Separation of a leaf extract by column chromatography

Aims of the experiment

- To produce a leaf extract.
- To demonstrate column chromatography as a method for separating substances according to their adsorption properties.
- To understand the separation principle of column chromatography using silica gel as the stationary phase.
- To explain the order of elution of various leaf pigments based on their molecular structure.
- To understand the structural associations between various classes of leaf pigments.

Principles

Column chromatography is a frequently used method for separating mixtures of substances in the laboratory. The substances are isolated based on their adhesion properties. It functions according to the same principle as other chromatographic methods, but in contrast to these, it is used less for identification and more for the separation and purification of substances.

The substances to be separated are transported on a mobile phase (solvent mixture) through a stationary phase (in this case silica gel). While the substances to be separated are passing over the stationary phase, they are continually absorbed (they adhere to it) or desorbed (they return to the solution). Substances with a high affinity to the stationary phase spend on average a longer period of time in the adsorbed state and less time in the solution. Therefore they pass more slowly through the column than substances with a lower affinity to the stationary phase.

With column chromatography, the stationary phase is located in a cylindrical tube with a discharge tap on the underside. The mobile phase trickles through the stationary phase by gravitational force or is pumped through the stationary phase using compressed air (*Flash* chromatography). The various eluates are collected in separate vessels. These can then be investigated for their exact composition, e.g. by thin-layer

Fig. 1: Set-up of the experiment. Left: Production of the leaf extract. Right: Column chromatography.

chromatography, or used for preparative purposes.

A further development of column chromatography is HPLC (*high performance liquid chromatography*). Here, the substances to be separated are pumped under high pressure through the stationary phase together with the solvent mixture. A detector is located at the end of the column which detects the impacting substance.

In the experiment described here, the stationary phase used is polar silica gel and the mobile phase a solvent mixture consisting of slightly polar acetone (20 % by vol.) and non-polar petroleum ether (80 % by vol.). Silica gel is a polymer consisting of units of silicic acid (see Fig. 2). These produce a network through condensation. As the condensation is not complete, randomly distributed hydroxyl groups arise. Because of its porous structure, silica gel is amply permeable to non-polar solvents. On the other hand, substances with polar groups adhere to the hydroxyl groups or interact with the bridging oxygen and are thus retained.

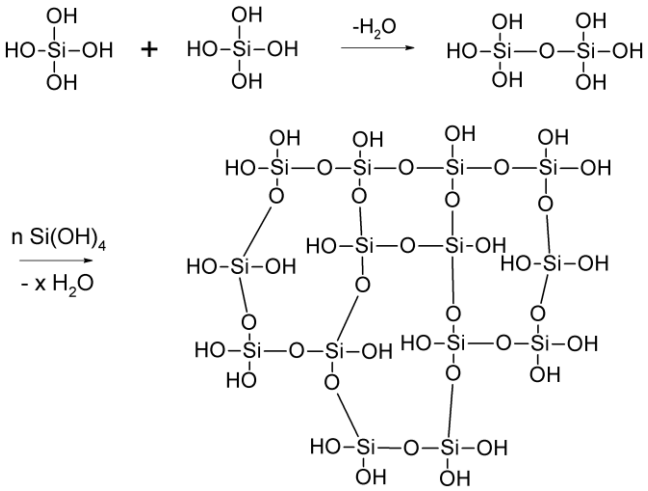


Fig. 2: Formation of silica gel by polymerisation.

Risk assessment

The solvent mixture used is a highly volatile and combustible substance that is harmful to health. For this reason, all stages of work in the experiment, including the production of the solvent mixture, must always be conducted in the fume cupboard. The apparatus must be kept away from fires.

A laboratory coat, protective glasses and gloves should be worn.

Acetone	
 **Signal word: Hazard**	**Hazard statements** H225: Highly flammable liquid and vapour. H319: Causes serious eye irritation. H336: May cause drowsiness or dizziness. EUH066: Repeated exposure may cause skin dryness or cracking. **Precautionary statements** P210: Keep away from heat/sparks/ open flames/hot surfaces. No smoking. P233: Keep container tightly closed. P305+P351+P338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.
Petroleum ether, boiling point range: 90°C - 110°C	
 **Signal word: Hazard**	**Hazard statements** H225: Highly flammable liquid and vapour. H304: May be fatal if swallowed and enters airways. H315: Causes skin irritation. H336: May cause drowsiness or dizziness. H411: Toxic to aquatic life with long-lasting effects. **Precautionary statements** P101: If medical advice is needed, have product container or label at hand. P102: Keep out of reach of children P103: Read label before use P210: Keep away from heat. No smoking. P260: Do not breathe vapour/aerosol. P262: Do not get in eyes, on skin, or on clothing. P243: Take precautionary measures against static discharge. P301+P330+P331: IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. P403+P233: Store in a well ventilated place. Keep container tightly closed.

Equipment and chemicals

For producing the leaf extract

1 Beaker, DURAN, 100 mL, nF 664 101
1 Porcelain mortar, 70 mm diam. 667 092
1 Pestle 100 mm long 667 091
1 Funnel, Boro 3.3, 100 mm diam. 665 005
1 Round filter Type 595, 150 mm diam., 100 pcs 661 038
1 Narrow-neck bottle, amber glass, 100 mL 661161
1 Warning notice according to GHS 661 0771
1 Filtration stand for 2 funnels 666 584
1 Sea sand, purified, 1 kg 674 8210
1 Acetone, 1 l 670 0410

Additionally required:
dried or fresh leaves/grasses

Column chromatography

1 Chromatography column 235 x 20 mm diam. ... 665 592
1 Dropper funnel, glass, 75 mL, SB 29, graduated 665 073
1 Beaker, DURAN, 100 mL, squat 664 101
1 Beaker, Boro 3.3, 600 mL, tall 602 012
1 Laboratory bottle to DIN, 500 mL, GL 45 ..... 602 347
1 Warning labels according to GHS 661 0771
1 Measuring cylinder 100 mL, plastic base 665 754
1 Measuring cylinder 500 mL, plastic base 665 756
1 Compact balance 200 g : 0.01 g 667 7977
1 Glass stirring rod, 500 / 8 mm diam., 10pcs 665 217
1 Powder funnel, PP, 100 mm diam. 665 025
1 Test tube rack, plastic, 18 mm diam. 667 050
10 Glass test tube, Fiolax, 16x160 mm, set of 10 . 664 043
9 Rubber stopper, solid, 14...18 mm diam. 667 253
1 Spoon-ended spatula, PP, 180 mm 666 966
1 Dropping pipette, 150 x 7 mm, set of 10 ..... 665 953
1 Rubber bulbs, set of 10 ..... 665 954
1 Stand base, V-shaped, small 300 02
1 Stand rod 47 cm, 12 mm diam. 300 42
2 Bosshead S 301 09
2 Universal clamp 0...80 mm 666 555
1 Petroleum ether, 90...110 °C, 250 mL 670 8200
1 Glass wool, 10 g 672 1000
1 Acetone, 1 L 670 0410
1 Silica gel 35-70 mesh, 500 g 661 058
1 Stopcock grease, 60 g 661 0821

Set-up and preparation of the experiment

Producing the leaf extract

1. Rub the dried leaves or grasses between your fingers. Place sufficient crushed plant material into the mortar to fill it about one third.

2. Add some sea sand and sufficient acetone (2-propanone) to fully saturate everything with extraction medium.

3. Using the pestle, grind the plant material in the mortar together with the sand for about five minutes.

4. Then add a few millilitres of acetone and grind for around a further two minutes.

5. Carefully place the funnel in the filtration stand (see Fig. 1). Place a beaker under the funnel exit.

6. Create a conical filter from the round filter paper and place it in the funnel.

7. Then moisten the filter with acetone and filter the contents of the mortar.

8. Allow the filtrate to evaporate for a short time in the fume cupboard in the dark. Then pour it into an appropriately labelled brown glass reagent bottle and attach the warning labels. Store this plant pigment in the dark.

Preparation of the column chromatography

Note: Before performing the experiment, grease the taps and check them for liquid flow.

Produce the solvent mixture consisting of 20 % by vol. of acetone and 80 % by vol. of petroleum ether by mixing 50 mL of acetone with 200 mL of petroleum ether in a laboratory bottle with a screw cap. It is necessary to use a bottle with a screw cap, as if left to stand in the air for a longer period, the composition of the solvent mixture can change in the case of highly volatile substances.

Cover the base of the chromatography column with some glass wool (press it down with a glass rod). Attach the column to the stand using a universal clamp. Place the large beaker beneath it. Weigh 15 g of silica gel into a 100 mL beaker. Pour a little solvent mixture onto the silica gel and stir well with a glass rod until a gel-like mass results. Then fill the column with this mass using a powder funnel. This will require a little solvent mixture to be added occasionally so that the silica gel sludge enters the column completely. Collect any residual liquid in the column in a beaker. Fill the column up to about 3 cm below the rim.

Performing the experiment

Attach the dropper funnel above the chromatography column. Allow solvent mixture to run out of the column until the liquid level reaches the upper edge of the gel. Now place a small amount of leaf extract onto the column using a dropping pipette. One to two millimetres of liquid above the column is sufficient. Open the tap and wait until the column has completely absorbed the leaf extract.

The addition of solvent mixture can now commence. Fill the dropper funnel with solvent mixture. Adjust the two taps such that the drop frequency is approximately equal for both. Should the column run dry, add a little more solvent mixture to the dropper funnel. Take care to note when the eluates of various colours reach the lower edge of the column so that they can be collected in test tubes.

Note: Optionally, the eluates collected can be investigated for purity by thin-layer chromatography using the same solvent mixture.

Observation

Bands of different colours can be identified in the column. The bands are orange, yellow, green and blue-green.

Result of the experiment

Leaf pigments enable photosynthesis to take place in plants. They belong to the light-collecting complexes in the thylakoid membranes of the chloroplasts. The centre of these complexes is formed by two molecules of chlorophyll a. The charge separation takes place on this chlorophyll pair which facilitates the oxidation of water to oxygen gas. Chlorophyll b, carotenes and xanthophylls have the function of extending the absorption spectrum of chlorophyll a so that more light can be absorbed. Carotenoids (carotenes and xanthophylls) also protect chlorophyll a from destruction through photo-oxidation.

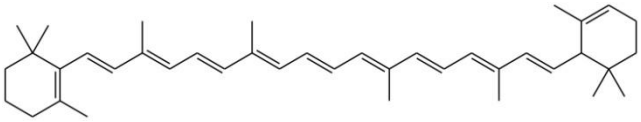
Various leaf pigments can be identified by column chromatography, depending on the type of leaves used.

Order of possible components:

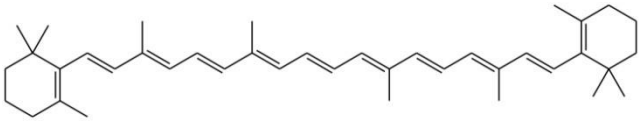
1. Carotenes: yellow-orange
2. Xanthophylls (lutein): yellow
3. Xanthophyll epoxide (violaxanthin): yellow
4. Chlorophyll a: blue-green (dark green)

5. Chlorophyll b: light green (yellow-green)
6. Neoxanthin: yellow

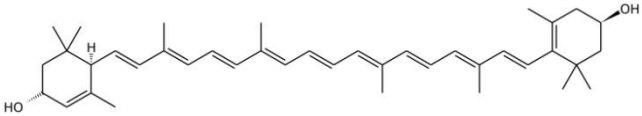
α-carotene (yellow-orange)



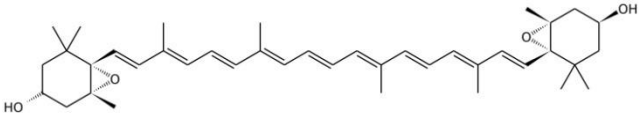
β-carotene (yellow-orange)



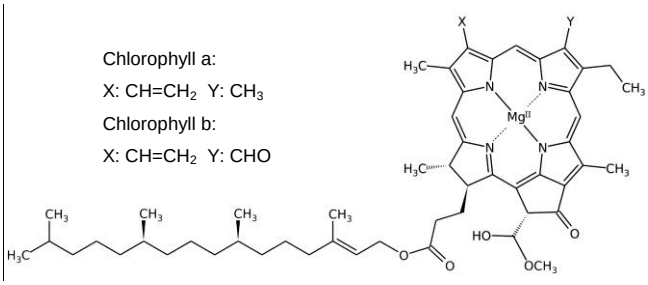
Lutein (yellow)



Violaxanthin (yellow)



Chlorophyll (green)



Neoxanthin (yellow)

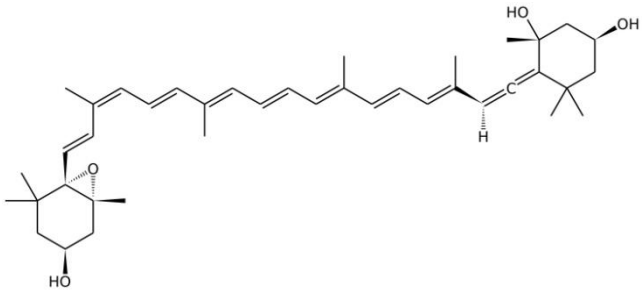
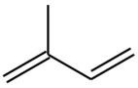


Fig. 3: Structural formulas and colours of leaf pigments.

The separation takes place based on the polarity of the substance to be separated. Completely non-polar leaf pigments, such as carotenes, are readily soluble in the solvent mixture and hardly interact with the hydroxyl groups of the silica gel. They migrate together with the solvent front.

Xanthophylls such as lutein are derived from the carotenes and together with these form the carotenoids. Both have the same basic structure, consisting of eight isoprene units (see Fig. 4).

Isoprene



Porphyrin

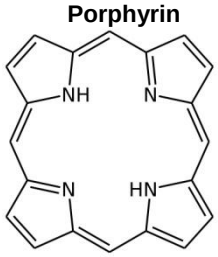


Fig. 4: Basic building blocks of leaf pigments.

In contrast to the carotenes, the xanthophylls contain oxygen-containing polar groups such as carbonyl, hydroxyl and epoxide groups. Their position in the separation depends on the number and type of polar groups. Hydroxyl groups lead to a stronger polarity than carbonyl or epoxide groups. They therefore interact more strongly with the hydroxyl groups of the silica gel.

The order of elution of the three xanthophylls lutein, violaxanthin and neoxanthin can be explained by this, as an example: Lutein has two hydroxyl groups. Violaxanthin has two epoxide groups in addition, which leads to a stronger interaction with the stationary phase. Violaxanthin therefore runs more slowly than lutein. Neoxanthin has only one additional epoxide group, but on the other hand a third hydroxyl group. As hydroxyl groups interact more strongly with the stationary phase than epoxide groups, neoxanthin runs the slowest of these three.

The chlorophylls a and b run between violaxanthin and neoxanthin. Their basic structure is derived from porphyrin (see Fig. 4), in the centre of which there is a magnesium ion coordinated by four nitrogen atoms.

The polarity of the chlorophylls stems on the one hand from the doubly charged positive magnesium ion in the centre of the porphyrin ring, and on the other from the carbonyl and ester functions of the side chains. Chlorophyll b runs more slowly than chlorophyll a, as it has an additional aldehyde group and therefore interacts more strongly with the stationary phase.

Cleaning and disposal

Place the chromatography column in the fume cupboard to dry. After drying, the silica gel can be removed from the column with a spatula and disposed of. Only fully dried silica gel may be disposed of in the waste.

It is not practical to reclaim the solvents by distillation, as the composition would change in this case. Remaining solvent mixture can be stored in a closed container for future experiments. Place used solvent in the collecting container for halogen-free solvents.

Degrease the taps of the glass equipment and store them separately.