Extraction of Photosynthetic Tissues: Chlorophylls and Carotenoids

BASIC PROTOCOL

The extraction of chlorophylls and carotenoids from water-containing plant materials requires polar solvents, such as acetone, methanol, or ethanol, that can take up water. These extracts must then be transferred to a solvent such as diethyl ether in order to be stored stably. Samples with very high water content, such as juices and macerated plant material, are usually freeze-dried first, and can then be extracted directly with diethyl ether. After extraction, solutions are clarified and diluted to an appropriate volume to measure chlorophyll content by UV-VIS spectrophotometry. Absorption coefficients and equations needed for quantitative determination are given in *UNIT F4.3*.

This protocol has been developed for extraction of chlorophyll from leaf samples, but it can also be used for other plant food samples. The authors recommend, however, that the beginner initially perform the procedure using green leaf samples before extending its use to other types of samples.

NOTE: Absorption in the red and blue maxima is highest in freshly isolated chlorophyll, and then decreases with time due to formation of allomeric chlorophyll forms and possibly destruction of chlorophylls (*UNIT F4.1*), particularly in the presence of light. This also applies to green pigment extract solutions of leaves and other plant tissues. Therefore, chlorophyll determinations should be carried out in dim light immediately after preparing the pigment extract solution.

Materials

Green leaf samples or other greenish plant tissue samples
MgO or $MgCO_3$
100% or 80% (v/v) acetone or diethyl ether, spectrophotometric grade
Hydrophobic organic solvent: diethyl ether, light petrol, or hexane, spectrophotometric grade
Half-saturated NaCl solution
Anhydrous Na_2SO_4

Rim-sharpened cork driller (optional)
Mortar and pestle
Aluminum dishes
100°C drying oven
Freeze dryer
5-ml graduated centrifuge tubes
Explosion-proof tabletop centrifuge or a cooling tabletop centrifuge
UV-VIS spectrophotometer
1-cm-path-length cuvette
25- or 50-ml separatory funnel
Water bath set below 35°C (optional)

Prepare samples and determine water content

1. For each sample to be tested, prepare three to five replicates weighing 6 to 12 mg each and place in a mortar.

   Because values may vary between different parts of leaves and fruits, three to five replicates must be taken from each sample to obtain a significant and reliable mean value.

   For leaf samples, a rim-sharpened cork driller can be used to punch samples from the leaf. A punch area of 0.6 to 0.9 cm^2 will give ~6 to 12 mg fresh sample weight, equivalent to 3

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to 6 mg dry weight. For dark green leaves, a single punch for each replicate is usually sufficient to obtain good absorbance readings (0.3 to 0.85 at 662 nm). Light green leaves may require two to three punches (12 to 36 mg fresh weight) per replicate, and yellowish green leaves may require four to five punches (24 to 60 mg fresh weight) per replicate to obtain a sufficiently high absorbance reading (>0.3 at 662 nm).

For other plant food samples, appropriate sample sizes will depend on chlorophyll content and water content. Samples with low chlorophyll content may require 100 to 200 mg per replicate. For samples with low chlorophyll content and high water content (e.g., inflorescences, fruit tissues, fruit juices), it is necessary to start with 2 to 3 g fresh weight sample and freeze dry it before extraction. Frozen food samples should also be freeze dried before extraction.

2. To determine the water content of the plant sample, take another four punches (24 to 48 mg sample) and weigh them. Place them in an aluminum dish, dry ~2 hr in a 100°C drying oven, and weigh them again. Subtract dry weight from original weight to obtain water content.

It is strongly recommended that the level of chlorophylls and carotenoids be determined on both a dry weight and a wet weight basis. The pigment values measured for a known leaf area can be converted to pigment values per gram dry weight sample with appropriate calculations. Dry weight and leaf area are reliable reference systems. Wet weight is less reliable since water content varies with storage.

Extract chlorophylls/carotenoids

3. Use the moisture content in step 2 to choose the appropriate extraction procedure, solvent, coefficients, and equations.

For one to five leaf punches per replicate, the water content coming from the plant tissue will account for <1% to 2% of the final 5 ml extract (step 6) and can be neglected. Perform extraction as described below and use the equations for 100% acetone (UNIT F4.3).

For 100 to 200 mg sample with very low chlorophyll, the water content in the final 5 ml acetone extract will exceed 2%. Perform extraction as described steps 4 and 5. Then, based on water content of the sample, adjust the solvent in step 6 with aqueous acetone to give a final of 5 ml of 80% aqueous acetone. Apply the equations for 80% acetone (UNIT F4.3).

For freeze-dried samples, extract the dry sample (~0.3 to 1 g dry weight starting from 2 to 3 g fresh weight) with diethyl ether and apply the equations given for diethyl ether (UNIT F4.3). Extraction of freeze-dried plant material with diethyl ether is performed by grinding in a mortar. It is also possible to use 80% acetone or 100% methanol, but diethyl ether has proved to be an excellent solvent for quantitative extraction of chlorophylls and carotenoids from freeze-dried plant material.

4. Add 100 to 200 mg MgO or $MgCO_3$ to the sample (step 1) to neutralize plant acids and prevent pheophytin *a* formation.

If high amounts of pheophytins are formed, the main absorption peaks near 660 and 662 nm would shift to other wavelengths and the green extract would change to a pale olive-green. A small amount of pheophytins (1% to 2%) does not significantly change the results.

5. Add 3 ml of 100% acetone (for ≤200 mg fresh sample) or diethyl ether (for freeze-dried samples with low chlorophyll content) and grind with a pestle.

An explosion-proof motor-driven grinder or steel or glass balls can also be used to grind the sample (see Critical Parameters).

6. Transfer the turbid pigment extract comprising chlorophylls and total carotenoids to a 5-ml graduated glass centrifuge tube. Rinse the grinding device with another 1.5 ml solvent, add to the centrifuge tube, and bring to exactly 5 ml with additional solvent.

7. Centrifuge 5 min at 300 to 500 × *g* in an explosion-proof tabletop centrifuge at room temperature.

   Slight cooling (10° to 15 °C) can be used.

Perform spectrophotometric analysis

8. Transfer an aliquot of the clear leaf extract (supernatant) with a pipet to a 1-cm-path-length cuvette and take absorbance readings against a solvent blank in a UV-VIS spectrophotometer at five wavelengths:

   750 nm (A_{750} = 0 for clear extract)
   662 nm (chlorophyll *a* maximum using 100% acetone)
   645 nm (chlorophyll *b* maximum using 100% acetone)
   520 nm (for extracts from green plant tissue, A_{520} should be <10% A_{662})
   470 nm (carotenoids).

   For other solvents, use the chlorophyll maxima, absorption coefficients, and equations found in UNIT F4.3 and in Lichtenthaler (1987).

   For green to dark green leaves, extracts made from 6 to 12 mg leaf sample give A_{662} values between 0.3 and 0.85. If $A_{662} \leq 0.3$ (e.g., with light green or yellow-green leaves), the procedure should be repeated using additional sample (see step 1).

9. Apply measured absorbance values to equations given for each solvent system in *UNIT F4.3* to determine pigment content (µg/ml extract solution). Multiply by 5 ml to obtain the total amounts of chlorophyll *a*, chlorophyll *b*, and carotenoids contained in the 5 ml extract.

   This represents the µg pigment in each replicate sample.

10. Determine the mean value from each set of replicates.

Store sample

11. Prepare a fresh extract from a larger amount of tissue, or pool the remainder of the replicate pigment extracts (step 7). Transfer to a 25- or 50-ml separatory funnel. Add 3 ml hydrophobic organic solvent (diethyl ether, hexane, or light petrol) and gently shake.

    In order to obtain an extract that can, under exclusion of light and water, be stored in a refrigerator for days and weeks, the pigments should be transferred to a hydrophobic organic solvent that gives a phase separation with water, such as light petrol (a mixture of hydrocarbons, boiling point 40° to 70°C), hexane, or diethyl ether.

    A 10- to 15-ml aqueous acetone extract solution (i.e., from three replicates) or a direct extract from a larger tissue sample can be extracted with 3 ml hydrophobic solvent.

    Rigorous shaking should be avoided as it causes formation of water-lipid emulsions that can only be broken down by centrifugation.

12. Add 10 to 15 ml half-saturated NaCl solution under continued gentle shaking until the hypophase (lower phase), which contains the original organic solvent (e.g., acetone), is ~50% aqueous and the chlorophylls and carotenoids are transferred to the organic epiphase.

    The half-saturated NaCl solution prevents emulsion formation.

    Under these conditions, chlorophylls a and b, carotenoids, and xanthophyll esters are transferred to the epiphase (upper phase), as described in German in Lichtenthaler and Pfister (1978) and briefly in English in Lichtenthaler (1987).

13. Transfer hypophase to a separatory funnel and extract the last traces of chlorophylls and carotenoids using 2 ml of the hydrophobic organic solvent used in step 11.

    The transfer of photosynthetic pigments from a larger extract in an aqueous organic solvent by a small amount of a hydrophobic organic solvent concentrates the pigments.

14. Combine the epiphases from the two extractions and wash once or twice with a small amount (e.g., 1 or 2 ml) of half-saturated NaCl solution.
15. Add 100 to 200 mg anhydrous Na_2SO_4 and decant the concentrated extract into a measuring flask. Close with a glass or Teflon stopper. For larger amounts of extract, concentrate the hydrophobic epiphase to a fixed volume (e.g., 5 or 10 ml) by evaporation in a water bath set below 35°C.
16. Store extract wrapped in foil at 4°C (stable for days or weeks).

For comparison with other plant samples, take 0.05 or 0.1 ml of this concentrated pigment extract, dilute to 5 ml with 100% acetone or diethyl ether, and determine the amount of photosynthetic pigments using the absorption coefficients and equations given in UNIT F4.3.

COMMENTARY

Background Information

Before the extraction of photosynthetic pigments, the plant material should be well defined, i.e., one should think in advance about the reference system for the chlorophyll (Chl) and carotenoid concentration (e.g., mg Chl/g dry weight or mg Chl/m^2 leaf area). The reference systems frequently used are: fresh weight, dry weight, or leaf area. Dry weight is a reliable reference system. However, fresh weight generally is not a suitable reference system because it includes the water content of plant tissue (leaves, fruits), which is highly variable. For this reason, the dry weight of a parallel plant sample must be determined, so that the pigment content can be expressed on a dry weight basis, providing a much more reliable reference system than fresh weight. For leafy food samples such as lettuce and spinach, leaf area is also an acceptable reference system.

Critical Parameters

Plant material

Usually the plant material can be directly extracted without any pretreatment. In cases of extremely high water content (e.g., juices, macerated plant material), the sample should be freeze-dried before extraction; otherwise, the lipid-soluble pigments cannot be adequately extracted. In addition, the absorption maxima of pigments in organic solvents are shifted towards longer wavelengths when water is present, and the absorption coefficients are considerably changed with increasing water content of the extract (Lichtenthaler, 1987). This requires some precautions to apply the proper equations for pigment determination.

Extraction procedure

Extraction of photosynthetic pigments can be carried out using a mortar and pestle or a motor-driven grinder, or by shaking the plant material with glass or steel balls. When using a conventional grinder with an electric motor, one should be aware of the danger involved in using inflammable organic solvents (e.g., acetone). In this case, a mortar and pestle are safer, and they are also easier to clean. It is advisable to add small amounts of MgO or $MgCO_3$ to neutralize plant acids that cause the formation of pheophytin *a* from Chl *a*. It is advisable to start pigment extraction with a small volume of solvent. When the plant material is well homogenized, add more solvent to give a final defined volume of the extract solution. If too much solvent is used, the pigments will be too dilute and absorbance readings will no longer be possible to obtain. Thus, 6 to 12 mg leaf material can easily be extracted with 3 ml acetone, re-extracted with 1.5 ml acetone, and then brought to a 5-ml final volume. After centrifugation for 5 min, 3 ml of the clear extract solution are placed into a cuvette for quantitative determination in the spectrophotometer.

Water-containing plant materials need to be extracted with polar solvents such as acetone, methanol, or ethanol that can take up water. Freeze-dried plant tissues and freeze-dried juices can be directly extracted with diethyl ether, which contains traces of water and is more polar than light petrol or hexane. Pure light petrol or hexane are less suitable, because more polar pigments, such as Chl *b* or xanthophylls, are only partially extracted from freeze-dried plant samples. A few drops of acetone or ethanol added to light petrol or hexane will, however, guarantee a complete extraction. This mixture will extract Chl *a*, Chl *b*, and all carotenoids—including xanthophyll esters and secondary carotenoids that are present in many fruits and juices—from the freeze-dried plant material.

Solvent

Chlorophylls and carotenoids are generally extracted with organic solvents. One should always apply purified solvents such as spectrophotometric grades, which are commercially available. This is an essential requirement. Organic impurities in standard-grade organic solvents considerably change absorption coefficients and wavelength maxima of the pigments. Because of the relatively high water content of intact plant material, one should use a solvent that mixes with water, such as acetone, ethanol, or methanol. However, chlorophylls are unstable in these water-containing solvents. Allomeric chlorophyll types that possess different absorption characteristics can eventually form. Moreover, part of Chl *a* can be broken down to pheophytin *a* (by removal of the central Mg atom), especially in the presence of plant acids from the vacuoles of extracted plant material (*UNIT F4.1*). Pheophytin *a* exhibits quite different absorption characteristics from Chl *a*. In addition, light photochemically destructs chlorophylls and carotenoids. Avoid chloroform, which is sometimes proposed as an extraction solvent for chlorophylls, because it not only is poisonous, but also contains hydrochloric acid, which partially transforms Chl *a* into pheophytin *a*. The formation of pheophytin *b* from Chl *b* usually does not occur during extraction, as this requires stronger acids.

Diethyl ether (spectrophotometric grade) is the best epiphase solvent, as it has a higher capacity to solubilize photosynthetic pigments than light petrol or hexane. When concentrated, pigment extracts are stored in strongly hydrophobic solvents (such as light petrol or hexane) in the refrigerator, but small turbid flakes containing polar pigments form. This does not occur with diethyl ether, which can contain some water. If diethyl ether has been used in the epiphase for extracting pigments from the hypophase, the epiphase can be stored in a refrigerator at 4° to 6°C for 1 or 2 hr in the separatory funnel. Under these conditions, water dissolved in diethyl ether at room temperature separates from the diethyl ether and this water hypophase can be discarded.

Final preparation of the extract

For the quantitative determination of Chl *a* and *b*, it is not necessary to separate these two pigments prior to spectrophotometric measurement (*UNIT F4.3*), as the absorbance of the extract is measured in the red region at the wavelength positions of both Chls. From these absorbance readings, Chl *a* and *b* are calculated by a particular subtraction method. This subtraction method also applies to the determination of total carotenoids (xanthophylls and carotenes, x + c) in a total pigment extract by measuring the absorbance at 470 nm, the main absorbance region of carotenoids (*UNIT F4.3*).

However, for an exact determination of the pigment concentration, the extract must be fully transparent to avoid obtaining values that are too high for Chl *b* and total carotenoids. In most cases, the homogenized plant extract contains colorless, undissolved, very fine, solid plant material, e.g., fibers and cell wall debris. These materials make the extract turbid and scatter light, rather than absorbing light. The scattered light increases from longer to shorter wavelengths (from red region to blue). Thus, the presumed absorbance signal measured in turbid solutions by the spectrophotometer is increased differentially for individual wavelengths (see Figure F4.3.4 in *UNIT F4.3*). Hence, the concentration of pigments, calculated from these incorrect absorbance values, are too high. Also, the ratio of Chl *a* to Chl *b* shifts from 2.7 to 3.2 to incorrect lower values of 2.1 to 2.6.

In order to have a transparent extract without turbidity, the homogenized extract should be centrifuged (5 min at 300 × *g*) or filtered. When using a centrifuge, one should cool the extract in order to keep evaporation as low as possible and avoid problems with inflammable organic solvents, such as acetone or diethyl ether. (Several companies make explosion-proof tabletop centrifuges, which should be used.) Filtering through fine glass filters (e.g., G3) at a reduced pressure (e.g., a water-wheel pump) is another, more time-consuming procedure. If used, one should ensure that no pigments are left in the filter. In addition, glass filters are quickly plugged by the fine plant debris and/or MgO or $MgCO_3$ powder added during extraction, requiring an intricate clean-up procedure. Paper filters are not suitable, as they retain only the larger plant particles and are permeable to finely ground plant debris. Special filters that retain finer plant debris and MgO or $MgCO_3$ powder take too much time to use, and the pigments may be partially oxidized or photochemically destroyed. Thus, for routine analysis, centrifugation is the method of choice. When transferring the centrifuged, clear pigment extract solution with a pipet into the spectrophotometer cuvette, great care should be taken not to disturb the sedimented debris.

It is important to note that chlorophylls are converted to pheophytins in the presence of acids (*UNIT F4.1*). Formation of a significant

amount of pheophytin due to unexpectedly high amounts of endogenous acids (which is very seldom the case) is observed as a change of the green pigment extract color to a pale olive-green. When this happens, add a drop of 25% HCl to the acetone extract, which will transform all chlorophyll *a* and *b* to pheophytin *a* and *b*. Then apply the absorption coefficients and equations given by Lichtenthaler (1987) for pheophytins. A small amount (1% to 3%) of pheophytins in the green extract solution does not significantly change the absorbance readings and can be tolerated. To avoid artifactual pheophytin formation, do not handle concentrated acids in the laboratory used for pigment extraction. Traces of gaseous acids in the air can convert chlorophylls to pheophytins before and during grinding and extraction.

Samples and extracts containing chlorophylls should be protected from light at all times. For storage and during analysis, extracts should be wrapped in aluminum foil.

Anticipated Results

Real pigment differences among leaf or other plant tissues of different light adaptation or developmental stages, and among fruit tissue of different maturation or senescence, occur on both a fresh and dry weight basis. Pigment differences showing up in just one reference system may not be real, as the reference system, such as dry weight (e.g., during fruit storage) or leaf area (e.g., shrinking during water stress or enlargement during leaf expansion) may have changed.

Time Considerations

One determination involving extraction with 100% acetone, centrifugation, and absorbance readings takes ~20 min. With experience, routine measurements of six samples can be completed in 40 to 60 min.

Literature Cited

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