Student Instructions

Chemistry of Autumn Colors: Quantitative Spectrophotometric Analysis of Anthocyanins and Carotenoids and Qualitative Analysis of Anthocyanins by Ultra-performance Liquid Chromatography–Tandem Mass Spectrometry

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Exploration of Red and Yellow Pigments of Plants

1.1 Background information

General information about anthocyanins and carotenoids

During late autumn, deciduous trees glow in red and yellow colors. Yellow and orange shades are caused by carotenoids that plants produce throughout the summer. The red pigments are called anthocyanins. The colors of these pigments are masked by chlorophyll in full-grown leaves, and become visible only, when the chlorophyll starts to decay to colorless degradation products.

In addition to leaves, anthocyanins are found in flowers, berries and fruits, where the color can range from red to blue and violet. The color depends on the pH of the solution. Anthocyanins have many different functions in plants. They act as antioxidants, protect the plant from excess UV radiation and take part in the plant’s chemical defense. Moreover, they have a special role in the reproduction of the plant, because the color of the compounds is used to attract pollinators and seed distributors.

Carotenoids are yellow, orange and red lipophilic compounds that can be found in almost all plants, and all parts of plants such as leaves, roots and fruits. Probably the best-known carotenoid is β-carotene, which can be found for example from carrot. Carotenoids assist chlorophyll in photosynthesis by absorbing effectively visible light. Animals need carotenoids, but cannot produce these compounds, so they must be obtained from diet. For example, the yellow color of egg yolk and yellow feathers of great tit are caused by carotenoids that originate from plant leaves eaten by insect herbivores.

Structures of anthocyanins and carotenoids

Anthocyanins are built from anthocyanidins, sugars and possibly acyl groups. Anthocyanidins are phenolic compounds, and their skeleton consists of two aromatic rings and one oxygen-containing ring. Anthocyanidins differ from each other by the number and position of hydroxyl and methoxy groups in the B-ring. Different sugars can be attached to the anthocyanin, but the most common ones are glucose and galactose. Hydroxyl groups of the anthocyanidin, and especially the sugar part (several C-O and O-H bonds) make the anthocyanin polar and therefore water-soluble.

Carotenoids are built from eight isoprene units, and they form a long hydrocarbon chain where almost all the double bonds between carbons are conjugated. The long hydrocarbon chain makes carotenoids non-polar and therefore lipophilic. Carotenoids can be divided to two groups: carotenes and xanthophylls where the difference is that xanthophylls contain oxygen.

Polar molecules have two properties: (a) at least one of the bonds have a difference in electronegativity (how strongly the atom draws the bonding electrons towards itself) between the bonded atoms and (b) the geometry of the molecule is asymmetric.
Cyanidin 3-O-glucoside

Lycopene

Violaxanthin

Figure 1. Cyanidin 3-O-glucoside is an example of anthocyanins. Lycopene and violaxanthin are carotenoids. Lycopene is an example of carotenes and violaxanthin an example of xanthophylls.

1.2 Extraction

The purpose of this experiment is to isolate anthocyanins and carotenoids from plant samples, such as autumn leaves or flower petals, by extracting them to an organic solvent. The solvent has two functions. Firstly, it should be able to dissolve the compounds of your interest. The ground rule is to use polar solvent for polar compounds, and non-polar solvent for non-polar compounds. For example, pure water would not dissolve the lipophilic carotenoids. Secondly, the solvent should help the plant cells to swell and increase the porosity of the cell walls, which enhances the solubilization of the compounds to the extraction solvent.

Before extraction, the plant samples must be dried in order to present the pigment concentrations in mg/g (how many milligrams of the pigment is in a gram of dried plant powder). Since plants contain different amounts of water, this makes the results from different plant samples comparable. To make the extraction as effective and quantitative as possible, the plant material should be ground into as fine powder as possible.

The extraction is best done in darkness and in low temperature to prevent degradation of the compounds. Low temperature (at around 4 °C) also eases the plant cells to swell and the cell walls become more porous. The extraction time affects the effectiveness of extraction, but extending the time from two to four or seven days has not been observed to have a significant effect. Too long extraction time might only reduce the result, as the compounds start to decompose.

During the evaporation of the solvent and storage, the samples should be protected from light, since especially carotenoids might start to decompose under the influence of light. In addition, storing the extracts in a freezer helps to keep the compounds intact.
Plant sampling and drying

Collect at least five fresh leaves from the chosen tree or bush. If the leaves are small, collect more. The leaves should be the same color. Dry the leaves right after the collection. Fully dry leaves feel crunchy and can be ground easily.

Equipment and reagents

- test tubes with caps
- graduated glass or pipette
- funnel and filter paper
- acetone-water (80/20, V/V)

Procedure

1. Grind or crush the leaves to as fine powder as possible in a resealable plastic bag.
2. Weigh 0.2 g of the “powder” to a test tube.
3. Add 8 ml of acetone-water (80/20, V/V) to the test tube and close it tightly with a cap. Shake the tube for approx. one minute.
4. Put the tube to a fridge for at least one night.
5. Filter the extract using a funnel and filter paper.
6. Evaporate the solvent using e.g. centrifugal vacuum concentrator
7. Cover the extract and put it to freezer to wait for the analysis step.
1.3 Separation of the compounds by liquid-liquid extraction

The extract obtained with acetone-water (80/20, V/V) should contain both anthocyanins and carotenoids. These compounds have chemically different properties, which is why they can be separated from each other by liquid-liquid extraction. Liquid-liquid extraction is a process, where two immiscible liquids are used as solvents, one of which dissolves some of the compounds in the sample, and the remaining compounds are dissolved in the other. The liquid-liquid extraction is usually performed in a separating funnel, in which the two layers formed by the solvents can easily be seen.

In this experiment, the liquids are 4 % aqueous formic acid, in which the water-soluble anthocyanins dissolve, and hexane, in which the lipophilic carotenoids dissolve. 4 % aqueous formic acid is heavier than hexane, so it falls to the bottom and the upper layer is hexane.

1.4 Spectrophotometric assay and UPLC-MS/MS analysis

In this experiment, the pigment concentrations are measured utilizing a UV/Vis spectrophotometer. A small amount of the sample solution is pipetted into a cuvette, which is put inside of the spectrophotometer. Light is led through the cuvette at a certain wavelength and intensity. When the light goes through the sample solution, the intensity of the light decreases, if the solution was able to absorb the light at some specific wavelength. The intensity of the light after the cuvette is measured with a detector. By comparing the original intensity and the intensity after the cuvette, it can be calculated, how much of the light was absorbed to the sample solution. The absorbance is directly proportional to the pigment concentration.

![Figure 2. Schematic representation of a spectrophotometer. Light at a specific wavelength (520 nm for example) and intensity (I₀) is led through the sample solution and the intensity of the light after the cuvette (I) is measured at the detector.](image)

The wavelength for the spectrophotometric assays has to be chosen according to the studied compounds. Suitable wavelength for carotenoids is 450 nm and for anthocyanins 520 nm, because on average, the compounds absorb at these wavelengths the most. The measured absorbance is converted to concentration using calibration curve. The anthocyanin and carotenoid concentrations of a sample can also be estimated by the eye. The darker the color, the higher the absorbance and therefore the concentration is higher.

The anthocyanins will be analyzed using liquid chromatography and tandem mass spectrometry (UPLC = ultra performance liquid chromatography, MS/MS = tandem mass spectrometry). First, small volume of the sample (e.g. 5 µl) is injected into the column with the eluent (0,1 % aqueous formic acid and acetonitrile), and the compounds are separated based on their polarities (figure 3). The column is
packed with polar particles that have been surfaced with non-polar sidechains. That retains the least polar compounds, while the most polar compounds go through the column easily. During the analysis, the eluent composition is gradually changed to less polar (acetonitrile percentage is increased) to elute the least polar compounds out of the column. Carotenoids would require a less polar solvent than acetonitrile to be properly eluted from the column, which is why they are not analyzed by LC-MS in this experiment.

The separated compounds are guided into the ion source, where the eluent is evaporated, and the compounds are ionized. With vacuum and voltage differences the compounds are guided into the vacuum region of the mass spectrometer, which measures their mass to charge ratios. In this experiment, the sugar moieties of the anthocyanins are already cleaved from the anthocyanins in the ion source. Thus, only the basic building block, the anthocyanin aglycone remains to be analysed by the mass spectrometer. Each of these so called precursor ions are then broken down in the collision cell of the mass spectrometer to smaller fragments that are characteristic to the precursor ion (figure 3). When the mass spectrometer detects for example the product ion characteristic to cyanidin, a signal will be observed in the chromatogram.

Based on the results of the UPLC-MS/MS measurement it can be concluded, which of the most common anthocyanidin classes are present in the anthocyanins of the plant sample. In other words, the goal is to be able to tell, if the sample contains delphinidin, cyanidin, pelargonidin, peonidin, petunidin or malvidin type anthocyanidin derivatives. Based on the literature, cyanidin derivatives are most abundant in nature. Does this apply to the selected samples?
Equipment and reagents

- pipettes
- Eppendorf tubes (5 ml and 2 ml volume)
- separating funnels
- 0.2 µm PTFE filters and syringes
- vials, caps and septumes
- cuvettes
- hexane
- 4 % aqueous formic acid
- technical ethanol

Procedure

Liquid-liquid extraction

Liquid-liquid extraction is performed in a fume hood! Use protective wearing, gloves and safety goggles when handling hexane and acidic solutions.

1. Take two Eppendorf tubes (5 ml volume). Mark them with your sample code (e.g. plant species and your initials). One of the tubes is for the anthocyanin sample (you can add e.g. letter a to the sample code) and the other one for the carotenoid sample.
2. Dissolve the sample first to the 4 % formic acid solution by pipetting 5 ml of it to the beaker where your dried extract is. Shake the beaker gently for approx. one minute to dissolve the water-soluble compounds. Make sure that the valve of the separating funnel is closed. Put the Eppendorf tube for anthocyanin sample underneath it. Add the water solution to the separating funnel.
3. Pipette 8 ml of hexane to the beaker and shake it again gently to dissolve the lipophilic compounds. Add this solution to the separating funnel as well.
4. Close the separating funnel and turn it carefully up and down couple of times. Open the cap and let the phases separate for a moment.
5. Open the valve carefully and drain around 4 ml of the lower phase to the Eppendorf tube.
6. Rest of the aqueous phase and some of the hexane phase is drained to a waste container. What should be left is around 5 ml of pure hexane phase.
7. Pour the hexane phase from the top of the separating funnel to the Eppendorf tube for your carotenoid sample.
8. Take a new Eppendorf tube and filter the aqueous phase to it with a 0.2 µm PTFE filter.

Preparing the sample for UPLC-MS/MS analysis

1. Take a small Eppendorf tube and vial for your anthocyanin sample. Mark them with your sample code.
2. Pipette 1 ml of 4 % formic acid solution to the Eppendorf tube. Add 0.5 ml of your filtered aqueous phase and shake the tube for a moment.
3. Transfer the dilution to a vial.
Spectrophotometric assay

Absorbances for the aqueous phases are measured at 520 nm and hexane phases at 450 nm. Before measuring the actual samples, the spectrophotometer has to be reset with a blank sample i.e. your sample solvent.

Preparation of the standards:
Stock solutions may be prepared in advance by the instructor. Calibration curve for carotenoids is done with β-carotene dilutions in 2,0; 1,0; 0,5 and 0,25 mg/ml concentrations. Calibration curve for anthocyanins is done with cyanidin dilutions in 40, 20, 10 and 10 µg/ml concentrations. Prepare 2 mg/ml β-carotene stock solution by dissolving 20 mg of β-carotene into 10 ml of acetone (β-carotene does not dissolve in hexane). Prepare 160 µg/ml cyanidin stock solution by dissolving 16 mg of cyanidin into 100 ml 4 % aqueous formic acid solution. Use the volumes in tables 1 and 2 to make the dilutions.

Table 1. Concentrations and volumes for β-carotene dilutions

| β-carotene concentration (mg/ml) | Volume of the stock solution (µl) | solvent (µl) |
|----------------------------------|----------------------------------|--------------|
| 1,0                              | 500                              | 500          |
| 0,5                              | 250                              | 750          |
| 0,25                             | 125                              | 875          |

Table 2. Concentrations and volumes for cyanidin dilutions

| Cyanidin concentration (µg/ml) | Volume of the stock solution (µl) | solvent (µl) |
|-------------------------------|----------------------------------|--------------|
| 40                            | 250                              | 750          |
| 20                            | 125                              | 875          |
| 10                            | 60                               | 900          |
| 5                             | 50                               | 1550         |

Measurement of the standards and samples:
1. Rinse the cuvette with a small amount of your sample and fill it with your sample.
2. Make sure that there are no fingerprints in the clear walls of the cuvette by wiping the walls with tissue paper. Measure the absorbance and mark the value down. **Note that the absorbance has to be in the linear photometric measuring range of the spectrophotometer. In practice, the absorbance should be less than three in most cases. If it is higher, the sample has to be diluted.**
3. Rinse the cuvette with ethanol and water.
4. Plot the calibration curves with e.g. Excel. Estimate the pigment concentrations from the graph or calculate the values using the equations. What are the pigment concentrations in mg/g dry weight?