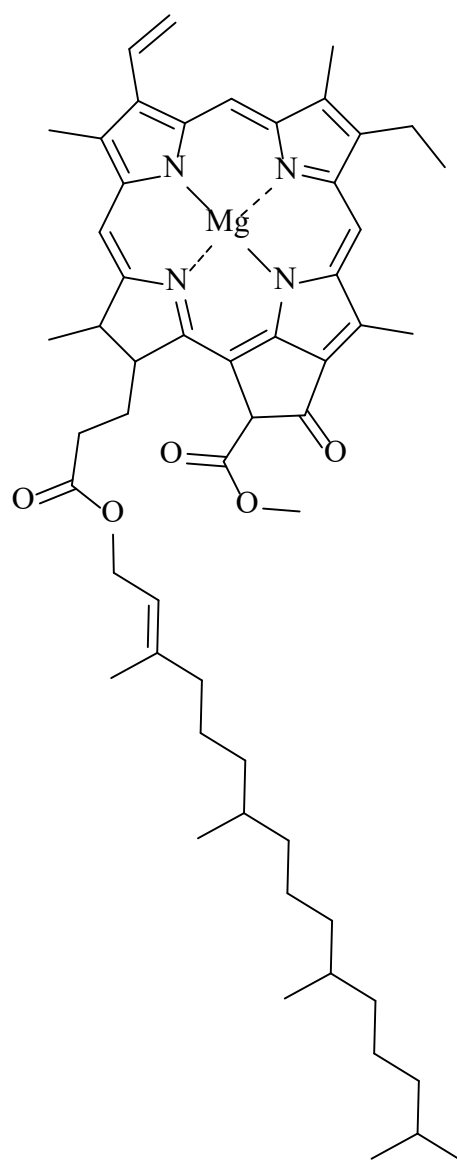


Experiment 9

Porphyryns



The Task

The first goal of this experiment is to extract a natural product (chlorophyll) from a green leafed plant (silverbeet). You then convert the chlorophyll to pheophytin, and investigate the latter's use as an optical sensor of metal ions.

Skills

At the end of the laboratory session you should be able to:

- make and use a fluted filter paper,
- use an ion exchange column,
- load, run and analyse a thin layer chromatography (TLC) plate,
- use a mortar and pestle.

Other Outcomes

- You will be able to understand the stick notation method of representing organic structures.
- You will develop an appreciation of the techniques used in organic chemistry and pharmaceutical science to extract useful materials from natural sources.
- You will develop an understanding of how thin layer chromatography is used to test sample purity.

The Assessment

You will be assessed on the quality of the thin layer chromatography plates you prepare. See Skill 10.



Introduction

The molecules in this experiment are shown as stick structures. See Skill 13 for an explanation. If you are enrolled in *Concepts in Biology* (BIOL1001 or BIOL1911) you may have already come across chlorophylls in a practical in that course. In Biology the emphasis is on their ability to absorb light and hence their effectiveness as photosynthetic pigments, whereas this experiment focuses on their ability to complex with metal ions.

Haem, vitamin B₁₂ and chlorophyll (see Figure 1) are all biologically important compounds that are composed of an organic molecule bound to a metal ion. They are examples of metal complexes, which consist of a metal ion surrounded by one or more ligand(s). A ligand is an ion or molecule that contains at least one atom, such as oxygen or nitrogen, that has the ability to bind to a metal ion through its lone electron pairs.

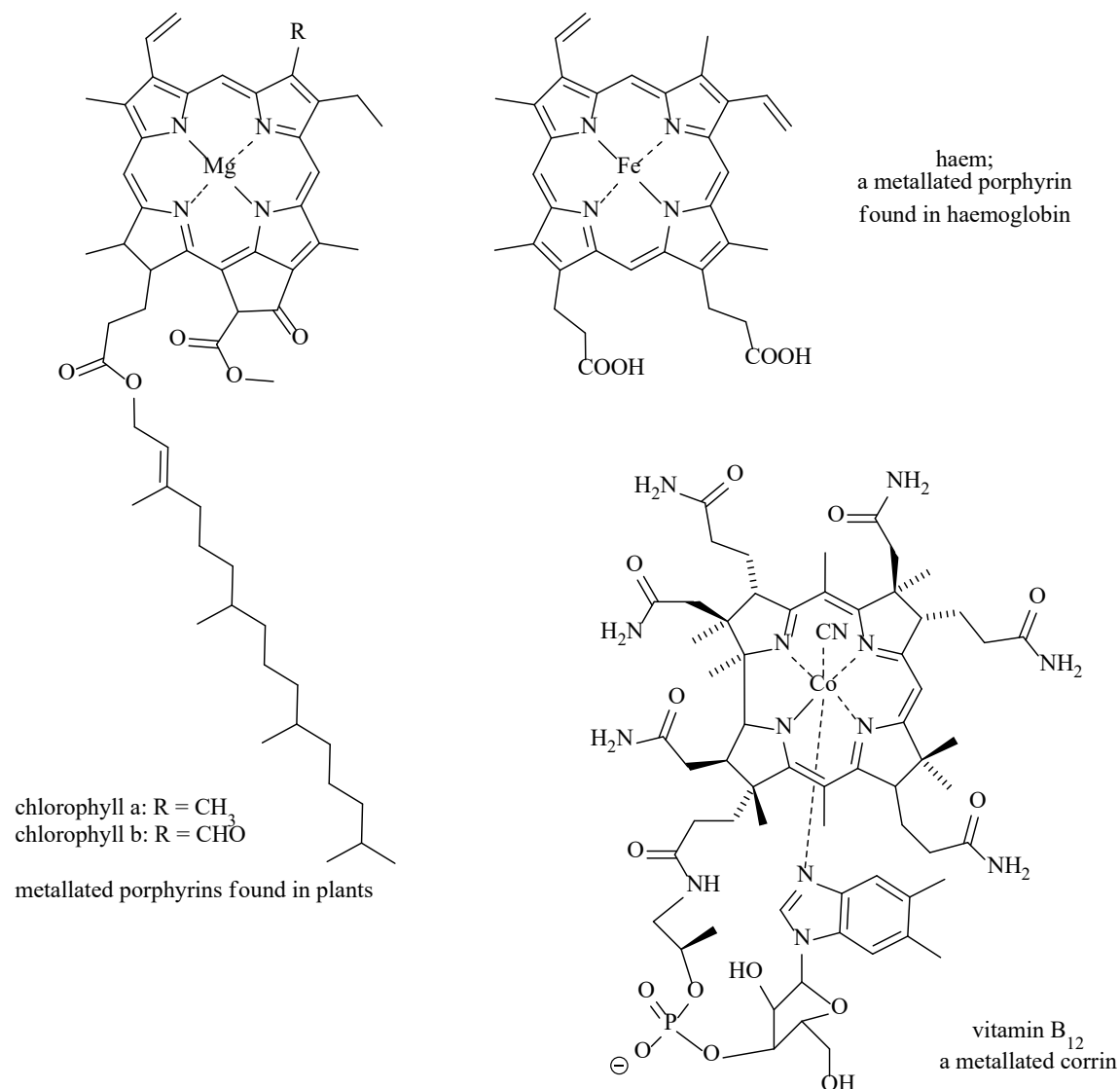


Figure 1. Naturally occurring porphyrins

All of the compounds shown above are known as metallated porphyrins or corrins. Porphyrins all contain the porphine functional group as a core (see Figure 2), but they have various substituents attached around the outside of the ring.

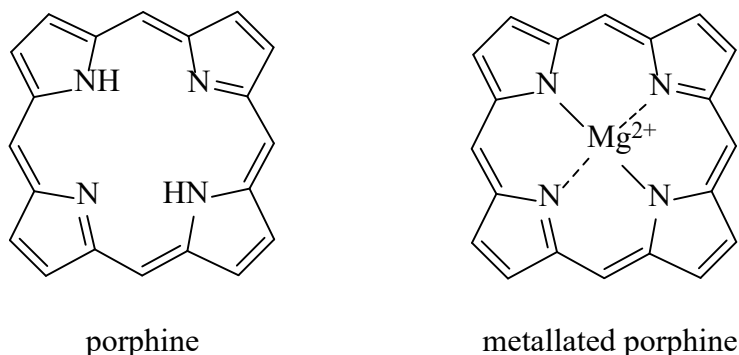
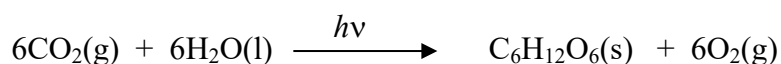


Figure 2. Porphine and its magnesium complex.

Photosynthetic Pigments

In green plants photosynthesis is a complex chain of redox (reduction-oxidation) reactions with the overall effect of reducing carbon dioxide to glucose and oxidising water to oxygen as shown below. Energy from light is used to drive the splitting of the water molecule to O_2 and H^+ ions, which takes place in chloroplasts (specialised organelles within plant cells).



The key function of chlorophyll molecules (concentrated in the chloroplasts) is to begin the sequence of reactions by capturing the energy of the light, converting it to chemical energy and passing this energy onto other species in the reaction chain *via* the movement of electrons and protons (H^+ ions). The ability to absorb light energy is essentially due to the system of alternating single and double bonds (conjugated π -system) of the chlorophyll porphyrin ring. The complexed magnesium metal ion acts to:

- help make the entire structure rigid, thus minimising the energy lost as heat *via* molecular vibrations;
- enhance the rate at which energy is transferred into the redox chain.

There are several similar but not identical chlorophyll molecules. Chlorophyll *a* is found in all green plants, chlorophyll *b* is found in all land plants and some green algae and chlorophylls *c* and *d* are found in other algae. In this experiment you will only be dealing with chlorophylls *a* and *b*.

Chlorophylls *a* and *b* strongly absorb blue and red light, but only weakly absorb green and yellow light. Consequently, when sunlight (white light) hits a leaf, the blue and red light is absorbed by the leaf and the green light is either reflected or passes through it entirely. Thus, the leaf appears green to our eyes.

Carotenoids are another class of pigment found in plants. They can be divided into carotenes (*e.g.* β -carotene), which only contain carbon and hydrogen, and xanthophylls (*e.g.* lutein), which are oxygenated derivatives (see Figure 3). The carotenoids are accessory pigments that also play a role in photosynthesis.

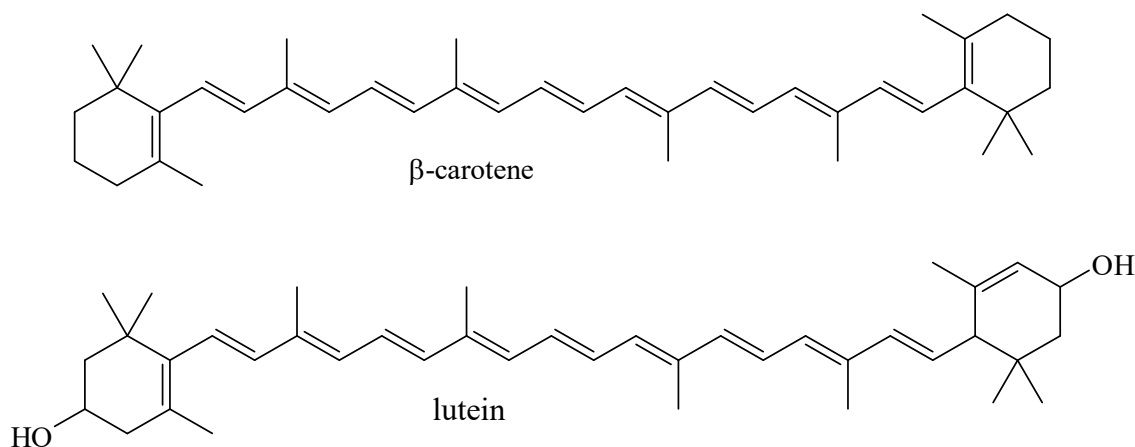


Figure 3. The structures of β -carotene and lutein.

In this experiment you will extract chlorophylls *a* and *b* from silverbeet leaves. You will then use an ion-exchange column to prepare the corresponding porphyrin ligands, pheophytin *a* and pheophytin *b*, by removing the magnesium ion from the chlorophylls (see Figure 4). You will then attempt to insert other metal ions into these ligands. To monitor the formation of new compounds you will observe any colour changes. Finally, to separate and identify the compounds you will apply the technique of thin layer chromatography (see Skill 10).

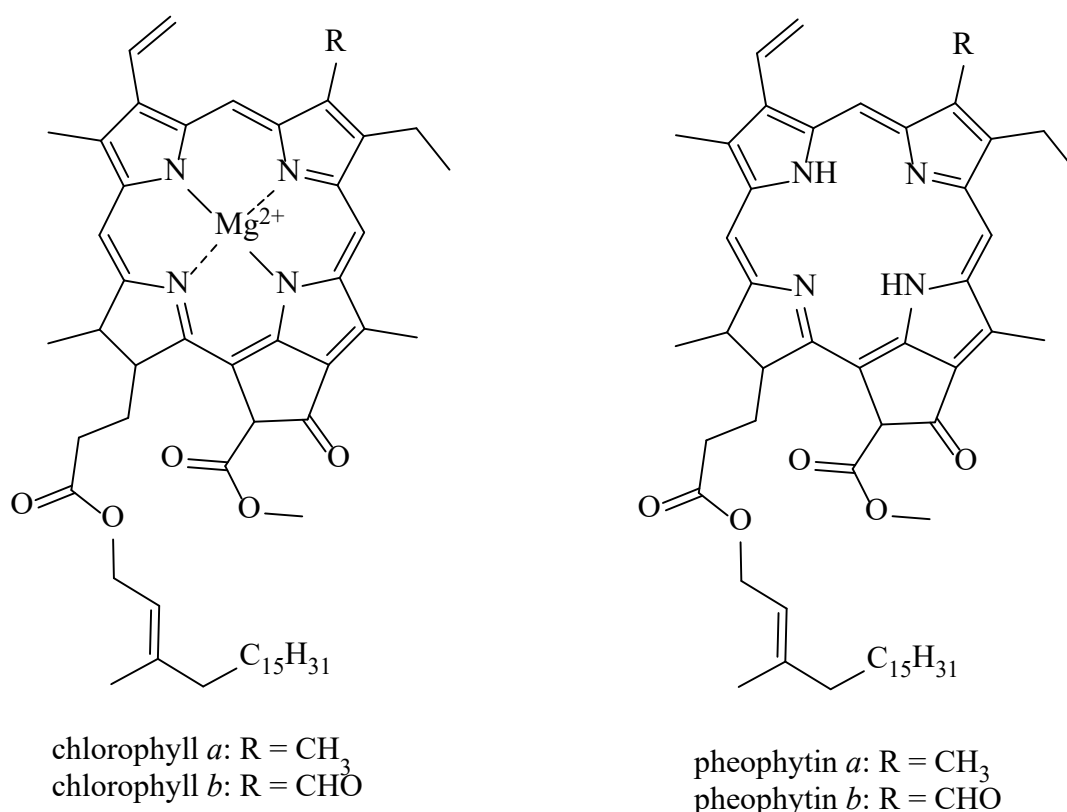


Figure 4. The structures of chlorophyll *a* & *b* and pheophytin *a* & *b*.

Chromatographic Techniques

In this experiment you will be using two chromatographic techniques: thin layer chromatography (TLC) and ion-exchange chromatography. Chromatographic techniques are used to separate the components of a mixture. They are based on differences in affinity of the components between a mobile phase (a solvent or a gas) and a stationary phase (a solid matrix). Separation can be on the basis of polarity (TLC), charge or charge density (ion-exchange chromatography), size, or a combination of these factors.

You will use ion exchange chromatography to separate the Mg^{2+} ion from chlorophyll and thus produce pheophytin. The ion-exchange columns contain Dowex-50W-X8, a cation-binding resin, as the stationary phase. The resin is made up of small spherical beads consisting of acidic SO_3H groups attached to an inert polymer. When a suitable solution is passed through the column, an exchange of ions occurs. The resin adsorbs Mg^{2+} ions in exchange for H^+ ions. Eventually the resin will become saturated with Mg^{2+} ions and the exchange of ions will cease. The activity of the resin can then be regenerated by flushing the column with HCl. This flushes the Mg^{2+} ions off the column again and replaces them with H^+ ions.

You will use TLC to determine which metal ions (apart from Mg^{2+}) are able to bind to the pheophytin ligand by observing the migration of metal complexes up the silica matrix of a TLC plate.

Safety

Chemical Hazard Identification

- acetone** – hazardous. Highly flammable, irritant, low to moderate toxicity.
- petroleum ether** – hazardous. Highly flammable, harmful, irritant.
- ethyl acetate** – hazardous. Highly flammable, irritant, low to moderate toxicity.
- methanol** – hazardous. Highly flammable, toxic, irritant.
- cyclohexane** – hazardous. Highly flammable, moderate toxicity, irritant.
- copper(II) chloride** – hazardous. Moderate toxicity, irritant.
- zinc chloride** – hazardous. Corrosive.
- cobalt(II) chloride** – hazardous. Toxic, irritant.
- iron(II) chloride** – hazardous. Moderate toxicity, corrosive, irritant.
- magnesium chloride** – non-hazardous. Low toxicity, irritant.
- magnesium sulfate** – non-hazardous. Low toxicity, low irritant.
- ion exchange resin** – hazardous. Low to moderate toxicity, irritant.

Risk Assessment and Control

Low risk.

Methanol is acutely toxic and must not be removed from the fume hood. Ensure your TLC plate is free of solvent before taking it to your desk.

Waste Disposal

Any remaining pheophytin solution needs to go into the “Non-Chlorinated Organic Waste” container in the fumehood. The metal ion solutions need to go into the “Heavy Metal” bucket in the fumehood. The solid silverbeet waste needs to go into the “Silverbeet Waste” container in the fumehood.

Experimental

This experiment is to be carried out in pairs.

Part A Extraction of chlorophylls from silverbeet

- (A1) Grind 5 g of silverbeet leaf with 5 g of magnesium sulfate (MgSO_4 , a drying agent) using a mortar and pestle until it becomes a dark green runny paste.
- (A2) Add 10 mL of acetone to the mortar and continue to grind until homogeneous.
- (A3) Add another 10 mL portion of acetone to the mortar and continue to grind until a thick green paste forms (1 - 2 minutes).
- (A4) Filter through a fluted filter paper (see Skill 6.1) in a short-stem funnel into a 100 mL conical flask.
- (A5) Wash the mortar and pestle with 2×5 mL portions of acetone and pour the washings into the filter paper.
- (A6) It may be necessary to aid filtration by gently agitating the residue in the filter paper with a glass rod. Take care not to break the filter paper. When filtration is complete, discard the filter paper and plant residue in the appropriate container in the fumehood.
- (A7) Put aside approximately 1 mL of your chlorophyll extract into a small labelled test tube - you will need this later for TLC analysis in Part E. Use the rest of the solution for Part B.

For your logbook:

Record the colour of the chlorophyll extract.

Part B Preparation of pheophytins

Your demonstrator will prepare ion-exchange columns for your group (one per bench) by flushing with 10 mL of hydrochloric acid solution (1.0 M) and then with 20 mL of deionised water followed by 10 mL of acetone. For the remainder of the laboratory session, each of you will need to wash the column with ~ 5 mL of acetone after you have used it.

(B1) Load the ion exchange column by gently pipetting the remaining chlorophyll extract onto the top of the column (see Figure 5). Keep topping it up until your extract is loaded. Collect the eluate (liquid dripping from the bottom of the column) into a 100 mL conical flask labelled “PHEOPHYTIN SOLUTION”.

(B2) When there is no more extract on the top of the column, flush the column with about 5 mL of acetone, collecting the eluate into the same flask as before. The column should be orange again and is ready for use by the next student.

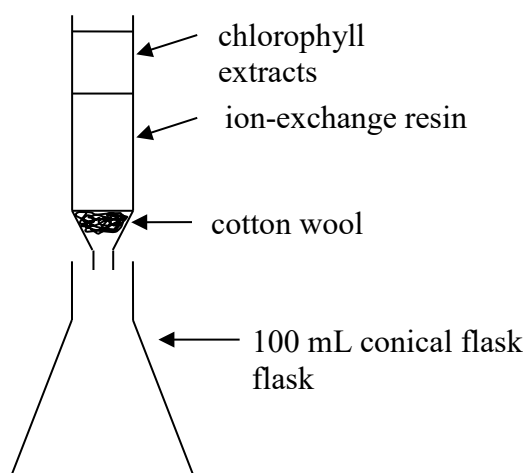


Figure 5: Set-up of the ion exchange column

(B3) Put aside approximately 1 mL of your pheophytin solution into a small labelled test tube for later analysis by TLC in Part E. Use the rest of your solution for Part C.

For your logbook:

Record the colour of your pheophytin solution.

Part C Adding other metal ions to pheophytins

(C1) In 4 large test tubes, appropriately labelled, add 0.15 g (approximately the tip of a nickel spoon - see the measured amounts at the front of the lab as a guide) of each of the following solids.

- ZnCl_2
- $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
- $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$
- $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$

(C2) To a fifth large labelled test tube add 0.5 mL of 2.5 M CuCl_2 solution.

(C3) Place 2 mL of your pheophytin solution from the ion exchange column into each of the test tubes and stir thoroughly with a glass stirring rod.

(C4) Keep your solutions for TLC analysis in Part E.

For your logbook:

Note the colour of each solution and compare with the original colour of pheophytin.

Using colour change as a possible indicator of metal binding, which ions would you suggest form complexes with pheophytin?

Part D Testing an unknown solution

(D1) Place 2 mL of your pheophytin solution from the ion-exchange column in a large test-tube. Add 0.5 mL of your unknown solution to the test-tube.

For your logbook:

Note the colour of the combined solution.

Based on its colour predict which metal ion is in the unknown solution.

Part E Analysis by TLC (Skill 10)

It is VERY IMPORTANT not to touch the silica layer of the TLC plate with your hands at any stage. Handle the plate by the edges only.

Preparation of the TLC Plate (Skill 10.1)

(E1) Collect a TLC plate that is 6 cm tall by 10 cm wide. Place the plate on a clean dry surface and, using a **lead pencil** and a ruler, draw a line 1.5 cm from the **long** (bottom) edge. Press lightly with the pencil so as not to damage the silica layer. On this line, mark points starting at 1.0 cm in from one side and then at 1.0 cm intervals to give 8 points. Using a pencil, lightly label the 8 points a, b, c, d, e, f, g & h. Put your initials at the top of the plate. Your plate should look like that in Figure 6.

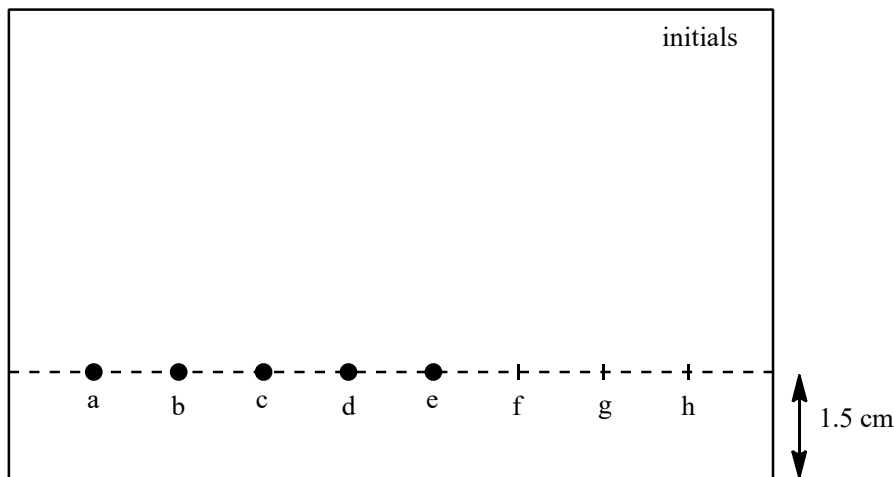


Figure 6: Preparation of the TLC plate

Loading the TLC plate (Skill 10.2).

(E2) When spotting the TLC plate, the aim is to have a small spot with a relatively large amount of solution on the plate. Practise spotting on a small practice plate as described in Skill 10.2 (2).

Spotting the TLC plate (Skill 10.2).

Many compounds are susceptible to decomposition and oxidation once loaded onto the plate, so it is good technique to load all samples as quickly as possible.

(E3) When you are confident in your spotting technique, spot the following solutions onto your TLC plate at the appropriate spots as described in Skill 10.2 (3) & (4). **You will need to use 4-6 touches for each analyte.**

- a) chlorophyll extract (from Part A)
- b) pheophytin solution (from Part B)
- c) CuCl_2 /pheophytin solution (from Part C)
- d) ZnCl_2 /pheophytin solution (from Part C)
- e) CoCl_2 /pheophytin solution (from Part C)
- f) FeCl_2 /pheophytin solution (from Part C)
- g) $\text{Mg}(\text{NO}_3)_2$ /pheophytin solution (from Part C)
- h) unknown solution (from Part D)

Developing the TLC plate (Skill 10.3)

The developing tanks are located in the fumehoods, out of direct sunlight. They already contain the appropriate mobile phase: 60% light petroleum, 16% cyclohexane, 10% ethyl acetate, 10% acetone and 4% methanol.

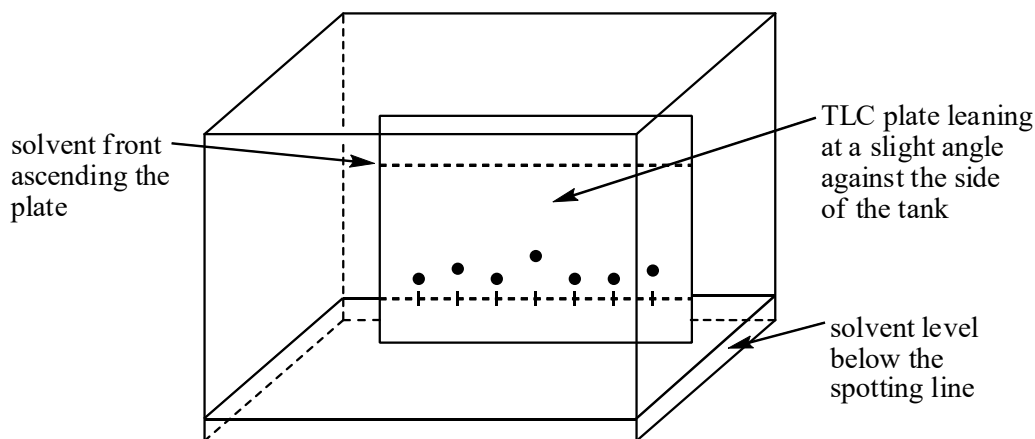


Figure 7: Solvent tank containing partially developed TLC plate

(E4) When all the spots are dry, place the plate into the solvent tank using a wooden peg to grip the plate at the very top. Make sure that the solvent level is below the baseline. Put the cover back on the tank. More than one plate can be put into a tank, but they must not touch each other. (See Figure 7.)

(E5) Allow the plate to develop until the solvent front is about 1 cm from the top (~5 minutes). Use a wooden peg to grasp the plate above the level of the solvent and remove it from the solvent tank. Immediately mark the position of the solvent front with a pencil and leave the plate on some paper towel in the fume hood to dry (~ 1 minute).

CAUTION: DO NOT REMOVE WET PLATES FROM THE FUME HOOD;

DO NOT LEAN INTO THE FUME HOOD TO MARK YOUR PLATE.

(E6) After the plate has dried, take it to your bench and rule a pencil line to show the solvent front.

Sketching and analysing the TLC plate (Skill 10.4)

The spots on the TLC plate can be characterised by their R_f values – a ratio of the distance the spot moves (x) relative to the solvent front (y). Figure 8 shows a developed TLC plate with two extracts, (A) and (B), similar to those in your extracts. It also includes a table of R_f values for the compounds that are contained in these two solutions for the solvent system you are using.

E9-12

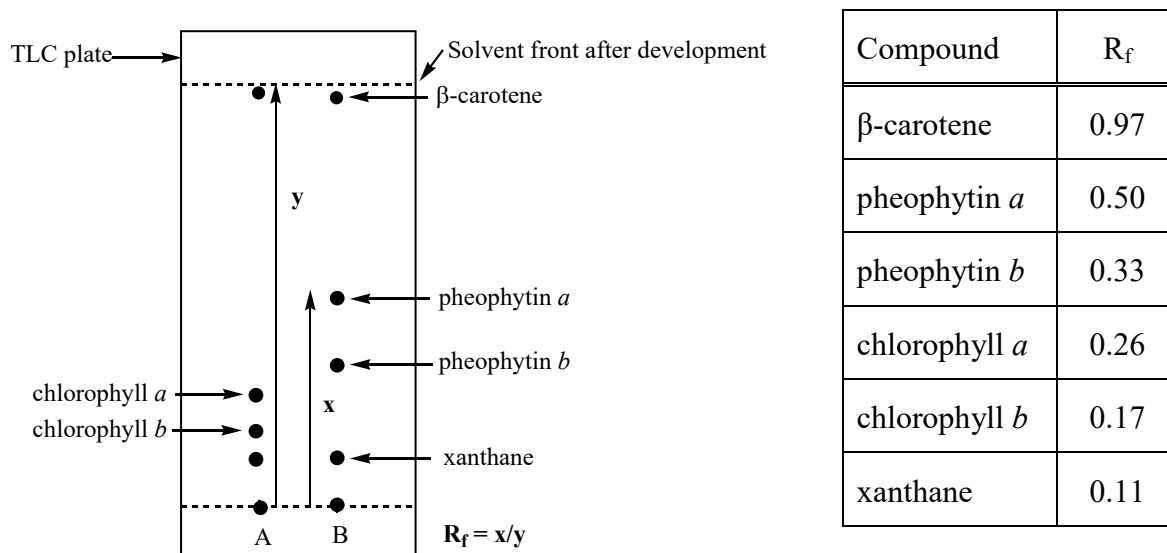


Figure 8: A diagram of a developed TLC plate for solutions A (spinach extract) and B (spinach extract after it has been processed), showing the different components in the solutions and a table of the various R_f values.

For your logbook:

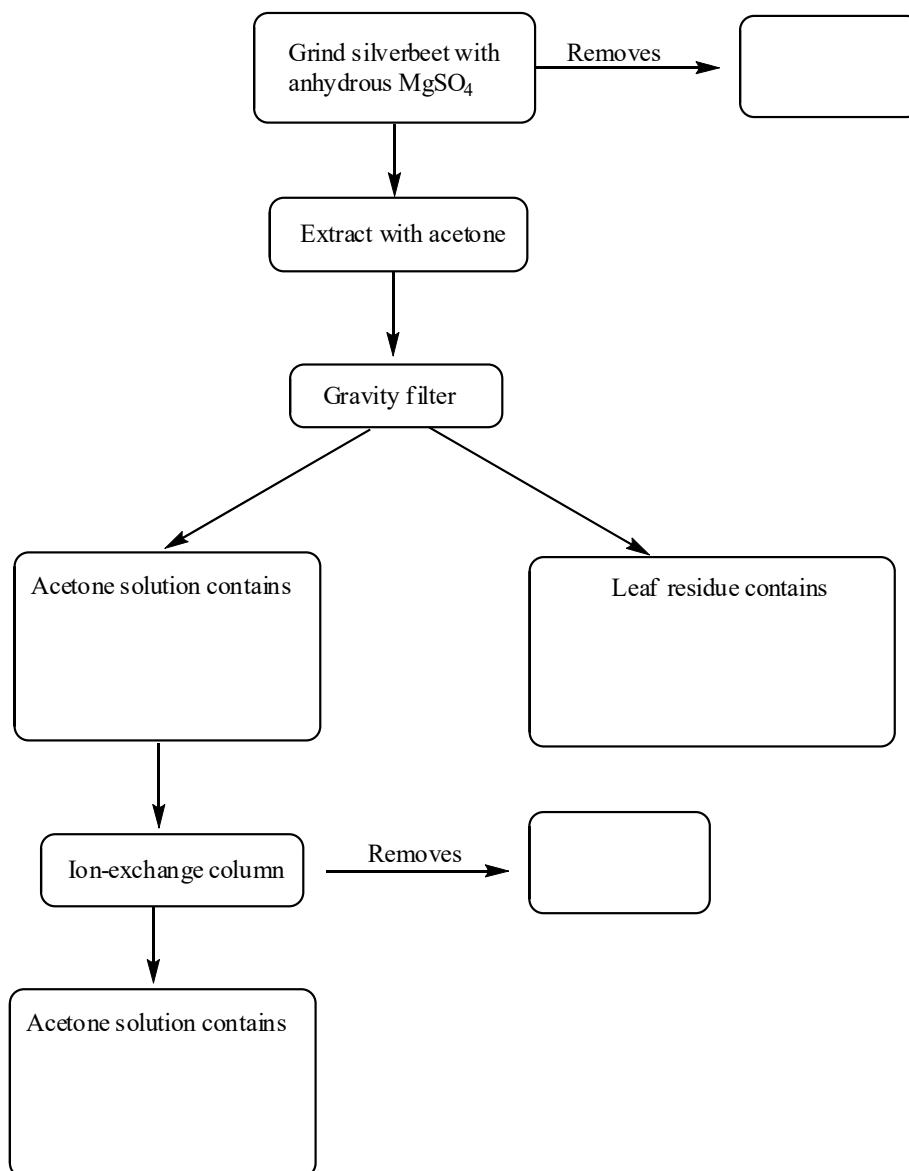
Record the colours and R_f values of the spots on your TLC plate.

Identify as many of the compounds as you can. Give as much detail as possible, justifying your assignment of each spot to a compound.

Make a sketch of your TLC plate in your logbook.

Group Discussion

A typical leaf of silverbeet would contain polysaccharides (such as cellulose and starch), water-soluble vitamins, pigments (such as chlorophyll, carotenes and xanthenes) and water. Below is a flowchart of your experiment today. What happens to the components of the leaf throughout the experiment? **Copy this diagram into your logbook and complete.**



From this experiment can you use the pheophytin ligand to detect all of the metal ions that you tested today?

What metal ion(s) can be detected?

What metal ion(s) did you determine to have in your unknown solution? Give reasons why?

What colour was the solution when cobalt was added to the pheophytin solution?

Did the TLC plate show the cobalt(II) ion had been inserted into the pheophytin?

What do you think could be happening?

[Other metals have been inserted into the pheophytin ligand, including the ones that we used today, however the ‘chemistry’ used to insert the metal ions is above first year chemistry. See reference - Michael Helfrich and Wolfhart Rüdiger, *Z. Naturforsch.* **47c**, pp 231-238 (1992).]

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