What makes autumn so colourful?

Into the world of pigments and colours in nature

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Summer School of Science 2018
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Abstract

The purpose of this project was to gain a better understanding of how and why plants change colour in autumn by studying the biology of pigments. Samples collected in nature served as an introduction to the pigments and proteins involved in photosynthesis. Ultimately, autumn-like conditions were simulated to identify the changes plants go through when changing colour.

1. Introduction

Autumn is the part of year when everything changes. There is less energy from the Sun, more rainfall and leaves change colour. Photosynthesis is an endothermic process and the energy needed for the reaction is obtained by absorbing light in chloroplasts. Chloroplasts contain different types of pigments which are materials that change the colour of reflected or transmitted light as the result of wavelength-selective absorption. Each pigment absorbs a different wavelength of light. This light energy is converted to chemical energy which is responsible for the growth of plants and cyanobacteria. To convert energy, the first step is to absorb that energy. Each compound that absorbs certain wavelengths of light also reflects certain wavelengths of light. Colours that human eyes can see are the light that is reflected from the surface of the object. Also, big role in this have proteins that are bound to some of the pigments. [1]

2. Aim

The main aim of this project is to get to answer the question of why autumn is so colourful, what it all affects, which pigments are bound to proteins. However, to get to these answers first the surface layer and cross-section of a leaf under a microscope needed to be

visualised. Also, another goal was to extract pigments from different plant sources and determine the absorption spectrum of crude extracts of plants.

3. Pigments in nature

As written in *Introduction*, pigments are materials in chloroplasts that change colour by absorbing and reflecting different wavelengths of visible light. Visible light is in the range from 400 nm to 700 nm. When this light encounters a pigment, parts of the spectrum are absorbed by the pigment. In nature, pigments can be found in plants and flowers, but also in the human body (skin, eyes, hair, etc.). Primary pigments in plants are chlorophylls (a and b), carotenoids and anthocyanins [2].

3.1. Materials and methods

3.1.1. Sample

The samples that were used in the experiments were freshly picked for the day. They were picked from an embankment between road and stream, a park and a garden in Požega, Croatia. The selected leaves were preferably multicoloured (e.g. green in the middle and red on the edges). Diseased leaves were also a good choice, due to their colour loss. The ones picked were maple (*Acer pseudoplatanus*), sweet cherry (*Prunus avium*) and grape (*Vitis vinifera*) (figure 1).

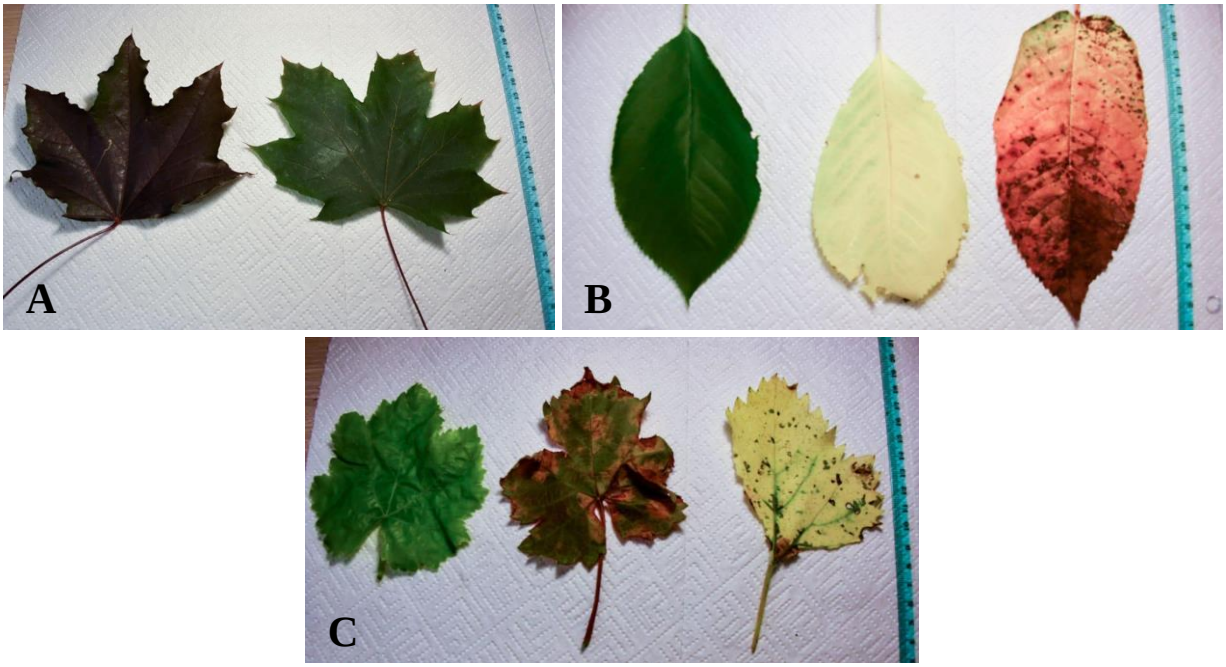


Figure 1. Examples of sampled leaves. Showing maple (A), sweet cherry (B) and grape (C).

3.1.2. Microscopy

All microscopy was done using several light microscopes. The samples were observed under 10X and 40X magnification. To cut the samples in small pieces that would let light through, a small portion of carrot was used to serve as a rudimentary microtome. The samples were put in it and then cut into fine pieces. The slices were placed on a microscope slide with a drop of water and the coverslip on top. The sample sat in water for 5 minutes whilst being observed every 50 to 60 seconds. The whole procedure was repeated two more times, except the first time acetone was added instead of water and the second time, hexane. Again, the sample sat in acetone and hexane for 5 minutes and was observed in the same intervals as with water. A fresh piece of the sample was cut for each solvent.

3.1.3. Extraction and separation of pigments (TLC)

For this experiment, samples needed to be prepared for extraction. Each leaf was torn into small pieces and 0.5 g was weighed for extraction. To extract the pigments from the leaf, some kind of solvent would need to "melt" the leaf, in this case acetone. First, small pieces of leaves were crushed with a mortar and pestle, then 1 ml of acetone was added and the crushing continued. The procedure was repeated two more times until 0.8 to 1.0 ml of extracted pigments was collected. This was done for all three leaf samples. When the crude extract is collected from the mortar, some small pieces of crushed leaves and even smaller material get picked up as well. To prevent that from corrupting the experiment, the collected crude extract was put in a centrifuge and centrifuged it on 5,000-10,000 RPM for 5 minutes. Now there is a clear separation of pigments and residue, from which the pigments can be pipetted out. This was the preparation for the thin-layer chromatography (TLC).

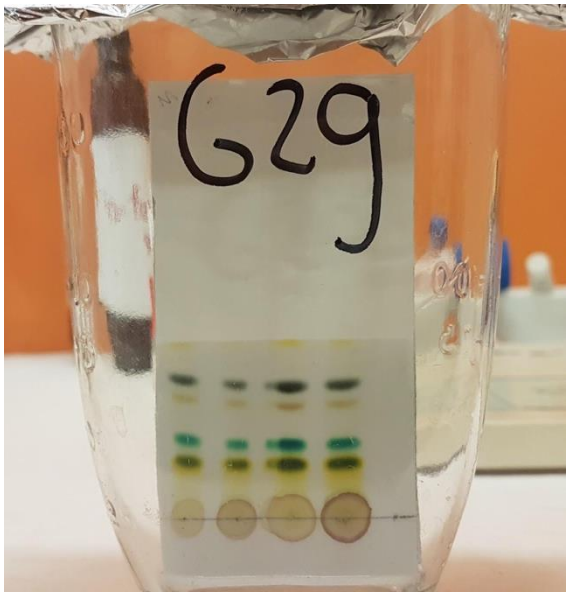


Figure 2. TLC chamber with plate inside.

The point of running the TLC is to separate different pigments from the whole pigment mixture and later identify them. Silica plates cut in rectangular shapes with dimensions 6 x 10 cm were used as the stationary phase. Then a baseline for the spotting was drawn 1.5 cm from the bottom of the silica plate with 4-5 spots marked 1 cm apart. Since silica is polar, a mixture

of highly non-polar organic solvents made of hexane, acetone, and water in 3:1:1 ratio was used as the mobile phase. Samples were spotted on the 4 or 5 marked spots in different amounts – some 20 μl (1X) and others 40 μl (2X). After that, the silica plate with spotted samples was put in a glass chamber and the solvent of hexane, acetone, and water was poured in until it got close to the baseline. Each TLC was run for 10 minutes. Finally, the retention factors (R_f values) were calculated for every band of pigment according to the formula:

$$R_f = \frac{pigment\ band\ distance\ from\ baseline}{solvent\ front\ distance\ from\ baseline} \qquad (1)$$

TLC works due to capillary action between organic solvent and the silica plate. As previously mentioned, the silica plate is polar and the organic solvent is non-polar which means that the polar compounds from the spotted samples make interactions with the stationary phase, resulting in a slower rise from the baseline in the non-polar mobile phase. On the other hand, the more hydrophobic non-polar compounds do not interact with the silica plate, resulting in a faster rise.

3.1.4. Absorption spectrum of pigments

An absorbance spectrophotometer is an instrument that measures the amount of light that passes through a sample. This instrument is designed to diffuse different wavelengths of light in order to determine the optimal absorption. In principle, the light from an incandescent bulb is refracted by a prism into a different portion of the spectrum, which passes through a narrow slit and hits the sample. The light that has passed through the sample is detected by a photocell that measures the absorbance value.

The absorbance of crude extract of plants was measured first. The sample was prepared by crushing 0.5 g of leaves with 0.5 ml of acetone using a mortar and pestle. Then the sample was centrifuged for 3 minutes at 1,000 g. Finally, 1.0 ml of a 50X dilution of the extracted supernatant in acetone was prepared and put inside a cuvette and placed inside the spectrophotometer along with a cuvette with 1.0 ml of acetone serving as a blank. After blanking the instrument, the absorbance was measured for wavelengths between 400 and 800 nm with regular intervals of 25 nm, as well as for 350 nm.

The absorbances of the different individual pigments were also measured. To begin, the desired pigment bands were scraped off of the TLC plate and diluted in 1.0 ml of acetone each. The rest of the procedure was the same as previously described.

3.1.5. Visualisation of protein-pigment complexes using PyMOL

PyMOL is visualisation software that allows the observation of the chemical structures of biomolecules [5]. Before working with the program, the photosystem proteins were overviewed using UniProt, an online database [6], in order to better understand the function and their sequence of amino acids, i.e. primary structure. Using another online tool called Clustal-Omega [7], different sequences of amino acids were compared. Finally, using PyMOL, the structure of the photosystem protein and its interactions with other molecules were studied. It

was observed that chlorophylls interact with the most hydrophobic part of the protein and the beta-carotene mainly bonds with the chlorophyll**.**

3.1.6. Isolation of membrane proteins (protein-pigment complexes)

Having identified what pigments can be found in different types of leaves, the next step was isolating the proteins these pigments are bound to within the cell, or rather the chloroplast (as described in *Introduction*).

First, the buffers presented in the table below were prepared:

Extraction buffer	Wash buffer	Solubilization buffer TYPE 1	Solubilization buffer TYPE 2
– 500 mM Tris-HCl pH 7.5 – 0.4 M sucrose 10 ml	– 50 mM Tris-HCl pH 7.5 – 10 mM NaCl – 5 mM $MgCl_2$ 10 ml	– 50 mM Tris-HCl pH 7.5 – 10 mM NaCl – 5 mM $MgCl_2$ – 10% Glycerol – 0.5% SDS 10 ml	– 50 mM Tris-HCl pH 7.5 – 10 mM NaCl – 5 mM $MgCl_2$ – 10% Glycerol – 0.1% Triton-X-100 10 ml

To begin the extraction procedure, 2 g of green and purple maple leaves were weighed and finely ground in a mortar and pestle with added acetone. The ground leaves were transferred into Eppendorf tubes and centrifuged at 500 g for 3 minutes. The supernatant was collected in a new tube and centrifuged again at 3,000 g for 10 minutes. The supernatant was removed and the pellet washed with 500 µl of wash buffer prepared earlier. It was then centrifuged at 10,000 g for 10 minutes and the same process was repeated once again before the supernatant was discarded and the pellet was resuspended using 50 µl of solubilization buffer type 1 and type 2. This mixture was incubated and later centrifuged again at 10,000 g for 5 minutes, after which the supernatant was collected.

To observe the isolated proteins, they were to be separated using native polyacrylamide gel electrophoresis (native PAGE). However, agarose 2% gel had to be used in place of the polyacrylamide gel, meaning the separation of Photosystems I and II would not be possible, but the whole protein-pigment complexes would be isolated instead. Equal amounts of green/purple maple leaf extract were added to each well.

3.2. Results

As leaves collected in nature vary in species, size and colour, results were not quantified, but important differences were noticed.

3.2.1. Microscopy

Stomata (figure 3) were noticeable on both sides of the leaf, but more numerous on the back of the leaf in all three species.

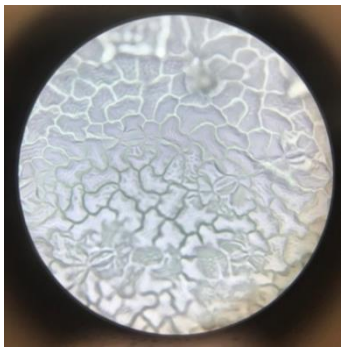
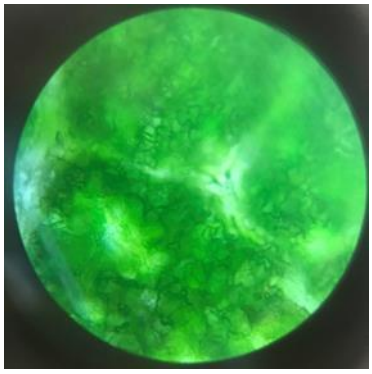
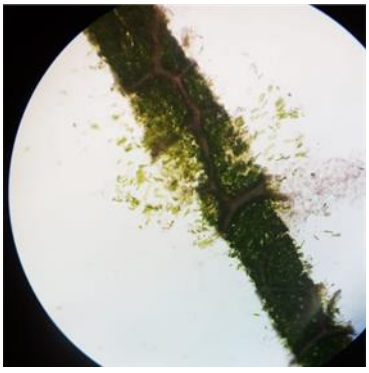
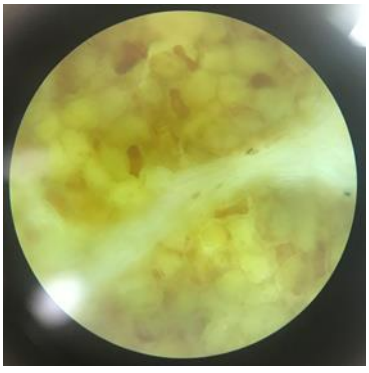
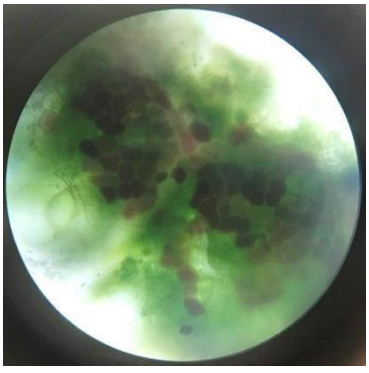
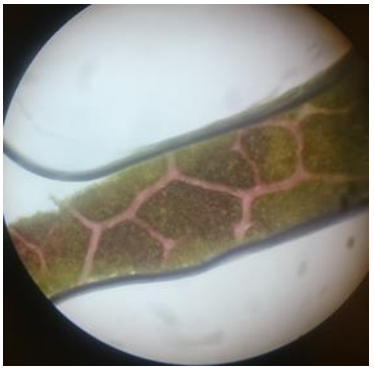


Figure 3. Stomata in leaf cross-section observed under light microscope.

Cherry leaves were found in green, yellow and orange colour; maple leaves in green and purple and grape leaf in green, yellow and red. Due to the quality of the pictures, not all types of these leaves are given.

In water

Table 1. Cross-sections of different leaves observed under light microscope.

Cherry leaf	Maple leaf	Grape leaf
Green		
		
Yellow	*Purple*	*Red*
		

The photographs of cross-sections in acetone and hexane are not as good, therefore results will only be described.

In acetone

Sections slowly started getting lighter in colour; however, colour did not change drastically in the middle of the section. After approximately five minutes, the sections shrank and became darker, especially on the edges.

In hexane

The changes were noticeably faster – colours became lighter almost immediately after exposure to hexane, even in the middle. The membranes of the cells seemed to disappear. Even before five minutes of immersion in hexane, edges turned almost black and the whole section shrank.

3.2.2. Thin layer chromatography

The pigments were best separated by a solvent made out of hexane, acetone, and chloroform in a ratio 3:1:1, as further described in *Materials and methods*. Some bands were curved up, not parallel with the baseline (figures 4 and 5), which is due to the shape of the chamber and how the solvent moved.

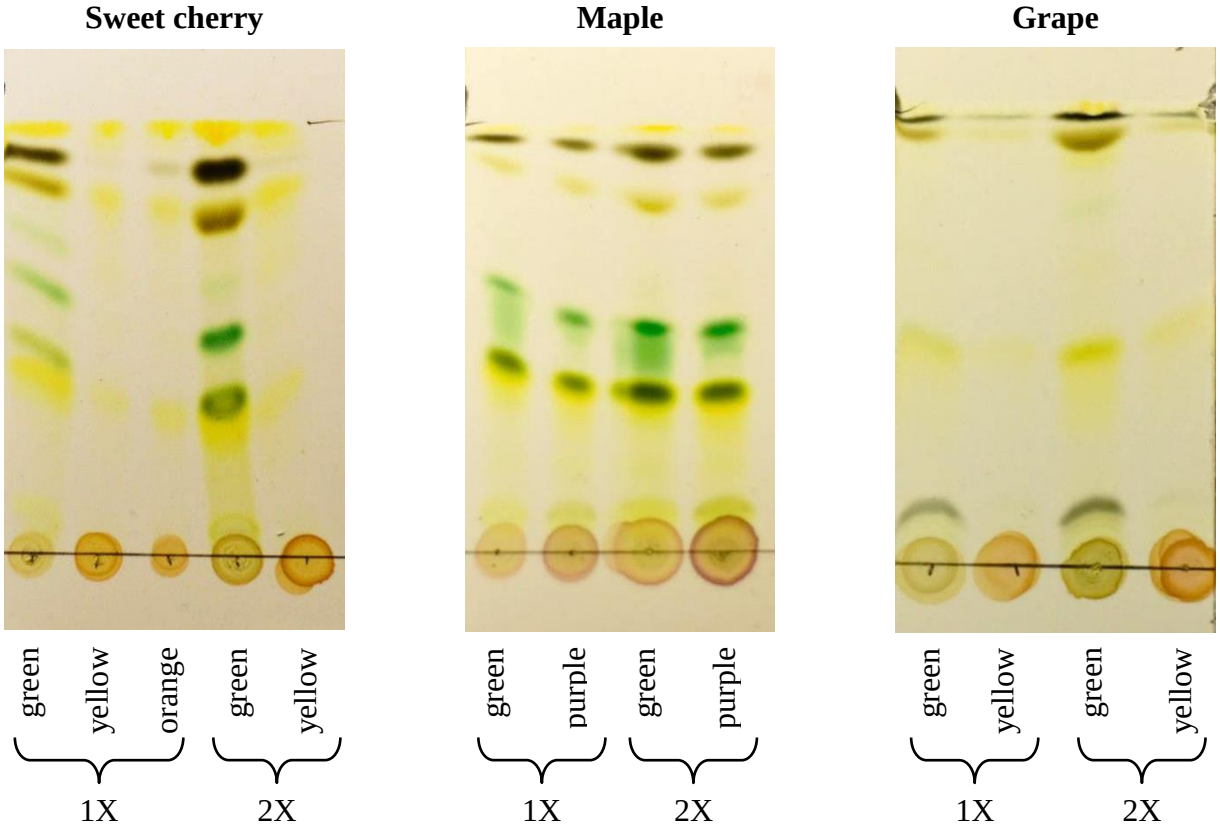


Figure 4. TLC of extracts from different leaf samples. Each colour has been spotted in two different concentrations (1X = 20 µl); orange cherry leaf extract was only spotted once.

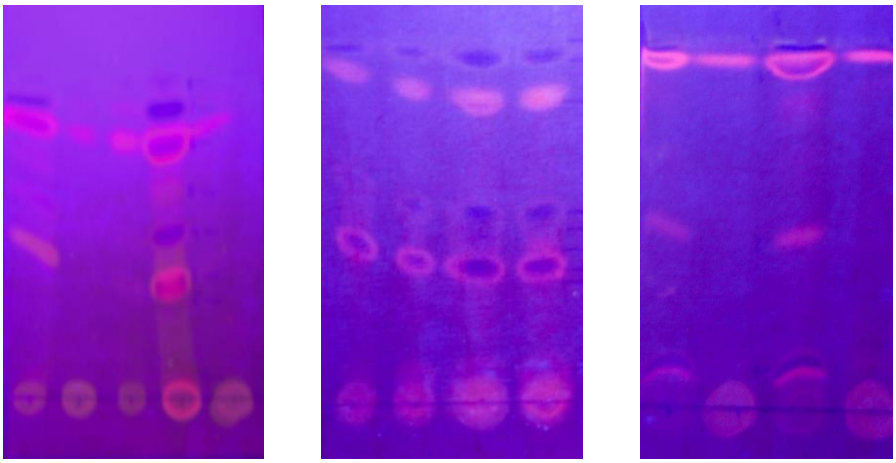


Figure 5. TLC plates shown in figure 4 (cherry, maple and grape respectively), seen under UV light.

TLC was repeated for extracts of green leaves only.

Retention factors were calculated for each distinguishable band using formula (1) (tables 2, 3 and 4) and later compared to results from literature and absorbance spectrum.

Table 2. R_f values for separated bands of pigment in green sweet cherry leaf extract.

Band no.	R_f
1	0.06
2	0.30
3	0.38
4	0.51
5	0.78
6	0.89
7	0.97

Table 3. R_f values for separated bands of pigment in green maple leaf extract.

Band no.	R_f
1	0.12
2	0.33
3	0.38
4	0.53
5	0.80
6	0.93
7	0.97

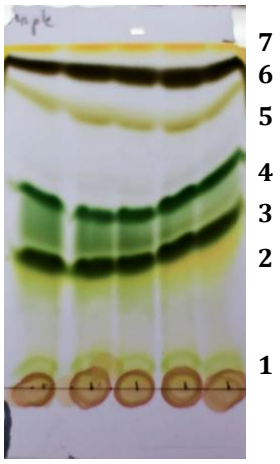
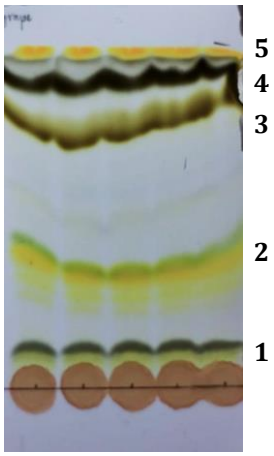


Table 4. R_f values for separated bands of pigment in green grape leaf extract.

Band no.	R_f
1	0.09
2	0.41
3	0.89
4	0.94
5	0.98



Separated bands and R_f values for cherry and maple are very similar, therefore absorbance of separated pigments was measured only for maple.

3.2.3. Absorption spectrum of pigments

Absorbances of crude extracts of leaves were measured as described in *Materials and methods* and plotted against their respective wavelengths (figure 6).

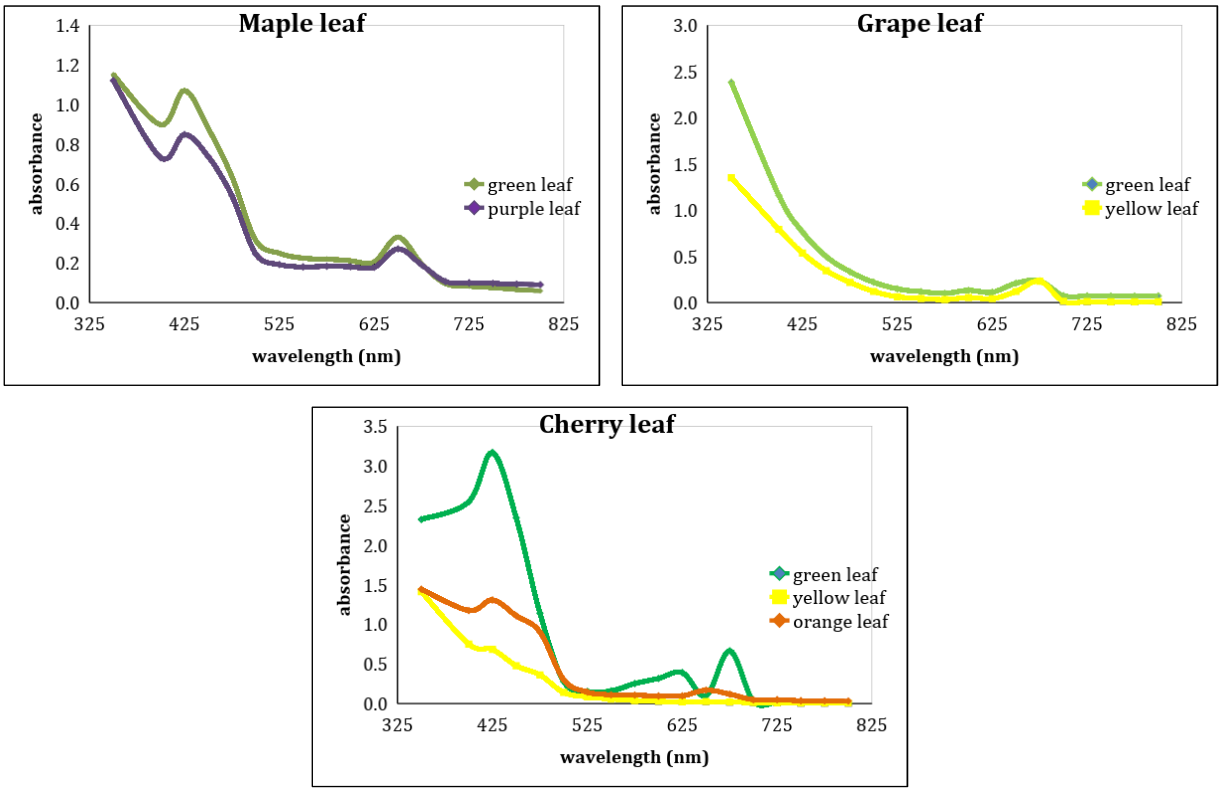


Figure 6. Absorbance spectra of crude leaf extracts.

When measuring the absorbances of separated pigments, only five bands from the maple and grape samples were analysed because some bands did not totally separate and others were too faint or undefined, as visible in tables 2, 3 and 4. Again, the absorbances were plotted against their respective wavelengths (figures 7 and 8).

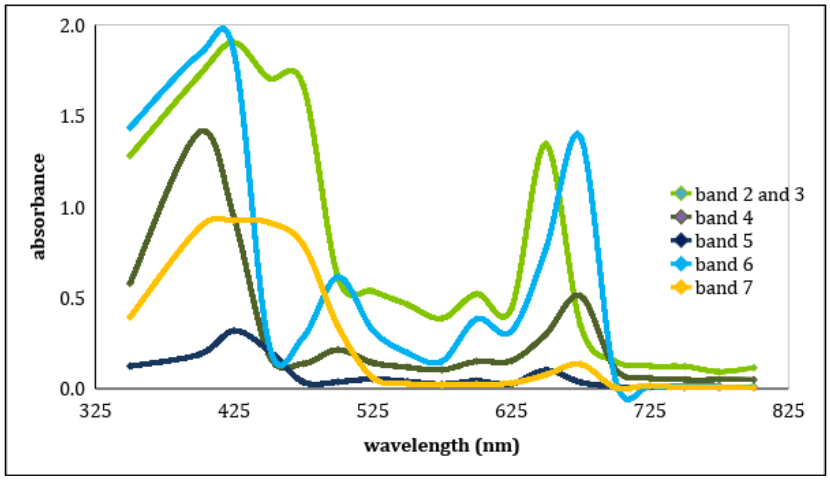


Figure 7. Absorbance spectra of separated pigments extracted from green maple leaves.

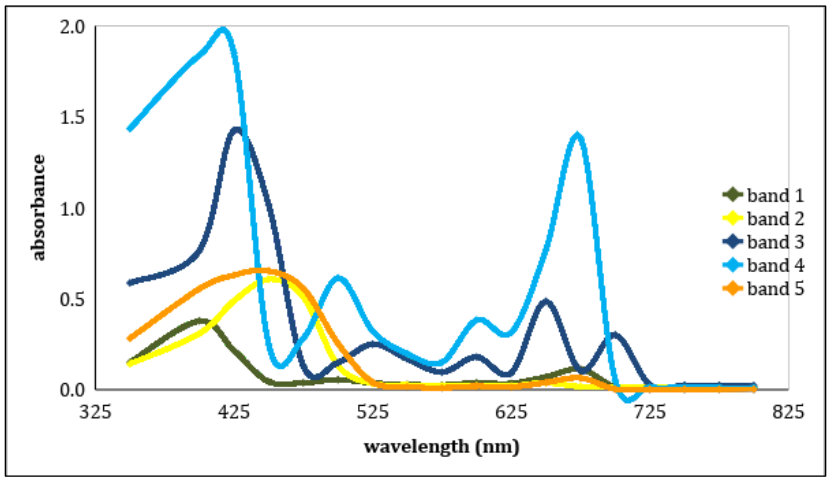


Figure 8. Absorbance spectra of separated pigments extracted from green grape leaves.

3.2.4. Isolation of membrane proteins (protein-pigment complexes)

As previously described, agarose 2% gel was used instead of polyacrylamide gel, thus Photosystems I and II were not separated, but the whole protein-pigment complex was isolated (figure 9). The results are more clearly visible with the first detergent even though the amount of supernatant added was constant for all wells.

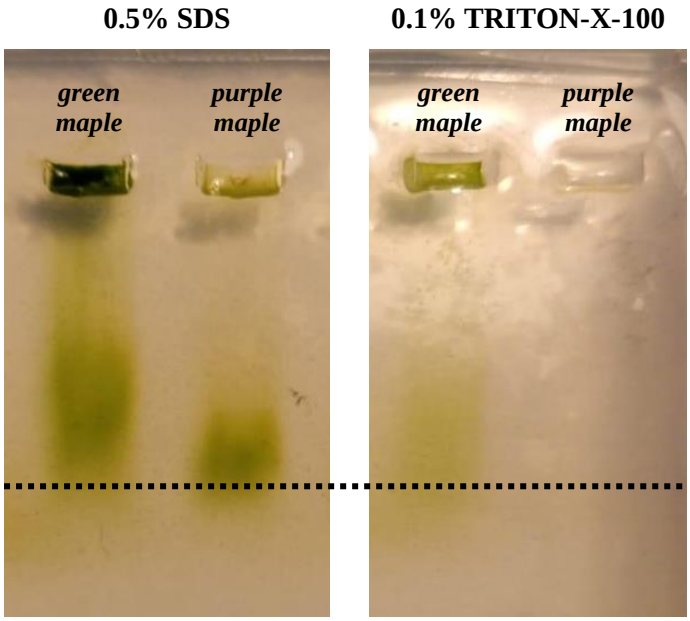


Figure 9. Isolated protein-pigment complexes.

3.3. Discussion

3.3.1. Microscopy

Observing cross-sections in water led to some important conclusions about the structure of leaves, especially the nature of chlorophyll and other pigments they contain. Free cells with green dots were evident in green leaves, which we interpreted as chloroplasts. An important

observation was that green pigments were still present in purple maple leaves, or yellow cherry and grape leaves.

Deformed cells were noticeable in cherry leaves. There were star-shaped cells even in the green leaves, whereas in yellow leaves they outnumbered healthy-looking cells (figure 10). As for the orange leaves, no chloroplasts or round cells were found; moreover, it looked like the cells were dead. It is very common for cherry trees to be infected with a virus – it is highly possible that this particular tree, as well as its leaves, was diseased.

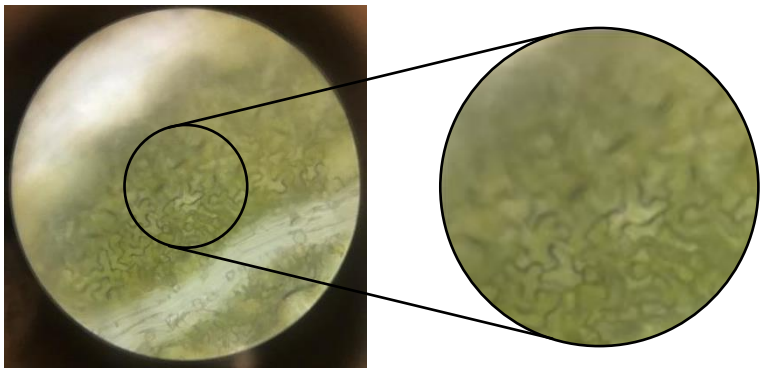


Figure 10. Diseased cells in yellow cherry leaf observed under LM.

Acetone and hexane were compared to see how fast the changes occur and to decide which solvent is better for extraction. In both cases, the section appeared lighter first and then darker in the end. Both organic solvents denatured the cell membranes, which led to leakage of chlorophyll and other pigments; as a result, the spreading and dilution of pigments presented as bleaching of colour. After five minutes everything is completely denatured and starts shrinking and concentrating again, which explains the darkening. Hexane is very hydrophobic; thus, water pushes it to the edge of the sample, which is why it denatures membranes faster and the lightening effect was visible within the first minute. Although hexane seemed to be working more rapidly, due to its high non-polarity, semi-polar and polar pigments would not extract well enough, so semi-polar acetone was the better option of extraction solvent in the next experiment.

3.3.2. Determination of pigments

As previously explained, acetone was used for extraction of pigments due to its semi-polarity. However, a mixture of differently polar organic solvents (hexane, acetone, chloroform in ratio 3:1:1) was used for TLC. Still, some more polar pigments were not expected to separate well. For instance, pigments in grape separated differently and more extract interacted with silica and stayed on the baseline, which resulted in fewer bands. Red and purple pigments, such as anthocyanin, are very hydrophilic and therefore these pigments did not move with the solvent we used.

The retention factors calculated for distinguishable pigment bands were compared to those reported by Maróti & Gabnai [8] and Quach et al. [9]; as a similar solution was used as the mobile phase in their research, results were not completely the same, but were very similar

and proportional nonetheless. Using that data and wavelengths at which certain pigments have absorption maxima, most pigments could be properly identified (tables 5 and 6).

Table 5. Pigments identified in green maple leaves based on R_f values and absorption maxima.

Band no.	R_f	Expected pigment	Absorption maxima (nm)	Reported pigment
1	0.12	/	/	/
2	0.33	Xanthophyll	425-475; 650	Xanthophyll and chlorophyll b
3	0.38	Chlorophyll b		
4	0.53	Chlorophyll a	400; 675	Chlorophyll a
5	0.80	Pheophytin b	425; 650	Pheophytin b
6	0.93	Pheophytin a	425; 675	Pheophytin a
7	0.97	Beta-carotene	400-475; 675	Beta-carotene

Table 6. Pigments identified in green grape leaves based on R_f values and absorption maxima.

Band no.	R_f	Expected pigment	Absorption maxima (nm)	Reported pigment
1	0.09	/	400; 675	Chlorophyll a
2	0.41	/	425-475	Xanthophyll
3	0.89	Pheophytin b	425; 650	Pheophytin b
4	0.94	Pheophytin a	425; 675	Pheophytin a
5	0.98	Beta-carotene	400-475; 675	Beta-carotene

Based on pigments identified in maple, and keeping in mind the aforementioned similarity of cherry and maple extracts, the same order of pigments can be reported for the green cherry leaf. The first band for maple, and cherry, was too faint for measuring absorbance and could not be identified as the R_f value did not correspond to any pigments in literature.

For maple, both retention factors and absorption maximum linked to same pigments. The second and third band were overlapping, and both pigments were extracted for spectrophotometry together. However, besides R_f values, which pointed to xanthophyll and chlorophyll b, absorbance confirmed the presence of those two pigments as the first maximum (425-475 nm) was characteristic for xanthophyll, and the second (650 nm) for chlorophyll b.

In grape, not all R_f values could be used to confirm pigments, but then again absorption spectrum helped us identify all bands as pigments. Additionally, chlorophyll b seemed to be missing. Even though some plants have only one type of chlorophyll, grape is unlikely to be one of them because of the presence of two types of pheophytin, which is actually degraded chlorophyll without central Mg^{2+} ion. In fact, a light green band was distinguishable overlapping with band 2 (xanthophyll), but it was too faint and thin to identify.

The concentration of each pigment was not possible to determine, but it could be compared through absorbance (figure 11). The same pigment, in this case chlorophyll a, absorbs less light in the grape leaf sample. Therefore, the concentration of pigment must be lower.

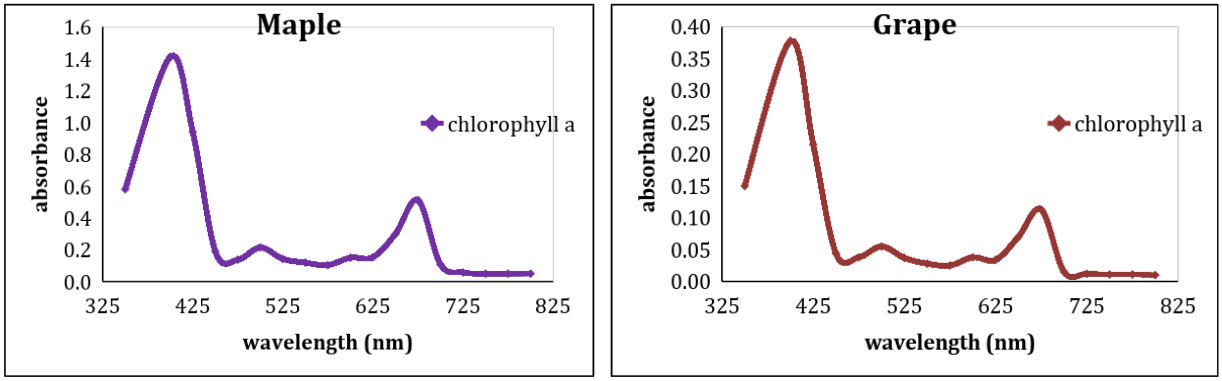


Figure 11. Absorbance spectra corresponding to chlorophyll a in green maple and grape leaves. The absorbance is lower overall in grape leaves, pointing to a lower concentration of pigment.

3.3.3. Isolation of membrane proteins

The lowest smudged green band, seen below the dotted line in figure 9, is free chlorophyll, which runs in front due to its small size; the other band is photosystem protein with chlorophyll still bound. Both bands are smaller and lighter in purple than in green maple, indicating that chlorophyll degrades along with the protein.

TRITON-X-100 did not solubilize the membrane proteins as well SDS, where the results are more visible. Another possible explanation of why the results are not as good with the second detergent as with the first one, is that TRITON-X-100 disrupted the membrane proteins and therefore denatured them.

3.3.4. Visualisation of protein-pigment complexes using PyMOL

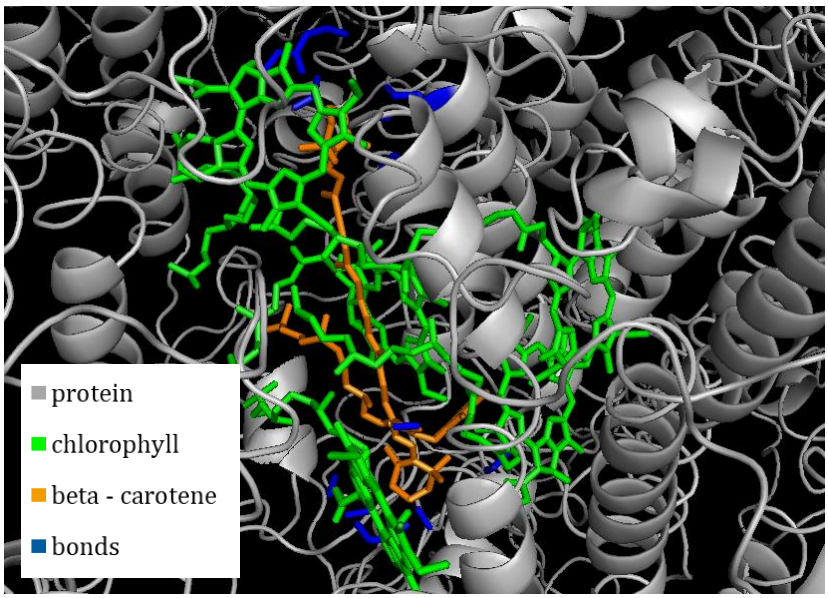


Figure 12. Components of Photosystem I visualised in PyMOL.

In figure 12, it is clearly visible that chlorophyll binds to the protein and to other pigments. Moreover, the specific amino acids chlorophyll usually binds to were visible. The results were as expected – chlorophyll mainly seemed to bind to non-polar amino acids, which indicates that chlorophyll is a non-polar pigment. As for other pigments, in this case beta-carotene, it only binds to chlorophyll. Therefore, the purpose of other pigments in plants should be to protect the chlorophyll from photodamage.

4. Simulation of autumn

To see how autumn affects plants, a basil plant was put in altered conditions to simulate autumn. First, it was placed in the fridge to simulate cooler temperatures. However, since photosynthesis is light-dependent and exposure to light decreases as we approach winter, depriving the plant of light seemed as a better choice. The simulation was followed by experiments to see whether there were any differences between the exposed and covered part of the plant.

4.1. Materials and methods

4.1.1. Sample

In order to simulate the autumn, one half of a basil plant was covered with aluminium foil and deprived of light for all but four hours every day. The other, control half of the plant remained uncovered and received light throughout the day. The simulation went on for four days.

4.1.2. Extraction and separation of pigments (TLC)

After four days, 1 g of leaf was taken from both the control and the experiment part of the plant to prepare the sample for TLC. They were ground with a solution of 10% hexane and 90% of acetone. The sample was further extracted for TLC using the procedure described in *Pigments in nature*. Then two different TLC plates were prepared: one qualitative and one quantitative. For the first plate, 20 µl of both samples was spotted; the second plate was spotted with three spots of 40 µl each of both the control and the experiment part. The stationary and mobile phases were the same as in previous experiments.

4.1.3. Absorption spectrum of pigments

Using the same procedure as described in *Pigments in nature*, bands 1 and 2 from the TLC plate were made into 100X dilutions in a 10% hexane solution in acetone. Measurements of absorbance were again done for the light of specific wavelengths (350 nm and 400-800 nm with 25 nm intervals), again using the same method as described in *Pigments in nature*.

4.1.4. Isolation of membrane proteins (protein-pigment complexes)

The methods for preparing the samples were the same as previously described for maple green and purple leaves in *Pigments in nature*. The same agarose 2% gel with 8 wells was used.

4.2. Results

4.2.1. Simulation of autumn

After four days, the plant showed evident change – the half that had been deprived of light was no longer upright; the leaves were thinner and more fragile to the touch, though there seemed to be no apparent change in colour (figure 13).

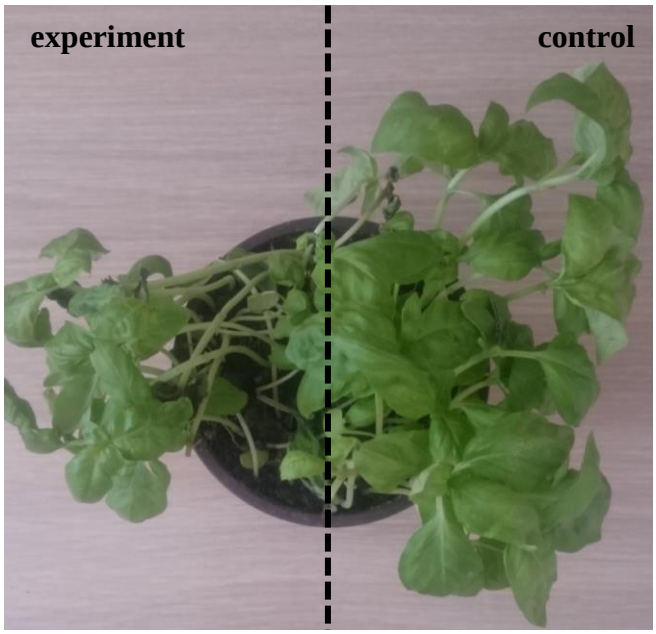


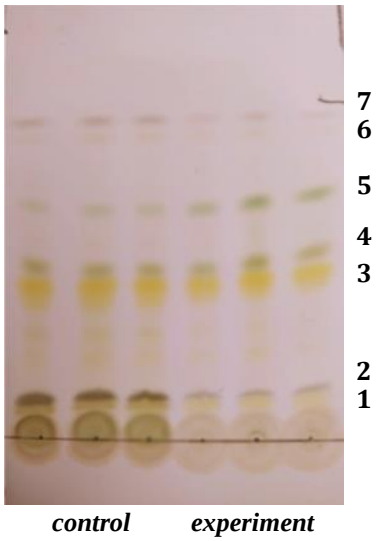
Figure 13. The result of light deprivation after four days.

4.2.2. Separation of pigments

Using the same methods as in previous experiments, thin-layer chromatography was performed on extracts from leaves that had been deprived of light as well as the control leaves. The same mixture of solvents (hexane, acetone and chloroform, 3:1:1) was used for separation. The resulting plate and the calculated R_f values are shown in table 7. The curvature of the bands on the right-hand side of the plate is, again, a result of the shape of the chamber.

Table 7. R_f values for separated bands of pigment in basil leaf extract.

Band no.	R_f
1	0.11
2	0.15
3	0.46
4	0.52
5	0.69
6	0.91
7	0.96



4.2.3. Absorption spectrum of pigments

Bands 1 and 2 (as seen on figure 14) were isolated and analysed as described in *Materials and methods*. The absorption spectrum for band 2 was ultimately scrapped as the values were too low and uniform, most likely due to the band being too light or undefined. Figure x+2 shows the absorption spectrum of band 1.

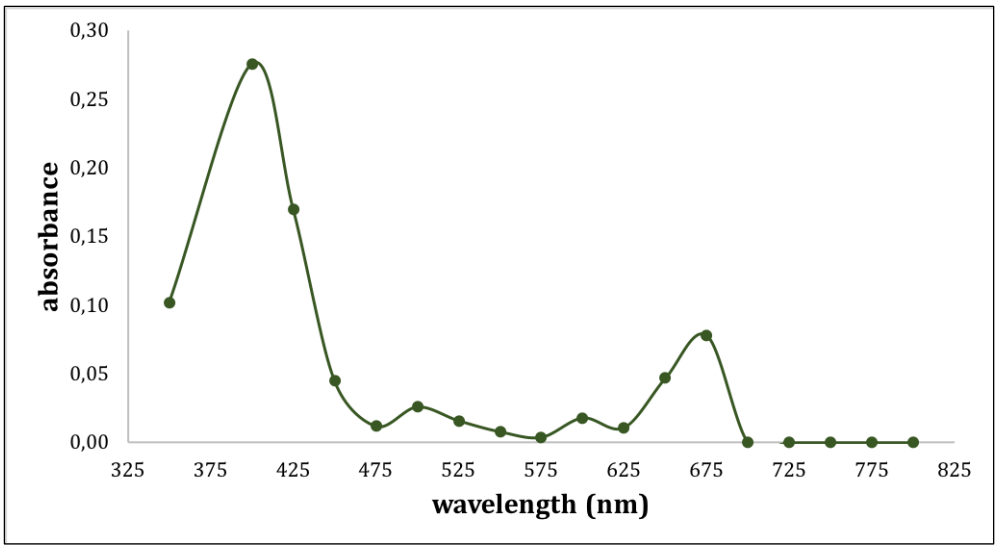


Figure 14. Absorbance spectrum of band 1 from basil leaf extract.

4.2.4. Isolation of membrane proteins

As previously described, agarose 2% gel was used for electrophoresis and the pigment-protein complex was isolated using two different detergents, of which the first performed better (figure 15).

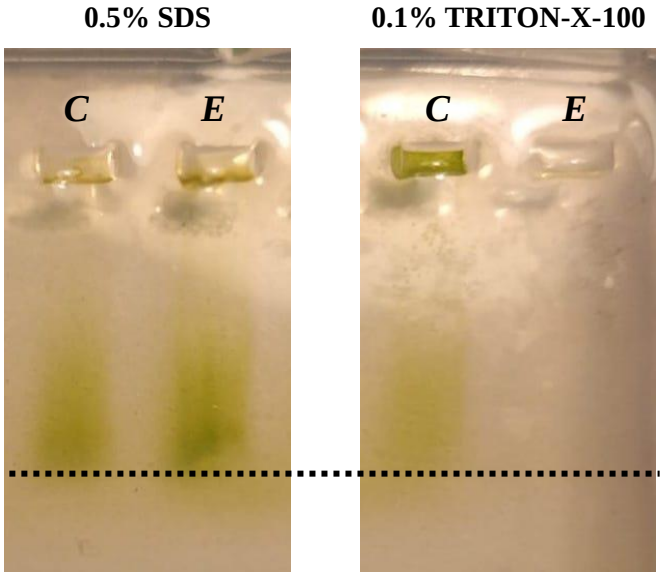


Figure 15. Isolated protein-pigment complexes from basil. C and E denote control and experiment respectively.

4.3. Discussion

4.3.1. Determination of pigments

Unlike the leaf samples from *Pigments in nature*, most pigment bands were not analysed using a spectrophotometer, meaning they could only be identified using their characteristic R_f values. When compared to literature [7], most of these bands come could not be identified as specific pigments based on retention factors. The absorbance maxima of band 1 could be used to confirm the presence of chlorophyll a (table 8).

Table 8. Pigments identified in basil leaves based on R_f values and absorption maxima.

Band no.	R_f	Absorption maxima (nm)	Expected pigment
1	0.11	400; 675	Chlorophyll a
2	0.15	/	/
3	0.46	/	/
4	0.52	/	/
5	0.69	/	Pheophytin b
6	0.91	/	Pheophytin a
7	0.96	/	/

The R_f values and appearance of bands obtained from basil samples most resemble those observed in green grape leaves. However, this observation alone cannot be used to determine which pigments can be found in basil leaves with certainty. Analysing the absorption of each observed band would resolve this issue.

Comparing the control and experiment samples as shown on figure x, there are some immediately noticeable differences. Bands 1 and 2 are darker and more saturated in the control samples than in their experiment counterparts. On the other hand, band 5 shows the opposite - it is darker in the experiment sample as opposed to the control. Since band 5 is expected to be pheophytin b based on retention factors [7] and band 1 is thought to be chlorophyll a, this could indicate that some of the chlorophyll in the leaves that were deprived of light degraded into pheophytin. As pheophytin is essentially a chlorophyll molecule without the central magnesium ion, this result implies the degradation of chlorophyll in autumn as a result of lower light exposure.

4.3.2. Isolation of membrane proteins

Analogously to the maple leaves from *Pigments in nature*, the lowermost green band in both the control and experimental sample is free chlorophyll which runs in front due to its small size. The darker green bands above it are the isolated pigment-protein complexes.

Both the free and protein-bound chlorophyll bands appear lighter in the experimental sample, although the difference is not as drastic as in the maple samples from *Pigments in nature*. This again indicates that proteins and chlorophyll both degrade in autumn.

5. Conclusion

In this project, we gained an overall idea of why and how leaves change colour in autumn. Having learned what pigments are present in plants and how they are stored within the cell, we concluded the variety of autumn colours in nature is a consequence of reduced light exposure and the resulting adjustments plants make to survive.

Acknowledgements

We would like to thank Nikolina Šoštarić and Dora Grbavac, the organizers of the Summer School of Science, for their continued support, as well as Katarina Varga from the Faculty of Science, Chemistry Department in Zagreb for providing us with the equipment we needed.

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