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Chlorophyll and Carotenoid Determination (after Lichtenthaler 1987),
a practical instruction.

Dear colleagues, the determination method for the photosynthetic leaf pigments, chlorophylls *a* and *b* and total carotenoids *x+c*, had been improved with freshly isolated pure chlorophylls and pure carotenoids (i.e. several regular leaf xanthophylls and carotenes). Their absorption spectra were measured in purest organic solvents using modern two-wavelength spectrophotometers which allowed to re-determine their specific absorption coefficients. The re-determined new equations for the quantitative determination of the photosynthetic pigments are found in the book series of *Methods in Enzymology*. Perhaps you have access to this book which is available in most international libraries. This is the method that most plant scientists apply for the quantitative determination of chlorophylls *a* and *b* in plant leaf extracts obtained with organic solvents such as acetone or methanol. This method allows to simultaneously determine the level of chlorophylls *a* and *b* and also that of total carotenoids *x+c* in the same leaf extract solution.

LICHTENTHALER, H.K. Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: Douce, R. and Packer, L. (eds.), *Methods Enzymol*. 148, 350-382, Academic Press Inc., New York 1987.

You best extract your leaves, e.g. small leaf disks (diameter 6 to 8 mm) obtained with a rim-sharpened cork driller, or any other green plant tissue with 100 % acetone in a mortar using quartz sand and a pestle. A tiny amount of MgO or $MgCO_3$ neutralizes plant acids and prevents the formation of pheophytin *a* from Chl *a*. Centrifuge your extract at room temperature for 3 to 5 min at 300 to 500 x g, so it will be ***fully clear***. The latter is an absolute requirement, otherwise you will obtain wrong pigment values. Then you determine the content of Chl *a*, Chl *b* and total carotenoids (*x+c*) in a two-wavelength spectrophotometer by using the equations given below. You measure the optical density, the absorption A, at 661.6 nm, at 644.8nm and at 470 nm, and then calculate the individual pigment amounts. These are indicated in µg per ml pigment extract solution. Then you need to consider the total extract solution you made. When you have 5 ml or 10 ml total extract solution, you need to multiply by 5 or 10, respectively.

For 100 % pure acetone the new equations are given here. For other solvents check the original literature reference in *Methods in Enzymology 1987.*

Chlorophyll a: $C_a = 11.24\ A_{661.6} - 2.04\ A_{644.8}$ (µg per ml solution)
Chlorophyll b: $C_b = 20.13\ A_{644.8} - 4.19\ A_{661.6}$ (µg per ml solution)

Total carotenoids: $C_{x+c} = (1000\ A_{470} - 1.90\ C_a - 63.14\ C_b) / 214$ (µg per ml solution)

If your spectrophotometer does not allow to set exactly to 661.6 nm use 662 nm and for 646.8 nm use 647 nm. Best results are obtained at absorbance readings between 0.3 and 1.0 for the red chlorophyll peak at 661.6nm. Avoid an absorbance below 0.3 because this yields wrong Chl *b* values.

You will find the same equations with more detailed instructions and with examples for leaf chlorophyll and carotenoid levels in:

LICHTENTHALER, H.K. and C. BUSCHMANN: Chlorophylls and carotenoids – Measurement and characterisation by UV-VIS. *Current Protocols in Food Analytical Chemistry (CPFA)*, (Supplement 1), F4.3.1 - F 4.3.8 (2001) (John Wiley, New York).

This paper gives in Table F4.33 examples of the Chl and carotenoid content in leaves, as well as the weight ratios Chl *a*/*b* and Chl *a*+*b* /carotenoids, also expressed as (*a*+*b*)/(*x*+*c*). Please determine the pigment content on a leaf area unit (mg Chl *a*+*b* per m^2 or as µg cm^{-2}) and also on a mg per 1g dry weight basis. Do avoid fresh weight as a reference system, because the water content of your leaf can change during the experiment. Therefore, fresh weight is not a valid reference system.

With the equations given in the 1987 *Methods in Enzymology* paper you can determine the levels of Chl *a* and *b*, as well those of total carotenoids (*x*+*c*) in the same pigment extract solution. 100% acetone is a very suitable solvent system for leaves. For isolated chloroplasts you better use 80 % acetone. Make sure that your extract solutions are fully clear, the best is to centrifuge the pigment extracts for 3 to 5 min at 300 x or 500 x g ! Pay attention: Any turbidity and light scattering in your extract solution yields wrong pigment values, in particular too low Chl *a*/*b* ratios are obtained, because the Chl *b* content in turbid solutions is estimated much too high. And the total carotenoid *x*+*c* levels are also fully wrong and much too high!

A further paper gives you information on the ways of extraction of Chls and carotenoids:

LICHTENTHALER, H.K. and C. BUSCHMANN: Chlorophylls and Carotenoids - Extraction, Isolation and Purification. *Current Protocols in Food Analytical Chemistry (CPFA)*, (Supplement 1), Unit F4.2.1-F4.2.6 (2001) (John Wiley, New York).

Examples for typical chlorophyll and carotenoid levels in green leaves are found in the following three papers:

LICHTENTHALER H.K. Biosynthesis, accumulation and emission of carotenoids, α-tocopherol, plastoquinone and isoprene in leaves under high photosynthetic irradiance. *Photosynth. Research* 92: 163-179 (2007).

SARIJEVA G., KNAPP M., LICHTENTHALER H.K. Differences in photosynthetic activity, chlorophyll and carotenoid levels, and in chlorophyll fluorescence parameters in green sun and shade leaves of *Ginkgo* and *Fagus*. *J. Plant Physiology* 164, 950 - 955 (2007).

SCHINDLER, C., REITH, P. and LICHTENTHALER, H.K.: Differential levels of carotenoids and decrease of zeaxanthin cycle performance during leaf development in a green and an aurea variety of tobacco. *J. Plant Physiol*.143, 500-507 (1994).

See and check typical chlorophyll and carotenoid levels also in these papers:

BABANI, F. and H. K. LICHTENTHALER: Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios. *J. Plant Physiol. 148*, 555-566 (1996).

SCHINDLER, C. and H. K. LICHTENTHALER: Photosynthetic CO_2 assimilation, chlorophyll fluorescence and zeaxanthin accumulation in field-grown maple trees in the course of a sunny and a cloudy day. *J. Plant Physiol. 148*, 399-412 (1996).

LICHTENTHALER, H.K. and F. BABANI: Light adaption and senescence of the photosynthetic apparatus: changes in pigment composition, chlorophyll fluorescence parameters and photosynthetic activity during light adaptation and senescence of leaves. In: Papageorgiou G. and Govindjee (eds.), *Chlorophyll Fluorescence: A Signature of Photosynthesis* (Chapter 30), pp.713- 736. Springer, Dordrecht, 2004.

Good luck with your measurements!
Karlsruhe, Germany, October 2010. Hartmut K. Lichtenthaler

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