Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy

The quantitative determination of chlorophyll (Chl) *a*, Chl *b*, and carotenoids in a whole-pigment extract of green plant tissue by UV-VIS spectroscopy is complicated by the choice of sample, solvent system, and spectrophotometer used. The various plant pigments absorb light in overlapping spectral regions, depending on the system selected. This unit discusses methods used to account for such overlap by applying equations for accurate quantitative determination of Chl *a*. Chl *b*, and total carotenoids in the same pigment extract of leaves or fruits. General information on the spectroscopic characteristics of Chl *a* and Chl *b*, their specific absorption coefficients, and their quantitative determination in a whole-pigment extract of green plant tissues can be found in Šesták (1971) and Lichtenthaler (1987). For Chl structures, see UNIT F4.1.

ABSORPTION MAXIMA

Figure F4.3.1 shows the absorption spectrum of isolated Chl *a* and Chl *b* in diethyl ether. Chl *a* and *b* absorb with narrow bands (maxima) in the blue (near 428 and 453 nm) and red (near 661 and 642 nm) spectral ranges. The isolated yellow carotenoids have a broad absorption with three maxima or shoulders in the blue spectral range between 400 and 500 nm (Fig. F4.3.2).

The absorption maxima of extracted pigments strongly depend on the type of solvent and, to some degree, on the type of spectrophotometer used. For example, with increasing polarity of the solvent, the red absorption maximum of Chl *a* shifts from 660 to 665 nm, and the blue absorption maximum from 428 to 432 nm. The same also applies to Chl *b*, which shifts from 642 to 652 nm and 452 to 469 nm (see, e.g., Fig. F4.3.3 and Table F4.3.1, and Lichtenthaler, 1987). These wavelength shifts of the absorption maxima are correlated with changes in the absorption coefficients used for the quantitative determination of Chls *a* and *b* and carotenoids. For these reasons, the absorbance readings of a pigment extract must be performed at the correct wavelength position, i.e., the maxima of pure Chl *a* and pure Chl *b* in a particular solvent. Moreover, the solvent-specific extinction coefficients have to be considered by applying the corresponding equations for calculation of the pigment content. Minor differences in the positions of the wavelength maxima also exist, depending on the spectrophotometer type used. Thus, the wavelength position can differ by 1.0 or 1.5 nm.

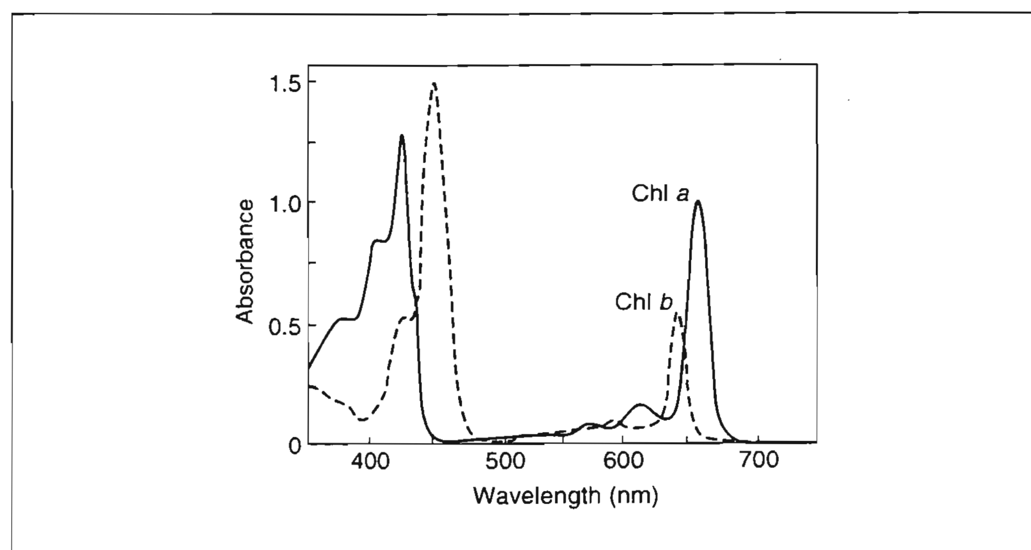

Figure F4.3.1 Absorption spectra of freshly isolated Chl *a* and Chl *b* in diethyl ether (pure solvent). The spectra were measured 40 min after extraction of pigments from leaves and 3 min after eluting the two Chls with diethyl ether from a TLC plate.

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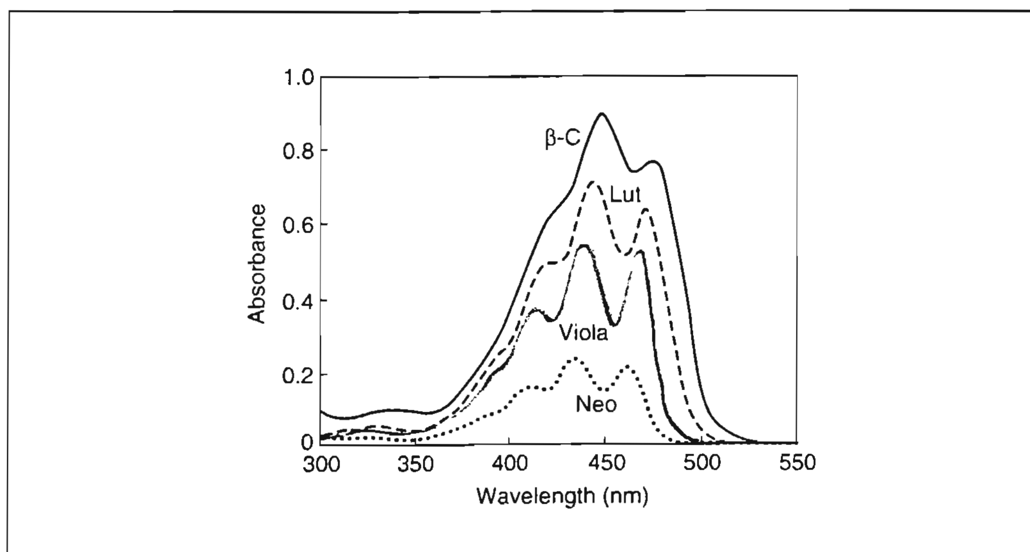

Figure F4.3.2 Absorption spectra of the major carotenoids of the photosynthetic biomembranes of green leaves of higher plants in diethyl ether (pure solvent). The carotenoids were freshly isolated from a pigment extract by TLC following Lichtenthaler and Pfister (1978) and Lichtenthaler (1987). β-C, β-carotene; Lut, lutein; Neo, neoxanthin; Viola, violaxanthin.

In order to perform spectroscopic measurements of green plant tissue extracts in the right maximum regions, one should determine the maximum red spectral position of pure Chl *a* and pure Chl *b* solutions with one's own spectrophotometer and compare them with those from the literature, given in Table F4.3.1. For a wavelength deviation of more than 1 nm, one should measure the absorbance of the pigment extract using these self-determined maxima rather than the literature values. The same equations for the particular solvent can be applied as long as wavelength positions differ by no more than 2 nm. At a deviation of >2 nm, either the spectrophotometer needs wavelength adjustment or a wrong, impure solvent has been applied. For the determination of carotenoids in the same extract solution, the wavelength position of 470 nm may be maintained, since a 1-nm shift has much less influence on the total carotenoid level than on the individual levels of Chls *a* and *b*.

ABSORPTION SPECTRA

The absorption spectrum of an extract of a green leaf containing a mixture of Chls *a* and *b* and total carotenoids (Fig. F4.3.4) is dominated by the absorption of Chl *a* at A_{428} (blue)

Table F4.3.1 Wavelength Maxima (A_{max}) and Specific Absorbance Coefficients (α)[a] of Chl *a* and *b* for Extracts in Different Organic Solvents

	Diethyl ether (water free)	Diethyl ether (pure)	Diethyl ether (water saturated)	Acetone (pure)	Acetone (with 20% water)	Ethanol (with 5% water)	Methanol (pure)
A_{max} Chl *a* [nm]	660.0	660.6	661.6	661.6	663.2	664.2	665.2
A_{max} Chl *b* [nm]	641.8	642.2	643.2	644.8	646.8	648.6	652.4
$\alpha_{(a)maxa}$	101.9	101.0	98.46	92.45	86.3	84.60	79.24
$\alpha_{(a)maxb}$	15.20	15.0	15.31	19.25	20.49	25.06	35.52
$\alpha_{(a)470}$	1.30	1.43	1.38	1.90	1.82	2.13	1.63
$\alpha_{(b)maxa}$	4.7	6.0	7.2	9.38	11.2	16.0	21.28
$\alpha_{(b)maxb}$	62.3	62.0	58.29	51.64	49.18	41.2	38.87
$\alpha_{(b)470}$	33.12	35.87	48.05	63.14	85.02	97.64	104.96
$\alpha_{(x+c)470}$	213	205	211	214	198	209	221

[a]Units of absorption coefficients are given in liter g^{-1} cm^{-1}. $\alpha_{(a)maxa}$ is the specific absorbance coefficient of Chl *a* at its red maximum; $\alpha_{(a)maxb}$ is the specific absorbance coefficient of Chl *a* at the red maximum of Chl *b*; $\alpha_{(a)470}$ is the specific absorbance coefficient of Chl *a* at 470 nm; $\alpha_{(x+c)470}$ is the specific absorbance coefficient of the sum of xanthophylls and carotenes at 470 nm.

and A_{661} (red). Chl *b* and the carotenoids absorb broadly in the blue region (400 to 500 nm).

A plant sample homogenized with an organic solvent is usually turbid and must be filtered or centrifuged to become fully transparent (see UNIT F4.2). Turbidity and light scattering lead to a higher absorption between 400 and 800 nm, with a slight but continuous increase towards shorter wavelengths (Fig. F4.3.5). Thus, measuring a turbid extract leads to an overestimation of the pigment levels, especially for Chl *b* and total carotenoids. Turbidity can be checked by measuring A_{750} and A_{520}. For a fully transparent leaf pigment extract, A_{750} should equal zero, since Chls *a* and *b* and carotenoids do not absorb in this region. A_{520} readings for extracts of green plant tissue should be <10% of the main Chl absorbance in the red maximum near 661 nm (diethyl ether) or 650 nm (ethanol), as shown in Figures F4.3.4 and F4.3.5.

ACCURACY OF SPECTROSCOPIC MEASUREMENTS

In order to have an exact spectroscopic measurement of absorbances, one must consider the absorbance range in which readings are made. Absorbance should be measured between 0.3 and 0.85. Leaf extracts with an absorbance <0.3 in the red region do not yield correct pigment values. There are several interfering factors, such as a base line that is not fully zeroed. Thus, values <0.3, whether read by the experimenter or given as digital values by the instrument, are not acceptable. Absorbance values >0.9 may indicate problems with the accuracy of the detector (e.g., a photomultiplier). Since the detector system examines the transmitted light of the cuvette, the absorbance is calculated from this value. When transferring the linear transmission unit to the logarithmic absorbance unit, the accuracy is exponentially reduced with rising values.

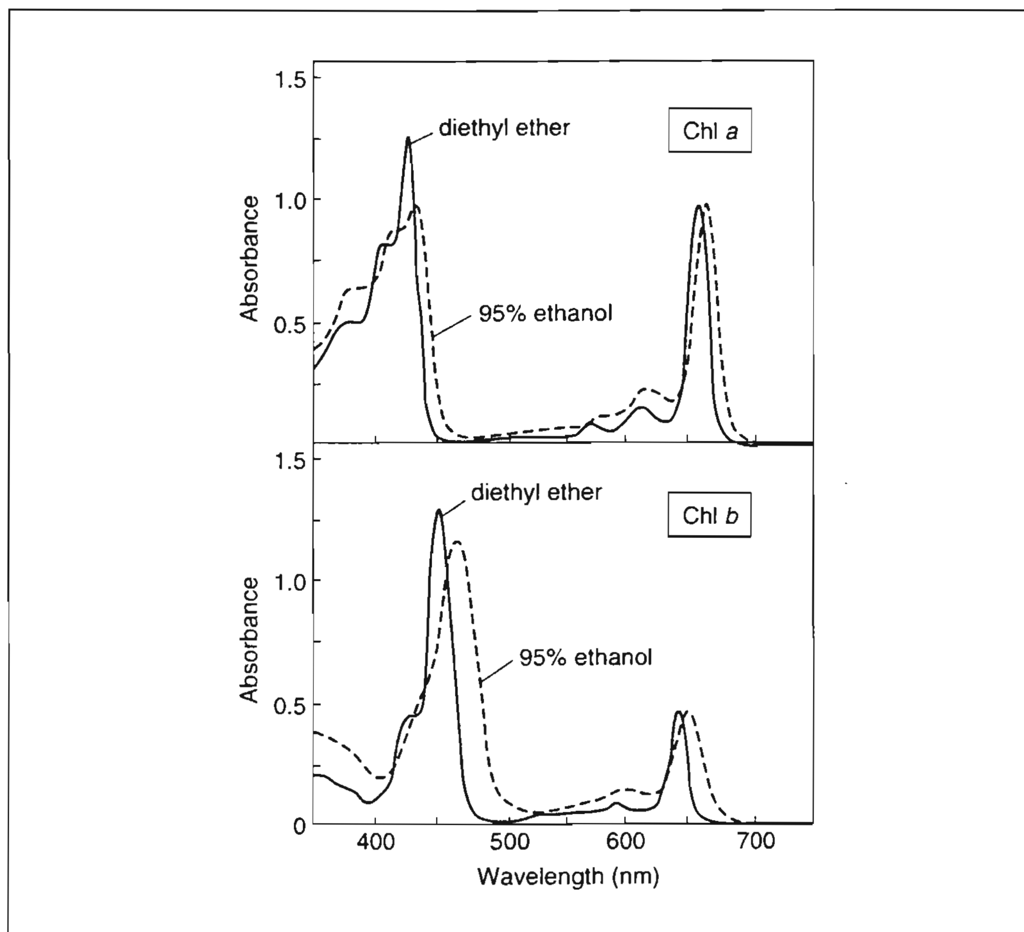

Figure F4.3.3 Differences in the absorption spectra of Chl a and Chl b in diethyl ether and 95% aqueous ethanol. For the more polar solvent (95% ethanol; broken line), the absorbance (extinction) in the blue and red absorption maxima of both Chls are decreased compared to values obtained using the less polar solvent diethyl ether (black), and the wavelength positions of the maxima are shifted to the right. For a better comparison, the absorbances in the red maxima were set at the same values.

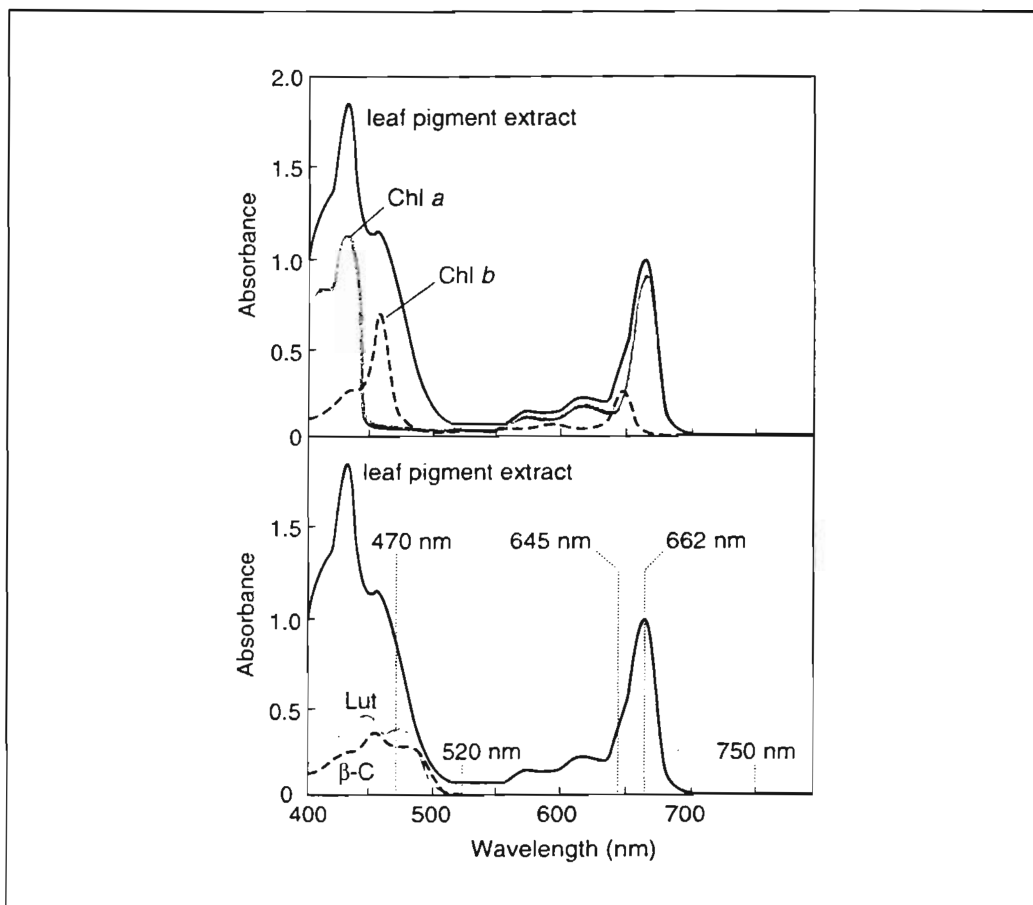

Figure F4.3.4 Absorption spectra of pigments from a green tobacco leaf extracted with 100% acetone. The leaf extract was measured directly after extracting the leaf. Chl *a*, Chl *b*, and the carotenoids β-carotene (β-C) and lutein (Lut) were measured after separation by TLC.

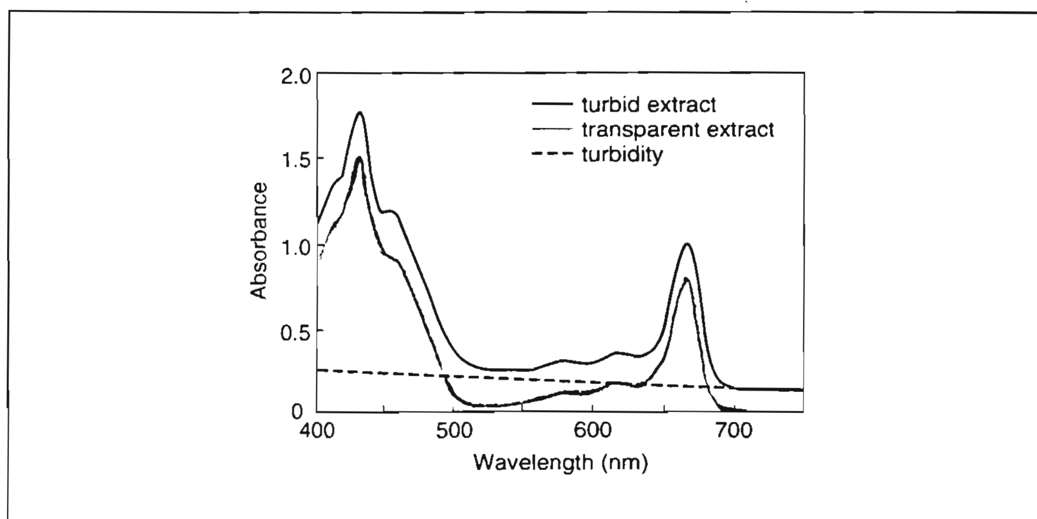

Figure F4.3.5 Absorption spectra of a leaf extract before (turbid) and after (transparent) centrifugation in 100% acetone. The difference spectrum between the two extracts represents the spectrum of turbidity.

For absorbance values <0.3, one should try to concentrate the extract (e.g., by evaporation), make a new extract using more plant material and less solvent, or extract the pigments in a separatory funnel into a small volume of a hydrophobic solvent in the epiphase. Various spectrophotometers are constructed to measure absorbance (extinction) values only up to 1.0 (i.e.. a transmittance of 10%). In such cases, an absorbance >0.85 is not suitable, and the extract solution should be diluted to obtain valid Chl *b* and carotenoid values. In both cases, care must be taken to ensure that the final volume of the extract solution is carefully recorded and considered in the calculation of total Chls and carotenoids.

The extinction coefficients and the equations used and established by Arnon (1949) are not correct. They provide only a rough estimate of Chl *a* and *b* levels and yield inaccurate Chl *b* values. and. consequently. incorrect values for the Chl *a/b* ratio. They have been redetermined by Lichtenthaler (1987) using the extinction coefficients of Smith and Benitez (1955) for pure Chl *a* and Chl *b* in diethyl ether, which were found to be correct in the red absorption maxima at 661 and 642 nm, respectively, for purified Chls. The relative absorptions of Chl *a* and Chl *b* at other wavelengths in other organic solvents have been redetermined using modern high-resolution spectrophotometers.

To exactly determine carotenoids by measuring A_{470}, one needs to know the exact level of Chl *b*. which (in contrast to Chl *a*) also absorbs considerably at this wavelength (Fig. F4.3.1). If Chl *b* is overestimated, the level of total carotenoids becomes too low, and vice versa. With the redetermined extinction coefficients, the new equations permit the determination of total carotenoids in addition to Chl *a* and Chl *b* in the same green tissue extract solutions.

QUANTIFACTION OF PIGMENTS

The basis for spectroscopic quantification of pigments is the Lambert-Beer law, which defines the absorbance of a solution with respect to the specific light absorption characteristic of an individual dissolved compound:

$$A = \alpha c_w d \text{ or } A = \varepsilon c_m d$$

where A is absorbance (dimensionless). α is the specific absorbance coefficient in liter g^{-1} cm^{-1}, ε is the molar absorbance coefficient in liter mol^{-1} cm^{-1}, c_w is the weight concentration in g $liter^{-1}$. c_m is the molar concentration in mol $liter^{-1}$, and d is the path length of the cuvette in cm, usually 1 cm.

This original Lambert-Beer law can only be applied for one isolated pigment. Absorbance coefficients taken from the literature (Table F4.3.1) are valid only for one substance (e.g., Chl *a*) using one solvent (e.g., 100% acetone) and one wavelength (e.g., 661.6 nm). Changes in substance, solvent, or wavelength lead to changes in the absorbance coefficient.

When the concentration of Chl *a* and Chl *b* is determined from a pigment extract containing both Chls, the equation derived from the Lambert-Beer law becomes more complex. The absorbance is then expressed as the sum of the absorbances of Chl *a* and Chl *b*. Thus. the absorbance of Chl *b* contributes to the absorbance of Chl *a* at the Chl *a* maximum, and vice versa:

$$A_{\max a} = A_{(a)\max a} + A_{(b)\max a} = \left(\alpha_{(a)\max a} \times c_{ma} \times d\right) + \left(a_{(b)\max a} \times c_{mb} \times d\right)$$

$$A_{\max b} = A_{(a)\max b} + A_{(b)\max b} = \left(\alpha_{(a)\max b} \times c_{ma} \times d\right) + \left(a_{(b)\max b} \times c_{mb} \times d\right)$$

The concentrations for Chl *a* (c_a) and Chl *b* (c_b) are then given by a different equation, where the specific contribution of Chl *b* to the Chl *a* maximum and of Chl *a* to the Chl *b* maximum are subtracted. The following equations contain the denominator z. a term formed from the four extinction coefficients of Chl *a* and Chl *b*. The light path length (usually 1 cm) is omitted here:

$$c_a = \left[\frac{\alpha_{(b)\max b} \times A_{\max a}}{z}\right] - \left[\frac{\alpha_{(b)\max a} \times A_{\max b}}{z}\right]$$

$$c_b = \left[\frac{\alpha_{(a)\max a} \times A_{\max b}}{z}\right] - \left[\frac{\alpha_{(a)\max b} \times A_{\max a}}{z}\right]$$

$$z = \left(\alpha_{(a)\max a} \times \alpha_{(b)\max b}\right) - \left(\alpha_{(a)\max b} \times \alpha_{(b)\max a}\right)$$

DETERMINATION OF TOTAL CAROTENOIDS

In an extract of plant material containing carotenoids (x + c = xanthophylls and carotenes) in addition to Chls, A_{470} (the carotenoid region) is determined as the sum of specific

absorbances for Chl a, Chl b, and total carotenoids:

$$A_{470} = A_{(x+c)470} + A_{(a)470} + A_{(b)470}$$

From this follows, according to the Lambert-Beer law:

$$A_{(a)470} = \alpha_{(a)470} \times c_a \times d$$

$$A_{(b)470} = \alpha_{(b)470} \times c_b \times d$$

$$A_{(x+c)470} = \alpha_{(x+c)470} \times c_{(x+c)} \times d$$

The concentration of carotenoids $c_{(x+c)}$ is then given by the following equation, which has been reduced using $d = 1$ cm:

$$c_{(x+c)} = \frac{A_{(a)470} - (\alpha_{(a)470} \times c_a) - (\alpha_{(b)470} \times c_b)}{\alpha_{(x+c)470}}$$

The concentrations for Chl a (c_a), Chl b (c_b), and the sum of leaf carotenoids (c_{x+c}) can be calculated with the following equations given for different solvents, where the pigment concentrations are given in µg/ml extract solution.

Diethyl ether (pure solvent):

$$c_a\,(\mu g/ml) = 10.05\,A_{660.6} - 0.97\,A_{642.2}$$

$$c_b\,(\mu g/ml) = 16.36\,A_{642.2} - 2.43\,A_{660.6}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.43\,c_a - 35.87\,c_b)/205$$

Diethyl ether (water free):

$$c_a\,(\mu g/ml) = 9.93\,A_{660.6} - 0.75\,A_{641.8}$$

$$c_b\,(\mu g/ml) = 16.23\,A_{641.8} - 2.42\,A_{660.6}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.30\,c_a - 33.12\,c_b)/213$$

Diethyl ether (water saturated):

$$c_a\,(\mu g/ml) = 10.36\,A_{661.6} - 1.28\,A_{643.2}$$

$$c_b\,(\mu g/ml) = 17.149\,A_{643.2} - 2.72\,A_{661.6}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.38\,c_a - 48.05\,c_b)/211$$

Ethanol with 5% (v/v) water:

$$c_a\,(\mu g/ml) = 13.36\,A_{664.1} - 5.19\,A_{648.6}$$

$$c_b\,(\mu g/ml) = 27.43\,A_{648.6} - 8.12\,A_{664.1}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 2.13\,c_a - 97.64\,c_b)/209$$

Acetone (pure solvent):

$$c_a\,(\mu g/ml) = 11.24\,A_{661.6} - 2.04\,A_{644.8}$$

$$c_b\,(\mu g/ml) = 20.13\,A_{644.8} - 4.19\,A_{661.6}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.90\,c_a - 63.14\,c_b)/214$$

Acetone with 20% (v/v) water:

$$c_a\,(\mu g/ml) = 12.25\,A_{663.2} - 2.79\,A_{646.8}$$

$$c_b\,(\mu g/ml) = 21.50\,A_{646.8} - 5.10\,A_{663.2}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.82\,c_a - 85.02\,c_b)/198$$

Methanol (pure solvent):

$$c_a\,(\mu g/ml) = 16.72\,A_{665.2} - 9.16\,A_{652.4}$$

$$c_b\,(\mu g/ml) = 34.09\,A_{652.4} - 15.28\,A_{665.2}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.63\,c_a - 104.96\,c_b)/221$$

Methanol with 10% (v/v) water:

$$c_a\,(\mu g/ml) = 16.82\,A_{665.2} - 9.28\,A_{652.4}$$

$$c_b\,(\mu g/ml) = 36.92\,A_{652.4} - 16.54\,A_{665.2}$$

$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.91\,c_a - 95.15\,c_b)/225$$

INTERPRETATION OF CHLOROPHYLL AND CAROTENOID CONTENT

The concentration of Chl a and b in plant material can be quantified with different reference systems. Reference systems currently in use include mg Chl a+b/m^2 leaf area (or µg/cm^2 leaf area), µg Chl a+b/g dry weight, and mg Chl a+b/g fresh weight (less suitable than dry weight).

When comparing results with those of other groups or with values obtained previously, the same reference system must be applied. Changes in Chl content should be demonstrated by means of a reference that does not change, otherwise an observed variation of data may not be due to changes in Chl concentration, but instead to changes in the reference system. For instance, an increase in Chl per fresh weight (in leaves or fruits) could be solely due to a decrease in fresh weight caused by water loss. In various cases, the number of leaves, cotyledon pairs, seedlings (shoots), or fruits may be the best reference system to follow changes in pigment levels, as these numbers do not change when dry weight or leaf area vary.

The weight ratio of Chl a and Chl b (Chl a/b ratio) is an indicator of the functional pigment

Table F4.3.2 Leaves with High Versus Low Chlorophyll *a/b* Ratios

High *a/b* ratio	Low *a/b* ratio
Greening of etiolated leaves (4.0-10)	Fully developed green leaves (2.5-3.5)
Sun leaves (3.0-3.8)	Shade leaves (2.4-2.7)
Leaves of C_4 plants (3.0-5.0)	Leaves of C_3 plants (2.5-3.5)

equipment and light adaptation of the photosynthetic apparatus (Lichtenthaler et al., 1981). Chl *b* is found exclusively in the pigment antenna system, whereas Chl *a* is present in the reaction centers of photosystems I and II and in the pigment antenna. Whereas the light-harvesting pigment protein LHC-I of the photosynthetic pigment system PS I has an *a/b* ratio of ~3, that of LHC-II of PS II exhibits an *a/b* ratio of 1.1 to 1.3. The level of LHC-II of PS II is variable and shows a light adaptation response. Shade plants possess much higher amounts of LHC-II than sun-exposed plants and, consequently, their *a/b* ratios are lower than in sun-exposed plants (Lichtenthaler et al., 1982, 1984). Thus, a decrease in the Chl *a/b* ratio may be interpreted as an enlargement of the antenna system of PS II. Some examples for high and low Chl *a/b* ratios in leaves of different developmental stages and in fully differentiated leaves grown at low light or high light conditions are given in Table F4.3.2.

The weight ratio of Chls *a* and *b* to total carotenoids (*a*+*b*)/(x+c) is an indicator of the greenness of plants. The ratio (*a*+*b*)/(x+c) normally lies between 4.2 and 5 in sun leaves and sun-exposed plants, and between 5.5 and 7.0 in shade leaves and shade-exposed plants. Lower values for the ratio (*a*+*b*)/(x+c) are an indicator of senescence, stress, and damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of Chls than carotenoids. Leaves become more yellowish-green and exhibit values for (*a*+*b*)/(x+c) of 3.5, or even as low as 2.5 to 3.0 as senescence progresses. Also, during chromoplast development in ripening fruits or fruit scales, which turn from green to yellow or orange or red, the ratio (*a*+*b*)/(x+c) decreases continuously and reaches values below 1.0.

Sun leaves of different trees exhibit average Chl *a*+*b* levels of 400 to 700 mg/m^2 leaf area (40 to 70 $\mu g/cm^2$) and shade leaves have 380 to 570 mg/m^2 leaf area (38 to 57 $\mu g/cm^2$). As sun leaves possess thicker cell walls, a lower leaf

Table F4.3.3 Examples of Chlorophyll and Carotenoid Levels and Pigment Ratios in Green Sun and Shade Leaves[a]

Leaf type		$a + b$ (mg/m^2)	x + c (mg/m^2)	$a + b$ (mg/g dw)	x + c (mg/g dw)	*a/b*	$(a + b)/(x + c)$
Fagus sylvatica (beech)	Sun leaves	510.8	126.4	6.29	1.56	3.22	4.04
	Shade leaves	450.1	85.8	12.01	2.29	2.65	5.25
Carpinus betulus (hornbeam)	Sun leaves	571.0	117.4	8.15	1.68	3.20	4.86
	Shade leaves	431.1	70.8	19.05	3.13	2.45	6.09
Populus nigra (poplar)	Dark green sun leaves	724.4	161.5	8.03	1.81	3.30	4.44
	Dark green shade leaves	568.2	109.2	12.41	2.39	2.74	5.20
	Green senescent leaves	351.5	87.4	5.00	1.24	3.08	4.02
	Yellowish-green senescent leaves	140.3	79.4	1.99	1.13	3.29	1.77

[a]Pigment levels given in mg/m^2 leaf area and in mg/g dry weight (dw). Values measured are those from fully developed leaves in June, 2000. Pigment levels within one leaf usually vary by <3%, and pigment ratios vary by <1%. Abbreviations: a + b: total chlorophylls a and b; x + c, xanthophylls and carotenes (total carotenoids).

water content (50% to 65% fresh weight), and higher dry weight than shade leaves, they exhibit on a dry weight basis a considerably lower Chl and carotenoid content than shade leaves (Table F4.2.3). The latter, in turn, possess a higher water content (68% to 85% fresh weight) and, consequently, a lower dry weight than sun exposed leaves.

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Presents a table (Table 7) of chloropyll, carotenoid, and vitamin E levels (in µg/g dw) of green leaf tissue, vegetables, green and red fruits (tomato, red pepper), and nongreen plant foods (carrots, cauliflower).

Lichtenthaler, 1987. See above.

Presents redetermined absorption coefficients for chlorophylls and total carotenoids, which allows the determination of all three in the same pigment extract of leaves or fruits

Šesták, 1971. See above.

Gives basic information on the measurements of chlorophylls in various spectroscopic instruments.

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