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Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy

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The extinction coefficients for chlorophylls *a* and *b* in diethylether (Smith, J.H.C. and Benitez, A. (1955) in Modern Methods of Plant Analysis (Paech, K. and Tracey, M.V., eds.), Vol. 4, pp. 143–196, Springer-Verlag, Berlin), used in this paper as primary standards, were verified, to within an error of less than 1%, by magnesium determination using atomic absorbance spectrophotometry. We also report the determination of accurate extinction coefficients for chlorophylls *a* and *b* in *N*,*N*′-dimethylformamide, methanol or buffered 80% aqueous acetone. Highly purified chlorophylls were used and methods were employed which not only minimize errors due to evaporation of the volatile solvents employed in their estimation but also eliminate variable micro-contamination by chlorophyll degradation products, a potential source of inconsistency between the extinction coefficients obtained in each of these three solvents. Using these new coefficients, expressed as both millimolar and specific coefficients, we have derived new simultaneous equations to obtain chlorophyll concentrations as nmol/ml and μg/ml, respectively. These equations were applied to data obtained with leaf discs from spinach and *Flindersia brayleyana* extracted with the three specified solvents and to a concentrated solution (in *N*,*N*′-dimethylformamide) of a chlorophyll *a* + *b* mixture added to the three solvent systems. The validity of these equations is proven by the consistency of the chlorophyll determinations and of the chlorophyll *a*/*b* ratios. New simultaneous equations, compatible with the equations derived for the three solvents, are presented for the assay of chlorophylls *a* and *b* converted to their cyclic hydroxylactone derivatives by extraction with alkaline pyridine reagent (2.1 M pyridine in 0.35 M NaOH). Most chlorophyll analyses in higher plants, including the chlorophyll content and chlorophyll *a*/*b* ratios of plant thylakoids and chlorophyll-protein complexes, have been obtained in 80% aqueous acetone with the much used simultaneous equations of Arnon (Arnon, D.I. (1949) Plant Physiol. 24, 1–15). For this reason we include conversion factors which correct these earlier data and make it compatible with data calculated with the simultaneous equations presented in this paper. The importance of these corrections to the formulation of meaningful models of the photosynthetic apparatus is demonstrated. Our results also indicate that grinding leaf discs with *N*,*N*′-dimethylformamide is a more reliable method for extracting all chlorophylls than shaking with this solvent for 24 h.

Introduction

Three different solvents, namely DMF [1], methanol [2] or buffered 80% aqueous acetone [3], were used in a search for a convenient extraction and assay procedure for Chls *a* and *b* in leaf discs from Queensland Maple seedlings (*Flindersia brayleyana*) grown under a variety

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Abbreviations: Chl, chlorophyll; DMF, *N*,*N*′-dimethylformamide.

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of conditions. It was found that the Chl *a/b* ratios, determined in buffered 80% aqueous acetone extracts of leaf discs from *F. brayleyana* using the simultaneous equations of Arnon [3] to determine the Chl *a* and *b* concentrations, differed significantly from the ratios obtained in DMF or methanol extracts using the simultaneous equations of Inskeep and Bloom [4] and of Böger [2], respectively. We have also noted that Chl *a/b* ratios determined in buffered aqueous acetone using the equations of Arnon [3] are drastically lower than those obtained with the equations of other workers [5–7].

In view of these considerations and to obtain reliable and consistent chlorophyll assays with the three specified solvents which are routinely used in these laboratories, we redetermined the extinction coefficients for Chls *a* and *b* in these solvents and derived from them three new sets of compatible simultaneous equations to calculate their concentrations. The coefficients used by Arnon [3] for Chl *a* and especially for Chl *b* were found to be low.

Porra and Grimme [8] earlier presented simultaneous equations for the assay of Chls *a* and *b*, converted to their cyclic hydroxylactone derivatives (see Fig. 2, structure IV in Discussion section) by extraction with alkaline pyridine, which were compatible with the equations of Arnon [3]. The original data [8] have now been reprocessed to give equations which are compatible with those presented for use with the other three extractants.

The past formulation of more accurate equations [5–7] than those of Arnon [3] does not appear to have diminished the use of the latter. This may be due to the failure to simultaneously provide conversion factors to quickly and conveniently correct earlier data coupled with a reluctance to forego the ability to compare newly gathered data with the old. To overcome such resistance, correction factors have been derived and presented in this paper. Replacement of Arnon's equations [3] by the new equations for use with buffered 80% aqueous acetone now enjoys the added advantage that DMF, methanol and alkaline pyridine can be used when alternative extractants are required and, with the equations provided in this paper, compatible results will be produced.

Experimental

Chemicals and reagents. Acetone, diethylether and methanol were all Analar grade reagents supplied by BDH Chemicals (Australia Pty.), Port Fairy, Australia. DMF was supplied by May and Baker, West Footscray, Australia. (DMF is a liver toxin which is readily absorbed through the skin: disposable gloves should be used for protection). Buffered aqueous acetone is 80% aqueous acetone containing 2.5 mM sodium phosphate buffer pH 7.8 to minimize conversion of chlorophylls to phaeophytins.

Preparation of pure chlorophylls. Chlorophylls were extracted from maize leaves with buffered aqueous acetone. Chls *a* and *b* were then separated and purified by column chromatography on sucrose [9] after precipitation with dioxane [10] as described by Porra et al. [11]. Chl *a* and *b* zones of the column were transferred to sintered glass funnels and the chlorophylls completely removed from the sucrose with diethylether by reduced pressure filtration. Diethylether was removed by evaporation under reduced pressure and the purified Chl *a* and *b* samples redissolved in petroleum spirit and rechromatographed on a fresh sucrose column as described above. Rechromatographed Chls *a* and *b* were again removed from the sucrose with diethylether and these ethereal solutions were kept in the dark under oxygen-free nitrogen at $-15\,^{\circ}C$ to minimize chlorophyll degradation by demetallation and oxidation reactions: the solutions were used for spectrophotometric determination of extinction coefficients within approx. 9 h. All preparative procedures were carried out in dim light to minimize light-associated degradation of chlorophylls.

Determination of extinction coefficients of Chl a and b in various solvents. To eliminate errors due to evaporative losses from all volatile solutions, absorbance measurements were made using a cuvette (1 cm light path) fused to a B14 socket with a total capacity of 7 ml when stoppered but marked with a 5 ml graduation line. To the cuvette was added exactly 5 ml of a diethylether solution of Chl *a* or *b* with absorbance at the major red peak between 0.7 and 1.0. The cuvette was immediately closed with a B14 stopper secured with two extension springs. The spectrum was recorded from 400 to 750 nm in a Hitachi model U3200 recording spectrophotometer with a corrected baseline. The spectrophotometer searched for all peaks; because spectra were automatically zeroed at 750 nm, all extinction coefficients are difference coefficients (i.e., peak maxima minus 750 nm). The cuvette was then opened under dim light, partly immersed in a warm water bath (approx. $55\,^{\circ}C$) and the diethylether removed by evaporation under a stream of oxygen-free nitrogen. The chlorophyll residue was then dissolved in a new replacement solvent such as DMF, cold methanol or buffered aqueous acetone and the volume adjusted to the 5 ml mark. The cuvette was quickly reclosed and secured with springs, the spectrum recorded between 400 and 750 nm and the peaks automatically recorded and measured.

For Chls *a* and *b* dissolved in Analar quality diethylether, containing approx. 0.2% H_2O, the major peak in the red region of the spectrum occurred at 660.8 and 642.6 nm, respectively. Smith and Benitez [12] found that the specific extinction coefficients (α) at these peaks are 100.9 and 62.0 $l \cdot g^{-1} \cdot cm^{-1}$, respectively: using molecular weights of 893.48 and 907.46 for Chls *a* and *b*, respectively, the corresponding millimolar ex-

tinction coefficients (ε'_{mM}) are 90.2 and 56.3 $l \cdot mmol^{-1} \cdot cm^{-1}$, respectively. The reliability of these extinction coefficients has been confirmed by Falk [13] who found that the spectrophotometric data of Smith and Benitez [12] were consistent with this data based on magnesium microanalysis with titanium yellow. In addition, we have also confirmed these extinction coefficients on the basis of magnesium content but determined by atomic absorption spectroscopy (see Results section).

Extinction coefficients (ε_{mM} or α) for Chl *a* and *b* in the new replacement solvent were then calculated as follows:

	α	ε_{mM}
Chl *a*	$100.9 \times \frac{A_1}{A_2}$	$90.2 \times \frac{A_1}{A_2}$
Chl *b*	$62.0 \times \frac{A_1}{A_3}$	$56.3 \times \frac{A_1}{A_3}$

where A_1 is the absorbance of each chlorophyll in the new replacement solvent at the peak maximum of either Chl *a* or Chl *b* in that solvent, and A_2 and A_3 are the absorbances of the Chl *a* and Chl *b* solutions, respectively, in diethylether at 660.8 nm (Chl *a*) and at 642.5 nm (Chl *b*), respectively.

Extraction of chlorophylls from leaf discs with DMF, methanol or buffered 80% aqueous acetone. Seedling trees (up to 1 m high) of well or poorly nourished *F. brayleyana* were grown in pots in a shade house. Spinach plants (*Spinacia oleracea*) were grown hydroponically in a glass house. For chlorophyll analysis, circular discs were cut with a 16 mm diameter punch (approx. 200 mm^2 surface) from both well nourished *Flindersia* and spinach leaves: for the pale green leaves of poorly nourished *Flindersia* plants, discs of 23 mm diameter (approx. 400 mm^2 surface) were used. These discs were extracted with DMF, cold methanol or buffered aqueous acetone by grinding with 2 ml of each solvent in a mortar with pestle. The homogenate, combined with a further three washings of the pestle and mortar (each of 1.5 ml) with the same solvent, was centrifuged at 2500 r.p.m. in an MSE bench centrifuge for 10 min. The pellet was then extracted with a further 1 ml of solvent in a Potter-Elvejhem homogenizer and the pooled supernatants adjusted to a final volume of 8 ml. The spectrum was recorded between 750 and 600 nm and the major red absorption peak automatically determined by the Hitachi Model U3200 recording spectrophotometer zeroed at 750 nm. The absorbance at the major red absorption peak of Chl *b* was also determined; Chls *a* and *b* and Chls *a* + *b* concentrations were then calculated using the equations described below in Table III.

Magnesium determination. The magnesium contents of Chls *a* and *b* were determined by atomic absorbance spectrophotometry using a Perkin Elmer atomic absorbance spectrophotometer (model AA 360). The method used is sensitive to 0.05 p.p.m.

Results

Confirmation of our primary standards by magnesium determinations using atomic absorption spectroscopy

The extinction coefficients of Chls *a* and *b* in diethylether, determined by Smith and Benitez [12], were used as primary standards in this work. The validity of these primary standards was confirmed by taking 10 ml of an approx. 20 μM solution of each chlorophyll in diethylether in a stoppered 10 ml graduated cuvette. The precise micromolar concentration was then predicted on the basis of the extinction coefficients of Smith and Benitez [12]. The magnesium was extracted exhaustively from the diethylether with 1.4% (w/v) perchloric acid and the pooled aqueous washings made up to a final volume of 10 ml. Complete conversion to phaeophytins *a* and *b* took approx. 30 and 120 min, respectively. The magnesium concentration was then determined by atomic absorbance spectroscopy using magnesium standards which were verified by complexiметric titration with EDTA using Eriochrome Black T as indicator. For two samples of Chl *a* the predicted concentrations were 12.97 μM and 16.75 μM and the magnesium concentrations were determined as 13.04 ± 0.25 μM and 16.67 ± 0.33 μM, respectively. Similarly, the predicted concentrations for two samples of Chl *b* were 12.97 μM and 15.31 μM and the magnesium concentrations were found to be 13.06 ± 0.25 μM and 15.32 ± 0.29 μM. The errors quoted here are maximum errors for individual determinations and include all errors associated with the atomic absorption determination, verification of the magnesium standard and volumetric manipulations. The magnesium determinations have confirmed the extinction coefficients of Smith and Benitez [12] for both Chls *a* and *b* in diethylether to within an error of less than 1.0%.

Extinction coefficients of chlorophylls and equations for determination of their concentrations in DMF, methanol or buffered 80% aqueous acetone

During our initial attempts to obtain accurate extinction coefficients, diethylether solutions (10 ml) of Chls *a* and *b*, with appropriate absorption of its major red peak (see Experimental), were prepared in a 10 ml graduated flask fitted with a ground glass stopper. Approx. 3 ml was transferred by Pasteur pipette to a Hellma (type 110QS) cuvette and tightly stoppered prior to spectrophotometric examination. When the contents of the cuvette were returned to the flask, a loss of about 0.6 ml of diethylether was noted. It seemed reasonable to assume that half the loss (0.3 ml) occurred during the transfer to the cuvette so that it could be anticipated that absorbance of the diethylether solution could be

TABLE I

Absorption maxima and Q_{RS} ratios for chlorophylls a and b in diethylether

The absorption spectra of Chls *a* and *b* dissolved in diethylether after rechromatography on sucrose columns were recorded on a spectrophotometer automatically zeroed at 750 nm (see Experimental). The Q_{RS} ratios, which represent the absorbance at the indicated peaks divided by the absorbance at the Soret peak, are compared with those of purified chlorophylls from other sources.

Maxima (nm)	Q_{RS} ratio		
	Ref. 12	Ref. 7	this work
Chl *a*			
660.8	0.767	0.794	0.794
615.4	0.124	0.125	0.123
577.4	0.070	0.069	0.065
532.4	0.032	0.035	0.030
429.0 (Soret)	1.0	1.0	1.0
409.8	0.648	0.651	0.650
Chl *b*			
643.0	0.355	0.369	0.366
594.2	0.073	0.074	0.075
454.0 (Soret)	1.0	1.0	1.0
430.0	0.359	0.370	0.363

overestimated by as much as 10% which would then lead to an underestimate of 10% in the extinction coefficients in the replacement solvents. The error would be somewhat mitigated if the replacement solvent was also very volatile. Such errors were overcome by using a 5 ml graduated cuvette fused to a B14 ground-glass socket and stopper (see Experimental).

Early studies with the graduated cuvette still failed to provide consistent Chls *a* and *b* concentrations and Chl *a*/*b* ratios for leaf discs extracted with the three solvents. Ethereal solutions of Chls *a* and *b* for these studies were freshly prepared from maize leaves just prior to each investigation with each of the solvents: this was done to minimize inconsistency due to variable microcontamination by chlorophyll degradation products arising from demetallation and oxidation reactions which can occur during storage if the pigments are not kept in the dark under oxygen-free nitrogen at −15 °C. In these early studies, Chls *a* and *b* were separated by only one passage down a sucrose column; a small but variable contamination of Chl *a* with pheophytin *a* and of Chl *b* with pheophytin *b* and Chl *a* was subsequently found to be responsible for this lack of consistency between results obtained in each solvent. To overcome this problem a second purification by sucrose chromatography was introduced (see Experimental) and by examining the ratios (Q_{RS}) of all absorption peaks to the Soret peak (Table I), the purity of the Chl *a* and Chl *b* samples produced by rechromatography was found to be better than that used by Smith and Benitez [12] and at least equivalent to that used by Lichtenthaler [7]: the lower ratios we have obtained for our Chl *a* at 532.4 and 409.8 nm indicate smaller contamination by phaeophytin *a* (cf. Ref. 12) and hydroxylactone derivatives (oxidation products) of Chl *a* (cf. Refs. 11 and 8), respectively. To eliminate inconsistencies due to variable microcontamination by the chlorophyll degradation products mentioned above, sufficient freshly purified Chls *a* and *b* were prepared to enable the extinction coefficients in all three solvents to be obtained from a single ethereal solution of each chlorophyll; the coefficients were obtained as quickly as possible (within approx. 9 h) during which time the ethereal solutions were kept in the dark and under oxygen-free nitrogen at −15 °C to minimize all degradation reactions including those mentioned above (see Discussion section). Each coefficient, given as both millimolar (ε_{mM}) and specific (α) extinction coefficients (Table II), is the

TABLE II

Corrected millimolar and specific difference coefficients for chlorophylls a and b in N,N′-dimethylformamide, methanol and buffered 80% aqueous acetone

As the spectrophotometer was zeroed at 750 nm, the extinction coefficients listed below are difference coefficients as shown in column 2. Each coefficient is the mean of three separate determinations. The percentage variation about the mean (m) is presented as $100 \cdot \sigma/m$, where σ represents the standard deviation. The percentage variation is presented (in brackets) beside the millimolar (ε_{mM}) coefficients but would be identical for the specific (α) coefficients which are derived from the same data.

Solvent	Wavelengths appropriate to the difference coefficients (nm)	Difference extinction coefficients			
		Chl *a*		Chl *b*	
		(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)	(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)
DMF	663.8 minus 750	79.29 (±0.34)	88.74	12.03 (±4.4)	13.26
	646.8 minus 750	18.62 (±1.47)	20.84	46.49 (±0.92)	51.23
Methanol	665.2 minus 750	71.43 (±0.91)	79.95	20.20 (±0.99)	22.26
	652.0 minus 750	31.65 (±0.66)	35.42	38.55 (±0.60)	42.48
Aqueous 80% acetone	663.6 minus 750	76.79 (±0.52)	85.95	9.79 (±0.51)	10.78
	646.6 minus 750	18.58 (±0.59)	20.79	47.04 (±0.34)	51.84

TABLE III

Corrected equations for the determination of chlorophylls in N,N'-dimethylformamide, methanol and buffered 80% aqueous acetone

These equations (1–18) are derived from the difference extinction coefficients presented in Table II; therefore all absorbance measurements at the indicated wavelengths must have the absorbance at 750 nm subtracted.

Equations for chlorophyll concentrations in nmol/ml		Equations for chlorophyll concentrations in μg/ml	
In DMF			
Chl a $= 13.43\,A^{663.8} - 3.47\,A^{646.8}$	(1)	Chl a $= 12.00\,A^{663.8} - 3.11\,A^{646.8}$	(10)
Chl b $= 22.90\,A^{646.8} - 5.38\,A^{663.8}$	(2)	Chl b $= 20.78\,A^{646.8} - 4.88\,A^{663.8}$	(11)
Chls $a + b = 19.43\,A^{646.8} + 8.05\,A^{663.8}$	(3)	Chls $a + b = 17.67\,A^{646.8} + 7.12\,A^{663.8}$	(12)
In methanol			
Chl a $= 18.22\,A^{665.2} - 9.55\,A^{652.0}$	(4)	Chl a $= 16.29\,A^{665.2} - 8.54\,A^{652.0}$	(13)
Chl b $= 33.78\,A^{652.0} - 14.96\,A^{665.2}$	(5)	Chl b $= 30.66\,A^{652.0} - 13.58\,A^{665.2}$	(14)
Chls $a + b = 24.23\,A^{652.0} + 3.26\,A^{665.2}$	(6)	Chls $a + b = 22.12\,A^{652.0} + 2.71\,A^{665.2}$	(15)
In buffered 80% aqueous acetone			
Chl a $= 13.71\,A^{663.6} - 2.85\,A^{646.6}$	(7)	Chl a $= 12.25\,A^{663.6} - 2.55\,A^{646.6}$	(16)
Chl b $= 22.39\,A^{646.6} - 5.42\,A^{663.6}$	(8)	Chl b $= 20.31\,A^{646.6} - 4.91\,A^{663.6}$	(17)
Chls $a + b = 19.54\,A^{646.6} + 8.29\,A^{663.6}$	(9)	Chls $a + b = 17.76\,A^{646.6} + 7.34\,A^{663.6}$	(18)

mean of three separate determinations. The percentage variation about the mean (see Table II) seldom reached ±1.0% and exceeded it only when absorbance measurements were made on steeply sloping segments of Chl *a* and Chl *b* spectra; data have been obtained (see below) which verify the reliability of all the coefficients.

Although it has been customary since Arnon's early work [3] to express chlorophyll concentrations in μg/ml, chlorophyll composition in modern photosynthesis research, especially that dealing with the composition and function of the various component complexes of the photosynthetic process, has been expressed in molar terms: for this reason, equations are presented in Table III expressing Chls *a* and *b* and Chls *a* + *b* concentrations in both nmol/ml (Eqns. 1–9) and μg/ml (Eqns. 10–18).

The effect of aqueous dilution of DMF and methanol on the calculation of chlorophyll concentrations and ratios

When undiluted solvents such as DMF or methanol

TABLE IV

Chlorophyll content and chlorophyll a/b ratios using three solvent systems for the extraction of three different types of leaves

Discs were taken from specific positions where L and R refer to left and right of the midrib and a, b and c to positions equally spaced from leaf tip to stem. The discs were 16 mm diameter (equals 200 mm^2 surface) for experiments with spinach and well nourished *Flindersia* but 23 mm diameter (equals 400 mm^2 surface) for the remaining experiment. The chlorophylls were extracted as described in Experimental and concentrations were calculated using equations 1–9 in Table III; consequently, Chl *a*/*b* are in molar terms (see Eqn. 19).

Leaf type	Solvent	Disc position	Chlorophyll content/unit leaf area ($\mu mol \cdot m^{-2}$)			Chl *a*/*b* ratios (*R'*)
			Chl *a*	Chl *b*	Chls *a* + *b*	
Spinach leaf	DMF	aL	294.5	79.0	373.5	3.72
	DMF	bR	285.9	76.8	362.7	3.73
	80% Acetone	bL	250.0	67.0	317.0	3.73
	80% Acetone	cR	278.8	75.2	354.0	3.71
	Methanol	cL	310.8	83.4	394.2	3.73
	Methanol	aR	288.4	73.1	361.5	3.94
Flindersia (well nourished)	DMF	aL	399.0	128.4	527.4	3.11
	DMF	bR	403.6	132.3	535.9	3.05
	80% Acetone	bL	382.1	123.3	505.4	3.10
	80% Acetone	cR	325.3	97.9	423.2	3.32
	Methanol	cL	408.3	135.2	543.5	3.02
	Methanol	aR	400.5	131.4	531.9	3.04
Flindersia (poorly nourished)	DMF	aL	85.8	25.2	111.0	3.41
	DMF	bR	107.9	32.0	139.9	3.38
	80% Acetone	bL	90.8	27.6	118.4	3.29
	80% Acetone	cR	88.3	25.3	111.5	3.42
	Methanol	cL	106.7	33.6	140.3	3.18
	Methanol	aR	103.2	31.0	134.2	3.33

are employed as extractants of chlorophylls from leaf discs using the assay conditions described in this paper (see Experimental), dilution by tissue water up to 10% can be expected. Stepwise increase of water up to 10% was found to decrease the extinction coefficients of both Chls *a* and *b* in both DMF and methanol by less than 0.5% thus having little influence on the calculation of chlorophyll concentrations or ratios.

The stepwise dilution of DMF solutions with water up to 10% has a stepwise effect on the position of the major red peaks of Chls *a* and *b*: the major red peak of Chl *a* moved from 663.8 to 664.6 nm and of Chl *b* from 646.8 to 647.6 nm. Thus, when the observable Chl *a* peak of a chlorophyll extract in DMF is located at 664.2 rather than 663.8 nm it is suggested that the second reading for the simultaneous equations for DMF in Table III also be read a further 0.4 nm towards longer wavelengths at 647.2 rather than 646.8 nm.

With methanol solutions, the major red peak maxima of Chls *a* and *b* were moved stepwise in the opposite direction toward shorter wavelengths but only by 0.2 to 0.4 nm; thus, if the observable Chl *a* peak in a chlorophyll extract in methanol has moved 0.2 nm towards shorter wavelengths it is suggested that the second wavelength in the simultaneous equations for methanol solutions (Table III) should also be read a further 0.2 nm towards shorter wavelengths.

Determination of chlorophyll concentrations and Chl a/b ratios in leaves extracted with DMF, methanol or buffered 80% aqueous acetone

To prove that our extinction coefficients (Table II) and the equations derived from them (Table III) will give reliable and consistent results, discs of spinach leaves and of well and poorly nourished *Flindersia* leaves were extracted with the three solvents by grinding in a mortar (see Experimental): using this technique, it was found that the re-extracted pellet (see Experimental) was no longer green. Concentrations of Chls *a* and *b* and of Chls *a* + *b* are expressed in $\mu mol/m^2$ of leaf area (Table IV). Chl *a/b* ratios (*R'*) are also presented in Table IV in molar terms: the relationship of this molar ratio (*R'*) to that expressed in terms of mass (*r*) is expressed in Eqn. 19 below:

$$R' = r\left(\frac{M_r(\text{Chl } b)}{M_r(\text{Chl } a)}\right) = 1.0156 \cdot r \qquad (19)$$

The chlorophyll content of the leaves varies somewhat depending on the position from which the disc was taken (see also Table VII) but the Chl *a/b* ratios remained remarkably constant over the entire leaf and in all three solvents. More compelling evidence that the equations also give consistent chlorophyll concentrations and ratios was obtained by adding 40 µl of a concentrated DMF extract of spinach chloroplasts to each of the three solvent systems (Table V). The consistency of these results indicates that our new chlorophyll-concentration equations in all three solvent systems (Table III), like the new extinction coefficients from which they are derived (Table II), are reliable while those of Arnon [3] are incorrect: our equations for use with aqueous acetone extracts, like those of Ziegler and Egle [5], Jeffrey and Humphrey [6] and Lichtenthaler [7], produce higher Chl *a/b* ratios than those of Arnon [3]. Our coefficients (Table II) must, of course, be consistent with those in diethylether [12], since the latter were used in their derivation (see Experimental). When using a full scale absorbance range of 0–1.0, other data (not shown) indicated that consistent results, including Chl *a/b* ratios, are more reliably obtained if the area of leaf tissue sampled or the volume of extractant used is adjusted to give Chls *a* + *b* concentrations not lower than about 6.5 nmol/ml (approx. 6.0 µg/ml).

TABLE V

Determination of chlorophyll concentrations and chlorophyll a/b ratios of a solution of a chlorophyll a and b concentrate diluted with three solvents

A concentrate of Chls *a* and *b* was prepared by extracting a spinach chloroplast pellet with DMF. An aliquot (40 µl) of this concentrate was diluted with 3.5 ml of DMF, methanol or buffered 80% aqueous acetone. The chlorophyll concentrations were calculated using Eqns. 1–9 of Table III.

Solvent	Chlorophyll concentration (nmol/ml)			Chl *a/b* ratio (*R'*)
	Chl *a*	Chl *b*	Chl *a* + *b*	
DMF	7.03	2.04	9.07	3.45
	6.98	2.04	9.02	3.42
Methanol	7.06	2.07	9.13	3.41
	6.94	2.07	9.01	3.35
Buffered 80% aqueous acetone	7.15	2.14	9.29	3.34
	7.22	2.12	9.34	3.41

Derivation of new simultaneous equations for use with alkaline pyridine as extractant

Porra and Grimme [8] derived simultaneous equations for assaying Chls *a* and *b* converted to their cyclic hydroxylactones by extraction with 2.1 M pyridine in 0.35 M NaOH at 60 °C for 15–30 min. They found that this procedure, which gave extremely reliable results [8], was exceedingly useful when chlorophylls were difficult to extract as in regreening nitrogen-deficient *Chlorella fusca* [14]; however, the extinction coefficients in this earlier work [8] were not calculated by comparison with diethylether solutions but with 80% aqueous acetone solutions using Arnon's earlier equations [3]. Using the new equations (Table III, Eqns. 7–9) for use with buffered aqueous acetone, the original data (see Ref. 8; pp. 261–262) were reprocessed. The new extinction

TABLE VI

Corrected millimolar and specific extinction coefficients for chlorophylls a and b in alkaline pyridine reagent

The data from Ref. 8 were reprocessed using Eqns. 7 and 8 of Table III to obtain corrected coefficients for the cyclic hydroxylactone derivatives of Chls *a* and *b* produced by addition of 2.1M pyridine in 0.35 M NaOH.

Section	Source	Wavelength (nm)	Extinction coefficient			
			Chl *a*		Chl *b*	
			(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)	(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)
A	ref. 8	419	131.9	–	65.5	–
		454	15.8	–	159.0	–
B	this work	419	136.4	152.7	93.5	103.0
		454	16.4	18.4	227.0	250.1

coefficients (Table VIB) for the Chl *a* and *b* hydroxylactone derivatives at 419 and 454 nm in alkaline pyridine were higher than the original values (Table VIA) from the original work [8]. These new extinction coefficients were used to derive new simultaneous equations (Table VII) to calculate concentrations of the Chls *a* and *b* derivatives expressed both in nmol/ml (Eqns. 20–22) and μg/ml (Eqns. 23–25). These new equations are now compatible with the other equations in Table III for use with the other three solvents.

Comparison of results obtained with the new and with Arnon's simultaneous equations

Arnon's simultaneous equations for determining Chls *a* and *b* were derived using the specific extinction coefficients (α) of Mackinney [15] in the same solvent. These coefficients with their millimolar equivalents shown in brackets are as follows:

Chl *a*: $\alpha^{663} = 82.04\ (73.30)$; $\alpha^{645} = 16.75\ (14.97)$

Chl *b*: $\alpha^{663} = 9.27\ (8.41)$; $\alpha^{645} = 45.60\ (41.38)$

Perusal of the corresponding coefficients in Table II shows that all Mackinney's coefficients are lower and especially that of Chl *b* so that the concentration of both chlorophylls calculated by the Arnon equations, especially that of Chl *b*, will be overestimated, and consequently the Arnon Chl *a*/*b* ratio will be low. For convenience, the Arnon equations expressing chlorophyll concentrations in μg/ml [3] have been converted, using millimolar extinction coefficients, to express all concentrations in nmol/ml and are presented below (see Eqns. 26–28):

$$\text{Chl } a = 14.21\,A^{663} - 3.01\,A^{645} \quad (26)$$

$$\text{Chl } b = 25.23\,A^{645} - 5.16\,A^{663} \quad (27)$$

$$\text{Chl } a + b = 22.22\,A^{645} - 9.05\,A^{663} \quad (28)$$

Using Mackinney's coefficients, the absorbances of solutions of Chls *a* and *b* mixtures of 5 : 1, 4 : 1, 3 : 1, 2 : 1 and 1 : 1 (all containing 10 nmol/ml of Chl *a*) were calculated at 663 and 645 nm. These absorbances were then inserted into Eqns. 7–9 of Table III to obtain the true total chlorophyll concentrations, $(\text{Chl } a + b)^T$. The relationship of $(\text{Chl } a + b)^T$ to the total chlorophyll derived from the Arnon equations, $(\text{Chl } a + b)^A$, is expressed below in Eqn. 29:

$$(\text{Chl } a + b)^T = 0.895(\text{Chl } a + b)^A \quad (29)$$

True individual concentrations of Chls *a* and *b* were then derived from $(\text{Chl } a + b)^T$ by using the true Chl *a*/*b* ratio, $(\text{Chl } a/b)^T$, obtained by using Eqns. 7 and 8 (Table III). A quicker and more convenient method to obtain $(\text{Chl } a/b)^T$ is by referral to Fig. 1 where the $(\text{Chl } a/b)^T$ ratio is plotted directly against the Arnon ratio, $(\text{Chl } a/b)^A$. Fig. 1 also shows the correction factor (Θ)

TABLE VII

Corrected equations for the determination of chlorophylls in alkaline pyridine reagent

Eqns. 20–25 below have been derived for the determination of Chls *a* and *b* and of Chls *a* + *b* converted to their cyclic hydroxylactone derivatives by solution in 2.1 M pyridine in 0.35 M NaOH.

Equations for chlorophyll concentrations in nmol/ml		Equations for chlorophyll concentrations in μg/ml	
$\text{Chl } a = 7.71\,A^{419} - 3.17\,A^{454}$	(20)	$\text{Chl } a = 6.89\,A^{419} - 2.84\,A^{454}$	(23)
$\text{Chl } b = 4.63\,A^{454} - 0.56\,A^{419}$	(21)	$\text{Chl } b = 4.21\,A^{454} - 0.51\,A^{419}$	(24)
$\text{Chl } a + b = 1.46\,A^{454} + 7.15\,A^{419}$	(22)	$\text{Chl } a + b = 1.37\,A^{454} + 6.38\,A^{419}$	(25)

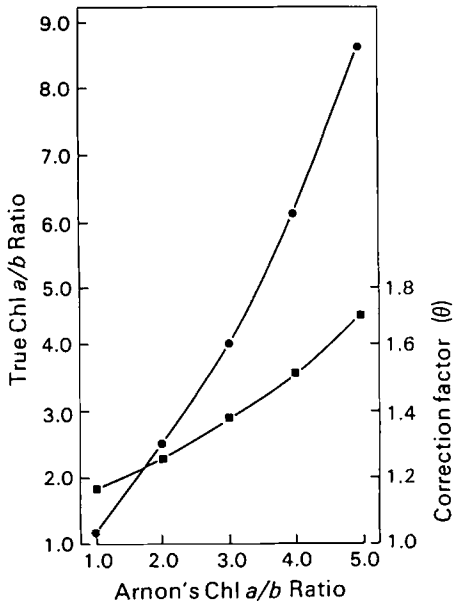

Fig. 1. True Chl a/b ratios (●) and correction factors (■) plotted against Arnon's Chl a/b ratios. The values of A^{663} and A^{645} for Chl $a+b$ solutions having Chl a/b ratios of 1.0, 2.0, 3.0, 4.0 and 5.0 were calculated using the coefficients of Mackinney [15] expressed in millimolar terms given in the text. These absorbance values were then inserted into Eqns. 7 and 8 (Table III) to obtain true Chl a/b ratios. The correction factor (Θ) is defined in Eqn. 30 (see text). After the true total chlorophyll has been obtained with Eqn. 29, the true Chl a/b ratios can be used with Eqns. 31 and 32 to obtain the correct concentrations of Chl a and b.

which is described in Eqn. 30 below:

$$\Theta = \frac{(\text{Chl } a/b)^{T}}{(\text{Chl } a/b)^{A}} \tag{30}$$

Thus, true Chl a and b concentrations may now be calculated using Eqns. 31 and 32 below:

$$(\text{Chl } b)^{T} = \frac{(\text{Chl } a+b)^{T}}{(\text{Chl } a/b)^{T}+1} \tag{31}$$

$$(\text{Chl } a)^{T} = \frac{(\text{Chl } a+b)^{T}(\text{Chl } a/b)^{T}}{(\text{Chl } a/b)^{T}+1} \tag{32}$$

The validity of the above equations for calculating true concentrations of total chlorophylls, of Chls a and b, and Chl a/b ratios was proven by comparison of the data obtained for leaf discs extracted with aqueous acetone (Table IV) with the same data processed with the Arnon equations. Eqns. 29, 31 and 32 as well as Fig. 1 may be used to correct chlorophyll concentrations and ratios expressed in either mass or molar terms.

An investigation of chlorophyll extraction procedures using DMF as extractant

Much has been made of the simple procedure described by Inskeep and Bloom [4] where intact leaf discs are shaken for 24 h with DMF. We compared the efficiency of this procedure with simple grinding and centrifugation (see Experimental) using discs from tough and leathery leaves of well nourished *Flindersia* plants. In earlier experiments (not shown) using the shaking technique, we found that the discs were still a very pale green colour and that subsequent grinding with DMF failed to extract more chlorophyll. Even when the discs were finely cut with scissors prior to shaking for 24 h, the leaf fragments often retained a pale green colour and no more chlorophyll could be extracted by subsequent grinding with DMF (Table VIII). These results with this modification of the technique of Inskeep and Bloom (Table VIII) showed that the recovery of total chlorophyll is almost 100% near the tip of the leaf but falls to about 84% closer to the stem; however, the Chl a/b ratios remain constant indicating that both chlorophylls are equally extractable by this solvent. Again, in

TABLE VIII

Comparison of chlorophyll extraction with N,N'-dimethylformamide from sliced leaf discs by shaking or by grinding with the solvent

Leaf discs were taken from specific positions of a well nourished *Flindersia* leaf where L and R refer to left and right of the midrib and a, b, c and d refer to positions equally spaced from leaf tip to stem. The discs were extracted with DMF by shaking the sliced leaf discs for 24 h with DMF or by grinding and centrifugation (see Experimental). Chlorophyll concentrations were calculated using Eqns. 1–3 of Table III. Av, average.

Position on leaf	Grinding in mortar		Position on leaf	Shaking for 24 h		Recovery $\frac{100 \cdot c}{C}$ (%)
	Chl $a+b$ (nmol/disc) (C)	Chl a/b ratio (R')		Chl $a+b$ (nmol/disc) (c)	Chl a/b ratio (R')	
aR	90.28	3.22	aL	88.43	3.11	98.0
bL	97.24	3.19	bR	90.57	3.23	93.1
cR	106.95	3.18	cL	90.08	3.22	84.2
dL	107.01	3.19	dR	90.79	3.16	84.8
		Av 3.19			Av 3.18	Av 90.02

Table VIII the total chlorophyll content of discs is lower towards the leaf tip while the Chl *a/b* ratios remain reasonably constant (cf. Table IV). In general, when DMF is employed, grindingn and centrifugation is more reliable (and faster) than the simple procedure which Inskeep and Bloom [4] found successful with soybean leaves.

Discussion

The purity of the chlorophylls used

Absorption spectroscopy was used to ensure that the chlorophylls employed were free of phaeophytin and hydroxylactone contamination (see Results section). Phaeophytin *a* has characteristic absorption bands at 532 and 505 nm and pheophytin *b* at 523 nm not possessed by the parent chlorophylls; similarly the hydroxylactone derivatives (oxidation products) of Chls *a* and *b* have absorption bands both in the ultra-violet and visible spectra not possessed by the parent chlorophylls [8]. If detected, the pheophytins [9] and hydroxylactones [11] can be removed by rechromatography on sucrose columns.

Other oxidation products of Chls *a* and *b*, the C-13^2-hydroxy (or methoxy) derivatives, are inseparable from the parent chlorphylls by sucrose chromatography and cannot be detected by absorption spectroscopy because their spectra [16–18], like those of the chlorophyll epimers [19], are virtually identical to the parent chlorophylls especially in the red region of the spectrum; thus, the error (if any) in the determination of extinction coefficients, even if caused by significant contamination by these hydroxy (or methoxy) derivatives, would be minuscule. Nonetheless, the presence of these compounds was monitored by reverse phase HPTLC on Merck RP-8 plates with a methanol/H_2O (98 : 2) solvent system: no contamination of Chl *b* by the C-13^2-hydroxy derivative could be detected after 9 h under oxygen-free nitrogen at $-15\,^\circ C$ and with Chl *a* it was estimated to be less than 5%. Consistent with this, no more than 5% of Chls *a* or *b* resisted conversion to 10 mg rhodochlorms in alkaline methanol as revealed spectrophotometrically.

The role of alkali, dephytylation and allomerization in the extraction and assay of chlorophylls

During these studies it was observed that methanol-extracted chlorophylls from leaf discs marginally more readily than DMF, but both these solvents were clearly more effective than buffered 80% aqueous acetone. This may account for the use of many methanol procedures, especially hot [2] or alkaline [20] methanol, in situations where the chlorophylls are difficult to extract (cf. Refs. 2 and 14). The use of alkali in such conditions is attractive, since it will hydrolytically release chlorophylls from their hydrophobic phytyl moieties, which are firmly embedded in the thylakoid membranes, thus releasing the chromophores into solution. Alkaline conditions, however, have the disadvantage that they render the chlorophylls susceptible to allomerization; that is irreversible oxidation by molecular oxygen.

Hynninen and Assandri [21] have proposed that allomerization involves the two types of oxidative reaction sequences described below [21] but other mechanisms have also been proposed [16]. The first type results in the formation of C-13^2-hydroxy (or methoxy) chlorophylls with absorption spectra virtually identical to the parent chlorophyll. The proposed sequence involves the removal of the proton at C-13^2 by base (OH^- or OCH_3^-) to give a carbanion which is then oxidized by oxygen to a carbonium ion prior to a rapid reaction with hydroxyl (or methoxyl) ions to produce C-13^2-hydroxy (or methoxy) chlorophylls [21]: structure I (Fig. 2) shows one stereoisomeric form of C-13^2-hydroxychlorophyll. The second type of allomerization sequence involves the formation, in alkaline solutions, of the C-13^1 enolate anion (see Fig. 2, structure II) which possesses a labile double bond between C-13^1 and C-13^2. This labile bond is susceptible to irreversible cleavage by molecular oxygen [21,22] which is incorporated to form an unstable rhodochlorin-type intermediate (Fig. 2, structure III) possessing formic, glyoxalic and propionic acid sidechains at C-13, -15 and -17, respectively [21]. This structure can subsequently break

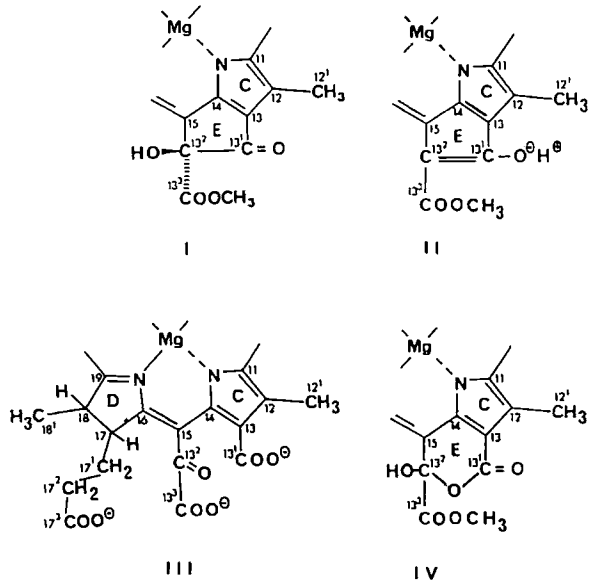

Fig. 2. The structural differences occurring in rings C, D or E of various chlorophyll derivatives involved in allomerization. Rings A and B are unaffected by allomerization. I, one stereoisomeric form of the allomerization product, C-13^2-hydroxychlorophyll; II, the enolate ion formed in alkaline conditions; III, the tricarboxylic magnesium-rhodochlorin product formed from II by reaction with oxygen (allomerization); and IV, the cyclic hydroxylactone derivative formed from III in alkaline pyridine. The tetrapyrrole numbering system and the use of the nomenclature term 'rhodochlorin' is consistent with the practice of the International Union of Biochemistry [27].

down to form a complex mixture of degradation products [21] including the cyclic hydroxylactones (Fig. 2, structure IV); thus, allomerization can make the assay of chlorophylls impossible. Many of the products of this second type of allomerization sequence have spectra quite different to the parent chlorophyll. While allomerization is well known to occur in methanolic solution [21,22], the consistency observed between he results of our methanol-extraction assays with those obtained using DMF and buffered aqueous acetone indicates that allomerization of the second type does not affect the results when the assays are performed rapidly with cold methanol in the absence of alkali as in this work.

Although the use of alkali in conjunction with methanol is normally avoided because of the opportunity for allomerization, Milner et al. [20] devised a simple colorimetric assay for total chlorophyll by extraction with 5% KOH in methanol at 63°C for 3 min: they anticipated only the release of 'saponified chlorophylls' from the membrane-embedded phytol moiety. While dephytylation occurred under their assay conditions, we have found (unpublished results) that allomerization of the second type occurred during the extraction of biological tissues. This complicated the spectra and may be the reason that this assay was used only to determine total chlorophyll and not the individual Chl *a* and *b* concentrations. Surprisingly, working with purified chlorophylls, we showed that the *iso*cyclic E rings of both Chls *a* and *b* were opened by hydrolysis but without the incorporation of oxygen (i.e., without allomerization) (cf. Ref. 21) to form two magnesium-rhodochlorins, known in the past, respectively, as Mg-chlorin e_6 and Mg-rhodin g_7 [23]: these compounds resemble structure III (Fig 2) but the glyoxalate sidechain at C-15 is replaced by an acetate. The formation of these two tricarboxyl compounds was proved by the occurrence of major red peaks at about 642 and 624 nm, respectively, in both KOH-methanol and diethylether [21,22]; also, the spectra in diethylether of the two Mg-free rhodochlorins, prepared by acidification with HCl, were identical to those of chlorin e_6 and rhodin g_7 [24]. Thus, it would appear that crude biological extracts contain a factor which enhances allomerization of chlorophylls.

Alkali has also been used in association with pyridine to extract and assay chlorophylls [8] in conditions where chlorophyll is difficult to extract [14]. This assay is again based on the removal of the chromophore from the thylakoid membrane by hydrolysing the phytyl–ester linkage. It is unusual, however, because the second type allomerization reactions occurring in this solvent stoichiometrically convert Chls *a* and *b* to their respective cyclic hydroxylactones (see Fig. 2, structure IV) by a mechanism described elsewhere (cf. Ref. 21). These photolabile cyclic hydroxylactones are stable for about 60 min at 4°C in foil-wrapped, stoppered tubes [8].

The experience gained here with DMF suggests that it may also be useful in some conditions where chlorophyll extraction is difficult: DMF has the advantage that it is not volatile or pungent and that chlorophylls are relatively stable in DMF in the dark at low temperatures [1].

Some biological implications of the corrections to Arnon's equations

The correction factors (Θ) which are required to correct the Chl *a*/*b* ratios obtained in 80% aqueous acetone extracts calculated by Arnon's equations are not trivial (see Fig. 1) ranging from 1.17 (for a ratio of 1.0) to 1.70 (for a ratio of 5.0). This means that the well established differences in Chl *a* and *b* content observed when higher plants and green algae acclimate to light [25] need correction. For example, sun plants or plants acclimated to high irradiance are reported to have Chl *a*/*b* ratios ranging from 2.8 to 3.4 but are, in fact, 3.8–4.8: shade plants and plants acclimated to low irradiance are quoted to have Chl *a*/*b* ratios of 2.1–2.3, which are actually 2.7–3.0. Moreover, the chlorophyll content and Chl *a*/*b* ratios of the isolated Chl *a*- and *b*-containing protein complexes have also been determined mainly in 80% aqueous acetone using Arnon's equations and, therefore, need correction. For example, the main light-harvesting Chl *a* and *b* protein of Photosystem II (LHC II) is generally accepted to have a Chl *a*/*b* ratio of 1.15 but, in fact, has a ratio of 1.38 which means that the percentage of Chl *b* is decreased from the previously accepted value of 46.6 to 42.0%. The corresponding pigment protein complex of light-harvesting complex I (LHC I) has a Chl *a*/*b* ratio of about 3.6 [26] which will actually be 5.25 so that the percentage of Chl *b* will be 16% rather than 21.7%. Thus, it becomes clear that the correction of earlier data is required to assess the true, but lesser, amount of total chlorophyll present and its correct distribution between Chls *a* and *b* before meaningful models of the photosynthetic apparatus can be proposed.

Concluding remarks

In the assay of a mixture of Chls *a* and *b* a major problem for the accuracy of the method is the measurement of the absorbance at the wavelength of Chl *b* which occurs on a steeply sloping segment of the spectrum of the mixture. Consequently, it is important to measure the position of the observable Chl *a* peak very carefully and maintain the appropriate wavelength interval (which separates the peaks) because this interval appears to be maintained at all relevant water concentrations. The appropriate interval is 17.0, 13.2 and 17.0 nm for the DMF, methanol and buffered 80% aqueous acetone procedures. Maintaining this interval is made considerably easier by the newest microprocessor controlled spectrophotometers which can locate a peak

to the nearest 0.1 nm and enable the operator to read absorbance at any wavelength accurate to the nearest 0.1 nm.

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