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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)