE VAPORS.**

11. Let the sprayed chromatogram dry and then place in the oven for about 3 minutes, or until spots are visible.

4. Circle spots with a pencil, as the ninhydrin color will slowly fade.

5. Mark the center of each spot, as best you can judge and record color of the spot.

To be handed in –

1. Developed chromatogram.

2. Data on each amino acid requested on the report sheet.

3. The number and identity (to the best of your ability) of the amino acids composing each unknown.

B. Procedure for felt-tip makers of ball-point pen inks

Using the same procedure as for amino acids (above), spot a fresh piece of filter paper with different brands of felt-tip marker and/or ball-point pen inks, all having the same color. Develop these spots and note whether 1) they consist of one, or several, components and 2) whether different brands of ink contain the same, or different, components. Development of this chromatogram can be done simultaneous with, or subsequent to, the amino-acid chromatogram using the same eluting solvent and the same beaker.

If the time permits, run a new chromatogram using inks of another color.

To investigate the effect of different solvents on the RF's (resolution) of the spots, your instructor might assign you other eluents, e.g. ammonia water, 2-propanol, acetone, petroleum ether, acetic acid, etc.

### C. Procedure for amino acids in orange and lemon juice.

The chromatography procedure is identical to that for amino acid identification, except the unknown amino acids will come from the orange and lemon juice. The procedure for obtaining the amino acids is outlined below.

Record the appropriate information from this chromatogram on the report sheet.

1. Squeeze fresh juice from an orange and lemon slice into separate beakers.
2. Pour a few ml of each juice into separate centrifuge tubes. The levels of the liquid in each tube should be matched.
3. Centrifuge then pour off solution into separate labeled test tubes.
4. Clean centrifuged tubes.
5. Prepare a chromatogram with two spots using these solutions.
6. Report stationary phase, moving phase,  $R_f$  for each amino present and the identity of as many amino acids as possible.

### D. Procedure for pigments in green plants.

The chromatography procedure for this experiment is similar to that for amino acid identification except the 'composition' of the eluting solution is changed and the pigments to be separated must be extracted from their normal location (the leaf itself). The extraction procedure is outlined below:

1. Place "green pigment" eluting solution in the 1000ml beaker. (see preparation of the development tank in part A).
2. cut away the stems and midribs from fresh spinach and chop into small pieces.
3. place 2 to 3 grams of the spinach into a small beaker and add 10 ml of acetone.
4. Stir the mixture with a glass rod and crush against the side of the beaker.
5. Pour off the acetone into a clean beaker and then add 10 ml of ethyl alcohol to the spinach.
6. Repeat step four with the alcohol.
7. Pour off the alcohol into the beaker with the acetone. This solution will be your sample for the chromatography of green plant pigments.

Chromatography of green plant pigments.

The following changes in the procedure for amino acid identification should be Noted:

1. The chromatogram will have only one spot.
2. Since plant pigments are colored, the pigments do not need the ninhydrin treatment.
3. Report stationary phase, moving phase, and  $R_f$  for each pigment

E. Procedure for a mixture of indicator dyes

A mixture of synthetic dyes may be separated by paper chromatography in the same manner similar to that for natural pigments except the eluting solution will have a different composition. A solution that can be used to separate the acid base indicators below is n-butanol saturated with 1.5 ammonia solution. Spot the strips as follows:

1. Bromthymol blue
2. Alizarian yellow
3. Bromcersol purple
4. Phenolphthalein
5. Phenol red
6. Unknown mixture

Report sheet for paper chromatography

Name \_\_\_\_\_ Lab section \_\_\_\_\_ Date \_\_\_\_\_

Staple chromatograms to report

A. Amino- Acid Chromatography

<u>Initial Spot No.</u>	<u>Material Applied</u>	<u>Distance moved by spot (s)</u>	<u>Developed color of spots</u>	<u>Distance Moved solvent</u>	<u>Rf of spot (s)</u>
1	_____	_____	_____	_____	_____
2	_____	_____	_____	_____	_____
3	_____	_____	_____	_____	_____
4	_____	_____	_____	_____	_____
5	_____	_____	_____	_____	_____
6	_____	_____	_____	_____	_____
7	_____	_____	_____	_____	_____
8	_____	_____	_____	_____	_____
9	_____	_____	_____	_____	_____

Unknown Letter \_\_\_\_\_

Amino acids Identified in Unknown \_\_\_\_\_

\_\_\_\_\_

Unknown Letter \_\_\_\_\_

Amino acids Identified in Unknown \_\_\_\_\_

\_\_\_\_\_

Unknown Letter \_\_\_\_\_

Amino acids Identified in Unknown \_\_\_\_\_

\_\_\_\_\_

B. Felt-Maker Ink Chromatography

Solvent used: \_\_\_\_\_

Color of Ink: \_\_\_\_\_

<u>Initial Spot No.</u>	<u>Brand name of felt Ball-point Ink Applied</u>	<u>Distance moved by spot (s)</u>	<u>Developed color of spot(s)</u>	<u>Distance Moved solvent</u>	<u>Rf of spot (s)</u>
1	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____
2	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____
3	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____
4	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____
5	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____
6	_____	_____ _____ _____	_____ _____ _____	_____	_____ _____ _____

Which, if any inks have common ingredients? \_\_\_\_\_

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C. Orange and lemon juice Chromatography

Stationary phase: \_\_\_\_\_ Moving phase: \_\_\_\_\_

<u>Initial Spot</u>	<u>Developed color of spot(s)</u>	<u>Distance moved by spot(s)</u>	<u>Distance moved by solvent</u>	<u>Rf of spot(s)</u>	<u>Amino acid present</u>
Orange Juice	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
Lemon juice	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____

D. Green-plant pigment chromatography

Stationary phase: \_\_\_\_\_ Moving phase: \_\_\_\_\_

<u>Developed Spot No.</u>	<u>color of spot</u>	<u>distance moved by spot</u>	<u>distance moved By solvent</u>	<u>Rf of spot</u>
1	_____	_____	_____	_____
2	_____	_____	_____	_____
3	_____	_____	_____	_____
4	_____	_____	_____	_____
5	_____	_____	_____	_____
6	_____	_____	_____	_____

E. Acid-base Indicator chromatography

<u>Initial spot Number</u>	<u>Indicator Applied</u>	<u>Distance moved by spot</u>	<u>Distance Moved by solvent</u>	<u>Rf of spot</u>
1	bromthymol blue	_____	_____	_____
2	alizarin yellow	_____	_____	_____
3	bromcresol purple	_____	_____	_____
4	phenol-phthalein	_____	_____	_____
5	phenol red	_____	_____	_____
6	unknown mixt. Number _____	_____	_____	_____
		_____	_____	_____
		_____	_____	_____

Dyes present in unknown \_\_\_\_\_

## Questions

1. Why must you use lead pencil, instead of a pen, to mark your chromatography paper?
2. Why should you avoid touching the surface of the paper to be used for amino-acid chromatography?
3. Where is the kitchen, or in the body, might one encounter mixtures of amino acids?
4. If two different substances have the same or nearly the same  $R_f$  values, it is difficult to separate (resolve) these materials in a mixture. How could this experiment be changed to separate such a mixture?
5. Give two or three practical examples where chromatography would be valuable analytical tool.
6. The  $R_f$  of a spot contains information regarding the attraction of the substance being chromatographed to the paper and the eluting solution. Using your data:
  - a) Which amino acid has the strongest attraction to the paper? (Explain your answer.)
  - b) Which amino acid has the strongest attraction to the eluting solution? (Explain your answer.)
7. What effect do other substances in a mixture have on the  $R_f$  of a specific substance?