Chapter 7

Spectrometric Assays for Plant, Algal and Bacterial Chlorophylls

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Summary

Chlorophyll (Chl) assays derive their importance from the essential role of Chls in the harvesting of solar energy and its transduction to biologically useful chemical energy (ATP) and reducing power (NADPH or NADH) during photosynthesis in higher plants, marine and aquatic algae, and in photosynthetic bacteria. Accurate determination of Chl *a* and *b* concentrations and of Chl *a*/*b* ratios has been an essential tool in photosynthesis research in higher plants and green algae. Spectrophotometric and spectrofluorimetric assays, relying on the characteristic absorption and fluorescence properties of the chlorophylls, will be described and accurate data presented for spectrophotometric assays in a wide variety of solvents.

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I. Introduction

The accurate assay of chlorophylls (Chls) *a* and *b* and determination of Chl *a/b* ratios is important in photosynthesis research. Photosynthetic reaction rates are frequently expressed per unit of Chl content. Accurate Chl *a* and *b* determinations also became important in attempts to produce accurate models of the various Chl-protein complexes located in thylakoid membranes of plant and algal chloroplasts. These complexes, light-harvesting complexes (LHCs) and reaction centers (RCs), have very different but characteristic Chl *a/b* ratios and their accurate determination has helped reveal how plants regulate the proportions, size and composition of these complexes to adapt to available light.

As a rapid alternative to quantitative HPLC methods, spectrometric assays have been developed which depend on either the characteristic absorption or the intense fluorescence of Chls. Further, special spectrophotometric and spectrofluorimetric techniques have been developed to determine Chl *b* concentrations in biological situations where Chl *a/b* ratios are so high that the Chl *b* Q_y absorption peak becomes indistinct.

II. Modern Spectrophotometric Assays of Chlorophylls *a* and *b*

Comar and Zscheile (1942) extracted Chls *a* and *b* with pure acetone, transferred them to diethylether and assayed both Chls spectrophotometrically, but without separation, using the simultaneous equation (Eq.) technique (Section IV). A one-step procedure was later devised in which Chls *a* and *b* were extracted with aqueous (aq) 80% acetone and, again, were both assayed without separation using simultaneous equations. (Arnon, 1949). In this solvent, the water-induced red-shift of the Chl Q_y absorption peaks by release of small amounts of cell sap during extraction became insignificant in the presence of 20% water. Arnon's method, therefore, was remarkable for its speed and simplicity. Unfortunately, it later proved to be grossly inaccurate (Vernon, 1960; Ziegler and Egle, 1965, Delaporte and Laval-Martin, 1971a,b; Lichtenthaler, 1987; Porra et al., 1989; Wellburn, 1994) because Arnon's simultaneous equations to determine Chl *a* and *b* concentrations were constructed using the flawed α extinction coefficients of Mackinney (1941). Thus, accurate extinction coefficients for Chls *a* and *b* were urgently required to obtain reliable simultaneous equations.

Abbreviations: aq – aqueous; DMF – N,N′-dimethylformamide; DMSO – dimethylsulfoxide; DV – divinyl; Eq. – equation; LHC I – light-harvesting complex I; MV – monovinyl; PS I and II – Photosystems I and II; RC – reaction center; The numbering and nomenclature systems for tetrapyrroles, approved by the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) Joint Commission on Biochemical Nomenclature, have been used throughout (see Fig. 1).

III. Choice of Extractant and Determination of Accurate Extinction Coefficients for Chlorophylls *a* and *b* in Such Solvents

Accurate millimolar (ε_{mM} = [l·mmol^{-1}·cm^{-1}]) and specific (α = [l·g^{-1}·cm^{-1}]) extinction coefficients for Chls *a* and *b* were obtained in aq 80% acetone, DMF, DMSO, chloroform and 100% methanol or aq 85% methanol containing both 2% KOH and 1.5mM sodium dithionite or 1.5mM dithionite alone (Table 1). The latter two solvents were used to extract Chls *a* and *b* from difficult-to-extract algae (Porra, 1990a,b). Alkali and dithionite presumably hydrolyze and reduce cell-wall proteins, respectively, allowing Chls to be extracted more readily with methanol (Thompson and Preston 1968); with KOH, however, Chls *a* and *b* are converted stoichiometrically to Mg-rhodochlorins (Mg-RChlns) *a* and *b,* and dithionite prevented their further conversion to hydroxylactone derivatives (Fig. 1). A 1:3 (v/v) methanol-chloroform mixture is an exceedingly efficient Chl extractant (Quail et al., 1976). In this method, Chls *a* and *b* are transferred to chloroform by aq. dilution and then determined by the appropriate simultaneous equations (Table 2); this extractant, however, is seldom used. Total extraction of Chls from leaf discs by prolonged immersion in DMF has been claimed (Moran and Porath, 1980) but rapid grinding of finely-cut leaf tissue in the chosen extractant with a pestle and mortar or Potter-Elvejhem homogenizor is the surest method and diminishes photo-oxidation of Chls during the prolonged immersion (Porra et al., 1989).

Of all these solvents, aq 80% acetone is strongly recommended because the Q_y absorption bands of the Chls *a* and *b* in this extractant are as sharply defined as in diethylether, DMF, DMSO and chloroform; however, aqueous acetone is less toxic than DMF, DMSO and chloroform. In methanol, an excellent Chl solvent, the Q_y peaks of Chls *a* and *b* are lower,

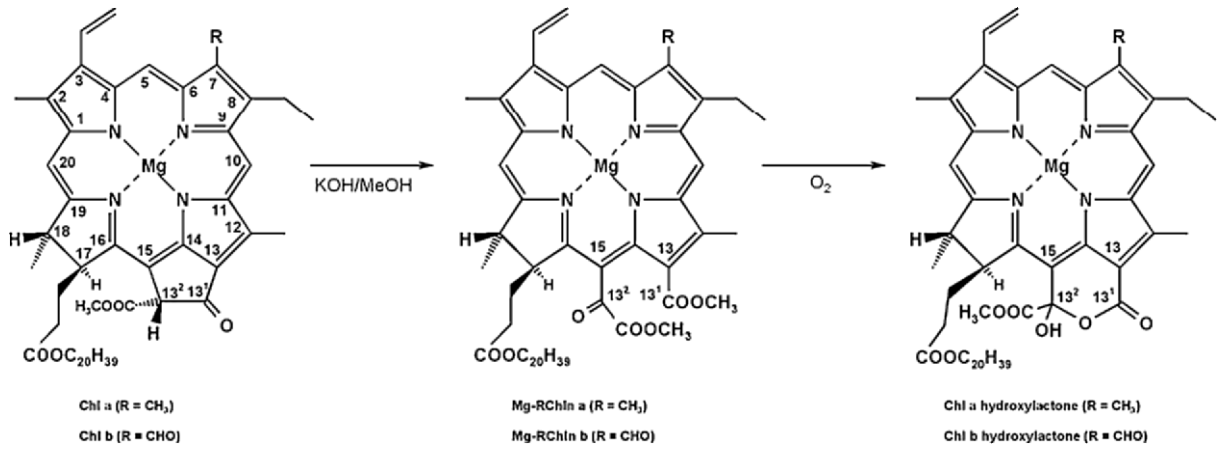

Fig. 1. The conversion of Chls *a* and *b* by alkaline methanolysis to Mg-rhodochlorins (Mg-RChlns) *a* and *b* by opening of isocyclic ring E and the subsequent formation of Mg-hydroxylactones *a* and *b* in the presence of O_2. Inclusion of oxygen into the hydroxylactone macrocycle is inhibited by reductants. The same reactions occur with the Mg-free Phes *a* and *b.*

Table 1. Accurate specific (α) and millimolar (ε_{mM}) extinction coefficients for Chls *a* and *b* in a variety of solvents and determined relative to the extinction coefficients of Smith and Benitez (1955) using the procedure described in Section III

		Difference Extinction Coefficients used in simultaneous equations in Table 2[a]				
		Chl *a*		Chl *b*		
Solvent	Wavelength [nm]	α [l•g^{-1}•cm^{-1}]	ε_{mM} [l•mmol^{-1}•cm^{-1}]	α [l•g^{-1}•cm^{-1}]	ε_{mM} [l•mmol^{-1}•cm^{-1}]	Reference
Buffered aq 80% acetone (pH 7.8)	663.6 646.6	85.95 20.79	76.79 18.58	10.78 51.84	9.79 47.04	Porra et al. (1989)
DMF	663.8 646.8	88.74 20.84	79.29 18.62	13.26 51.23	12.03 46.49	Porra et al. (1989)
DMSO	665.1 649.1	86.73 22.51	77.49[b] 20.11[b]	12.53 43.16	11.37[b] 39.17[b]	Wellburn (1994)
Chloroform	665.6 647.6	90.42 18.74	80.79[b] 16.74[b]	8.26 47.47	7.50[b] 43.08[b]	Wellburn (1994)
Methanol	665.2 652.0	75.95 35.42	71.43 31.65	22.26 42.48	20.20 38.55	Porra et al., (1989)
Aq 85% Methanol with dithionite	664.0 650.0	76.26 31.11	68.18 27.81	20.03 40.62	18.19 36.88	Porra (1990b)
Aq 85% Methanol in 2% KOH with dithionite[c]	640.0[c] 623.0[c]	53.61 19.38	47.93 17.32	7.92 19.49	7.18 17.69	Porra (1990a)

[a] The extinction coefficients are difference coefficients as the extinction at 750 is subtracted to correct for non-specific absorption. [b] These ε_{mM} coefficients in DMSO and chloroform were calculated from the α coefficients of Wellburn (1994) using Eq. (5) (Section IV). [c] The coefficients in alkaline methanol refer to Mg-rhodochlorins (Mg-RChlns) *a* and *b* as alkali opens ring E of the Chls (Fig. 1).

flatter, broader and closer together (Table 1) making the peaks more difficult to accurately measure. Diethylether is too volatile and inflammable to be safely used in routine Chl assays and is, therefore, not included in Table 1.

The updated list of accurate Chl *a* and *b* extinction coefficients in Table 1 were all determined relative to the specific (α) coefficients of Smith and Benitez (1955) in diethylether which are 100.9 for Chl *a* at 662 nm and 62.0 for Chl *b* at 644 nm: these coefficients were shown, by Mg determination using atomic absorption spectrometry, to have an error of less than

Table 2. Recommended simultaneous equations ('post Arnon equations') for assaying Chls *a* and *b* in various solvents using the accurate extinction coefficients presented in Table 1 which were calculated relative to the accurate coefficients of Smith and Benitez (1955) in diethylether.

Solvent	Interval between Chl *a* and *b* Q_y peaks (nm)[a]	Eqns for Chl *a* and *b* concentrations [nmol/ml]	Eqns for Chl *a* and *b* concentrations [µg/ml]	Reference
Buffered aq 80% acetone (pH7.8)	17.0 (i.e. 663.6–646.6)	[Chl *a*] = 13.71 $E^{663.6}$ – 2.85 $E^{646.6}$ [Chl *b*] = 22.39 $E^{646.6}$ – 5.42 $E^{663.6}$ [Chl *a*+*b*] = 19.54 $E^{646.6}$ + 8.29 $E^{663.6}$	[Chl *a*] = 12.25 $E^{663.6}$ – 2.55 $E^{646.6}$ [Chl *b*] = 20.31 $E^{646.6}$ – 4.91 $E^{663.6}$ [Chl *a*+*b*] = 17.76 $E^{646.6}$ + 7.34 $E^{663.6}$	Porra et al. (1989)
DMF	17.0	[Chl *a*] = 13.43 $E^{663.8}$ – 3.47 $E^{646.8}$ [Chl *b*] = 22.90 $E^{646.8}$ – 5.38 $E^{663.8}$ [Chl *a*+*b*] = 19.43 $E^{646.8}$ + 8.05 $E^{663.8}$	[Chl *a*] = 12.00$E^{663.8}$ – 3.11$E^{646.8}$ [Chl *b*] = 20.78$E^{646.8}$ – 4.88$E^{663.8}$ [Chl *a*+*b*] = 17.67$E^{646.8}$ + 7.12$E^{663.8}$	Porra et al. (1989)
DMSO	16.0	[Chl *a*] = 13.95 $E^{665.1}$ – 4.01 $E^{649.1}$ [Chl *b*] = 27.59 $E^{649.1}$ – 7.16 $E^{665.1}$ [Chl *a*+*b*] = 23.58 $E^{649.1}$ + 6.79$E^{665.1}$	[Chl *a*] = 12.47 $E^{665.1}$ – 3.62 $E^{649.1}$ [Chl *b*] = 25.06 $E^{649.1}$ – 6.50 $E^{665.1}$ [Chl *a*+*b*] = 21.44 $E^{649.1}$ + 5.97 $E^{665.1}$	Wellburn (1994)
Chloroform	18.0	[Chl *a*] = 12.84 $E^{665.6}$ – 2.02 $E^{647.6}$ [Chl *b*] = 24.0 $E^{647.6}$ – 4.99 $E^{665.6}$ [Chl *a*+*b*] = 21.98 $E^{647.6}$ + 7.85 $E^{665.6}$	[Chl *a*] = 11.47 $E^{665.6}$ – 2.0 $E^{647.6}$ [Chl *b*] = 21.85 $E^{647.6}$ – 4.53 $E^{665.6}$ [Chl *a*+*b*] = 19.85 $E^{647.6}$ + 6.94 $E^{665.6}$	Wellburn (1994)
Methanol	13.2	[Chl *a*] = 18.22 $E^{665.2}$ – 9.55 $E^{652.0}$ [Chl *b*] = 33.78 $E^{652.0}$ – 14.96 $E^{665.2}$ [Chl *a*+*b*] = 24.23 $E^{652.0}$ + 3.26 $E^{665.2}$	[Chl *a*] = 16.29 $E^{665.2}$ – 8.54 $E^{652.0}$ [Chl *b*] = 30.66 $E^{652.0}$ – 13.58 $E^{665.2}$ [Chl *a*+*b*] = 22.12 $E^{652.0}$ + 2.71 $E^{665.2}$	Porra et al. (1989)
Aq 85% methanol with dithionite	14.0	[Chl *a*] = 18.36 $E^{664.0}$ – 9.06 $E^{650.0}$ [Chl *b*] = 39.94 $E^{650.0}$ – 13.85 $E^{664.0}$ [Chl *a*+*b*] = 30.88 $E^{650.0}$ + 4.51 $E^{664.0}$	[Chl *a*] = 16.41 $E^{664.0}$ – 8.09 $E^{650.0}$ [Chl *b*] = 30.82 $E^{650.0}$ – 12.57 $E^{664.0}$ [Chl *a*+*b*] = 22.73 $E^{650.0}$ + 3.84 $E^{664.0}$	Porra (1990b)
Aq 85% methanol in 2% KOH with dithionite[b]	17.0	[Chl *a*] = 24.45 $E^{640.0}$ – 9.93 $E^{623.0}$ [Chl *b*] = 66.25 $E^{623.0}$ – 23.94 $E^{640.0}$ [Chl *a*+*b*] = 56.32 $E^{623.0}$ + 0.51 $E^{640.0}$	[Chl *a*] = 21.87 $E^{640.0}$ – 8.88 $E^{623.0}$ [Chl *b*] = 60.14 $E^{623.0}$ – 21.74 $E^{640.0}$ [Chl *a*+*b*] = 51.26 $E^{623.0}$ + 0.13 $E^{640.0}$	Porra (1990a)

[a] If an individual spectrophotometer records a different Chl *a* peak, the wavelength of the second reading should maintain the indicated interval. [b] These equations refer to Chls *a* and *b* after stoichiometric conversion to Mg-RChlns *a* and *b* by alkali which opens ring E (Fig. 1)

1% (Porra et al., 1989). Individual stock solutions of Chls *a* and *b* in diethylether were assayed using the Smith and Benitez (1955) coefficients. By removing the diethylether from measured volumes of the stock solutions by evaporation under O_2-free N_2 and replacing with identical volumes of the specified solvents, accurate α and ε_{mM} coefficients were calculated from the recorded absorption spectra (Table 1).

IV. Reliable Simultaneous Equations for the Accurate Assay of Chlorophylls *a* and *b*

A. Equations to Determine Chlorophylls a and b in Solvent Extracts of Plant and Algal Cells

Determining Chl *a* and *b* concentrations with simultaneous equations, correctly assumes that no other pigments in the extracts, such as carotenoids or other Chls and their derivatives, absorb at the Q_y maximum of either Chl *a* or Chl *b*; thus, Eqs. (1) and (2), in which the constants are the α coefficients for Chls *a* and *b* in aq 80% acetone (Table 1), are valid:

$$E^{663.6} = 85.95 \bullet [\text{Chl } a] + 10.78 \bullet [\text{Chl } b] \quad (1)$$

$$E^{646.6} = 51.84 \bullet [\text{Chl } b] + 20.79 \bullet [\text{Chl } a] \quad (2)$$

[Chl *a*] and [Chl *b*] represent Chl *a* and Chl *b* concentrations in g/l, and $E^{663.6}$ and $E^{646.6}$ are the extinctions of the aq 80% acetone extract at 663.6 and 646.6 nm, respectively. By standard mathematical techniques, these equations transform to the simultaneous Eqs. (3) and (4) (Table 2).

$$\text{Chl } a \ (\mu\text{g/ml}) = 12.25 \bullet E^{663.6} - 2.55 \bullet E^{646.6} \quad (3)$$

$$\text{Chl } b \ (\mu\text{g /ml}) = 20.31 \bullet E^{646.6} - 4.91 \bullet E^{663.6} \quad (4)$$

To obtain Chl *a* and *b* concentrations in nmol/ml, simultaneous equations must be generated replacing the specific (α) coefficients with millimolar (ε_{mM}) coefficients which are calculated from α coefficients as follows:

$$\varepsilon_{mM} = \alpha \bullet Mr \bullet 10^{-3} \quad (5)$$

where Mr for Chls *a* and *b* are 893.48 and 907.46, respectively.

Table 2 presents an updated list of reliable simultaneous equations to determine Chl *a* and *b* concentrations in a variety of solvents, either in nmol/ml or µg/ml, which were generated from the accurate extinction coefficients in Table 1: if a spectrophotometer records a wavelength for a Chl *a* peak differing from that in the relevant Eq. (say by 0.5 nm), the wavelength of the second extinction reading must change 0.5 nm to maintain the correct interval (Table 2). Using bandwidths of 2 nm or less, preferably between 0.1–0.5 nm, produce sharper absorption peaks and more accurate extinction coefficients (Wellburn, 1994).

The simultaneous equations in Table 2 can be recommended together with those published by other laboratories that had also detected the inaccuracy of the Arnon equations (Vernon, 1960; Ziegler and Egle, 1965, Delaporte and Laval-Martin, 1971a,b; Lichtenthaler, 1987; Porra et al., 1989; Wellburn, 1994): all such equations, designated here as 'post-Arnon equations', are reliable as they were all constructed with accurate extinction coefficients determined relative to the accurate coefficients of Smith and Benitez (1955) in diethylether. The equations for use with DMF (Moran and Porath, 1980; Moran, 1982; Inskeep and Bloom, 1985) and DMSO (Barnes et al., 1992) were derived from coefficients calculated relative to the faulty coefficients of Mackinney (1941) and should be replaced by the appropriate equations in Table 2.

B. High Chlorophyll a/b Ratios: A Problem for the Simultaneous Equation Method

Because of the very large overlap of the Q_y bands of Chls *a* and *b*, the absorption maximum of the small proportion of Chl *b* present when the Chl *a/b* ratio is high becomes a barely perceptible shoulder at ~647 nm on the rising absorption of the Chl *a* which peaks at ~664 nm. True Chl *a/b* ratios (Chl a/b^T), determined by post-Arnon equations, reach values of 11–14 in PSI-enriched fractions of chloroplast thylakoid membranes (Section V) but can be even higher in Chl *b*-deficient mutant tissues (Chunaev et al., 1991). Chl *a* and *b* determinations at such high Chl *a/b* ratios can be more accurately made spectrophotometrically by converting Chl *b* to its oxime which, like Chl *a,* has a peak at ~664 nm and then recording the increase in extinction at this wavelength: for details see Ogawa and Shibata (1965). Alternatively, Chl *b* can be determined under standard acidification conditions by

spectrophotometrically recording its slow demetallation rate (Laval-Martin, 1985): demetallation of Chl *a* is almost instantaneous. Both these methods have been extensively reviewed (Porra, 1991). Elegant spectrofluorimetric methods are suitable for Chl *a* and *b* determinations in the Chl *a/b* ratio range of 10–220 (Talbot and Sauer, 1997) while the method of Meister (1992) extends this range downwards to ~1.3 (Section IX.D).

V. The Unacceptable Errors and Consequences of Using the Arnon Equations

The errors reported in the extinction coefficients used by Arnon (1949) to construct his simultaneous equations are very large (Porra, 1991, 2002): for Chl *a* the errors are 19.43 and 4.55% at 663 and 645 nm, respectively, and for Chl *b* are 14.01 and 12.03% at the same wavelengths. It is illogical, therefore, that many workers still use Arnon's equations after so many laboratories (Vernon, 1960; Ziegler and Egle, 1965, Delaporte and Laval-Martin, 1971a,b; Lichtenthaler, 1987; Porra et al. 1989; Wellburn, 1994) have not only reported them unreliable but also published reliable post-Arnon equations.

Arnon's equations lead to erroneous Chl *a/b* ratios, designated Chl a/b^A, which are always lower than the true Chl a/b^T ratios determined by post-Arnon equations: the error increases alarmingly as the proportion of Chl *a* increases (see Fig. 2). When constructing Fig. 2, it was found that the true total Chl, $[\text{Chl } a+b]^T$ was always $0.895[\text{Chl } a+b]^A$. Thus, with the true total Chl and the true ratio from Fig. 2 it became a simple matter to correct the results of Arnon's equations and obtain true Chl *a* and *b* concentrations (Porra et al., 1989).

The absurdity of retaining the Arnon equations is apparent when the Chl a/b^A ratio range of 6.0–6.5 reported for PS I-enriched fractions of fragmented chloroplasts (Sane et al., 1970; Andersson et al., 1976) corresponds to a true Chl a/b^T range of 11.4 to 13.4 (Fig. 2) and when the Chl a/b^A ratio for the LHC I protein of 3.6 (Thornber, 1986) corresponds to a true Chl a/b^T value of 5.25.

Chl a/b^A ratios approximating 1.0 incur small errors (Fig. 2) which, however, significantly affect modeling. Using Arnon's assay, Butler and Kühlbrandt (1988) calculated for each LHC II pigment polypeptide a total, $[\text{Chl } a+b]^A$, of 15 Chls and a Chl a/b^A of 1.15 indicating 8 Chl *a* and 7 Chl *b*; however, this equates to $[\text{Chl a+b}]^T$ of 13.4 Chls and Chl a/b^T of 1.42 (Fig. 2) indicating 8 Chl *a* but only 6 Chl *b.* With the aq.80% acetone equations of Porra et al (see Table 2), Kühlbrandt et al (1994) found a $[\text{Chl } a+b]^T$ of 14 Chls and a Chl a/b^T = 1.3 also suggesting 8 Chl *a* and 6 Chl *b* but their electron crystallography results, at 3.4 Å resolution, showed only 12 Chl sites. Both recent high-resolution X-ray crystallography studies, by Liu et al. (2004) and Standfuss et al. (2005), unequivocally show that LHC II contains 8 Chl *a* and 6 Chl *b,* thus unambiguously confirming the

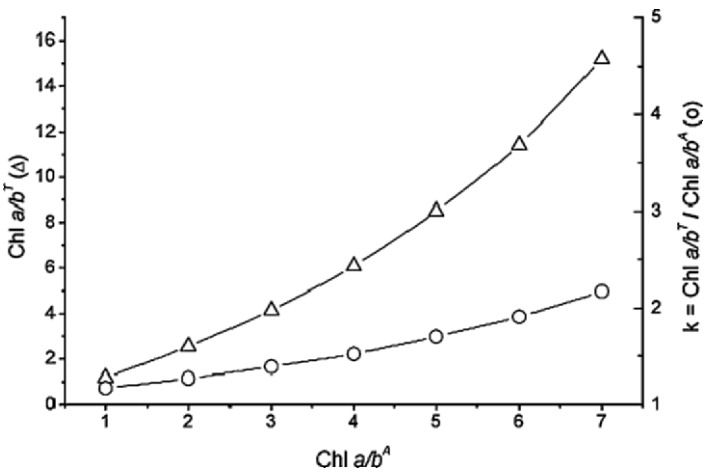

Fig. 2. The plot of true Chl a/b^T ratios against Arnon's Chl a/b^A ratios in aq 80% acetone (open triangles) and of the correction factor (k) against Chl a/b^A ratios (open circles). While $[\text{Chl } a+b]^T$ is simply $0.895[\text{Chl } a+b]^A$, the complex quadratic equation best fitting the first plot of Chl *a/b* ratios is:

$$\text{Chl } a/b^T = 0.593 + 0.4594 \text{ Chl } a/b^A + 0.2294 (\text{Chl } a/b^A)^2$$

accuracy of the 80% aq. acetone equations of Porra et al. (1989) in Table 2

VI. Other Spectrophotometric Assays for Chlorophylls *a* and *b* in Association with Their Derivatives or Other Pigments

Listed below are at least three other spectrophotometric assays employing simultaneous equations which are available for the determination of Chls in the presence of Chl derivatives including protochlorophyll or protochlorophyllide, PChl(id), and pheophytins (Phes). For a full description of these assays, the readers are referred to the original authors (see below) and an earlier review (Porra, 1991).

A. Chlorophylls a and b Together with Total Carotenoids

An assay for this combination of pigments has been developed in aq 80% acetone, DMF and DMSO (Lichtenthaler and Wellburn, 1983; Lichtenthaler, 1987 Wellburn, 1994)

B. Chlorophylls a and b Together with Protochlorophyll(ide)

Brouers and Michel-Wolwertz (1983) have developed an assay for mixtures of these three pigments in aq 80% acetone. PChlid is an intermediate in Chlid *a* biosynthesis (Benz and Rüdiger, 1981) and PChl may arise by oxidation of ring D of Chl *a* but such an oxidase is unknown.

C. Pheophytins a and b

Phe mixtures have been assayed in aq 90% acetone by Laval-Martin (1985) and in diethylether, methanol or aq 80% acetone by Lichtenthaler (1987). Phe(ides) *a* and *b* may arise by enzymic or non-enzymic demetallation of Chl(ides) *a* and *b.* Phe *a* is present in minute amounts in RC II.

VII. Spectrophotometric Assays for Chlorophylls in Chlorophyll *c*-containing Algae

The Chls *c* are an ever-increasing group of Mg-phytoporphyrins functioning as algal antenna Chls in association with Chl *a* or Chls *a* and *b.* The Chl *c* pigments, like other Chls, possess a fifth ring (*iso*-cyclic ring E) but ring D of the Mg-phytoporphyrins is not reduced at C-17 and -18 as in Chls *a* and *b* which are Mg-dihydrophytoporphyrins, also known as Mg-chlorins (see Chapter 1, Scheer; Chapter 2, Senge; Chapter 3, Zapata et al.). As discussed in these chapters, Chls *c* differ markedly in structural complexity having Mr values between 611 (Helfrich et al., 1999) and 1313 (Garrido et al., 2000). The possible biogenetic relationships between members of the Chls *c* group and with the biosynthesis of Chl *a* has been discussed (Porra, 1997; Porra et al., 1997; Chapter 18, Larkum).

HPLC systems interfaced to spectrophotometric or spectrofluorimetric detectors have been extensively used for separation and quantification of carotenoids and Chls of phytoplankton: these methods have been extensively reviewed in a recent book on phytoplankton pigments (Jeffrey et al., 1997). Further, Jeffrey et al. (1999) discuss new HPLC systems permitting the separation of divinyl Chls *a* and *b* and new polar and non-polar Chls *c* (see Chapter 3, Zapata et al. and Chapter 8, Garrido and Zapata).

The most highly recommended simultaneous equations for spectrophotometric determinations of Chls *c,* in association with other Chls, have been extensively reviewed (Porra, 1991; Jeffrey et al., 1997).

VIII. Spectrophotometric Data for the Assay of Bacteriochlorophylls

There are many BChls and they are classified into two categories: Mg-dihydrophytoporphyrins, which include BChls *c, d,* and *e,* and Mg-bacteriochlorins including BChls *a, b* and *g* (Scheer, 1991; Chapter 1, Scheer; Chapter 2, Senge). While Mg-dihydrophytoporphyrins have one reduced ring, ring D (like plant Chls *a* and *b*), the Mg-bacteriochlorins are Mg-tetrahydophytoporphyrins with two reduced rings, rings B and D. For the structures of these pigments and their esterifying alcohols, see Scheer, 1991 and Chapter 1 (Scheer). The spectrophotometric properties and the assay of these pigments has been reviewed by Oelze (1985), Scheer (1988), Porra (1991, 2002) and Richards (1994): an updated list of these properties is presented in Table 3. Currently, no simultaneous equations exist for the determination of these pigments when they occur in combinations with other Chls. Millimolar (ε_{mM}) extinction coefficients from these reviews and other papers are

Table 3. Spectroscopic properties and millimolar extinction coefficients of BChls. The Mg-bacteriochlorins, BChls *a*, *b* and g are grouped together as are the Mg-chlorins, BChls *c*, *d* and *e*

BChl	Solvent	Millimolar Extinction Coefficients Peak wavelengths in nm; [ε_{mM}]						Soret:Q_y	Reference
A:	**Mg-Bacteriochlorin type pigments**								
BChl *a*	Diethylether	358.5 [73.4]	391.5 [48.1]	530 [2.7]	577 [20.9]	697 [9.1]	773 [91.2]	0.80	Smith and Benitez (1955)
		358 [85.4]	391 [52.8]		575 [22.1]	697 [11.5]	772 [96.0]	0.89	Weigl (1953)
		357 [73.4]	392 [47.1]		573 [22.0]		770 [96.0]	0.76	Sauer et al., (1966)
	Acetone	358 [65.7]			576.5 [19.4]		770 [69.2]	0.94	Sauer et al., (1966)
	Methanol						772 [60.0]		Cohen-Bazire et al., (1966)
	Ethanol	365.5 [58.5]			607 [15.2]		773 [62.0]	0.94	Sauer et al., (1966)
BChl *b*	Diethylether	368 [81]	408 [78]		578 [25]	676 [18]	794 [100]	0.81	Steiner (1981)
							794 [106]		Scheer and Steiner (1985)[a]
	Acetone	368 [94]	407 [82]		580 [27]		794 [100]	0.94	Baumgarten (1970)
BChl *g*	Acetone	364.8	404.8		566.4		761.6 [76]		van de Meent et al., (1991)
B.	**Mg-Chlorin type pigments**								
BChl *c*	Diethylether	412 [72.6]	432 [142]			622 [15.9]	660 [91.0]	1.56	Stanier and Smith (1960)
(809)[b]	Acetone	413 [71.8]	433 [115.5]			625 [13.7]	662.5 [74.9]	1.54	Stanier and Smith (1960)
	Methanol	419 [69.7]	435 [79.1]			620 [14.6]	670 [69.6]	1.14	Stanier and Smith (1960)
BChl *d*	Diethylether	406 [69]	425 [115.6]	530 [2.7]	575 [6.8]	612 [12.5]	650 [89.9]	1.28	Stanier and Smith (1960)
(792)[b]	Acetone	406 [69.6]	427 [99.7]	530 [3.1]	575 [7.3]	612.5 [13.0]	654 [77.6]	1.28	Stanier and Smith (1960)
	Methanol	411 [60.6]	427 [66.1]			612.5 [14.3]	659 [65.2]	1.01	Stanier and Smith (1960)
BChl *e*	Acetone		462 [185.0]				649 [48.9]	3.78	Borrego et al., (1999)
(835.1)[c]	Acetone/MeOH (7:2)		466 [155.6]				651 [41.4]	3.76	Borrego et al., (1999)
	Methanol		476 [130.7]				660 [35.5]	3.68	Borrego et al., (1999)
	Ethanol		469 [142.3]				654 [41.0]	3.47	Borrego et al., (1999)

[a] Re-determined by Scheer and Steiner (cf. Oelze 1985). [b] These Mr values were employed to calculate ε_{mM}values from the specific α extinction coefficients of Stanier and Smith (1960). [c] These ε_{mM} values were calculated by Borrego et al., (1999) using an Mr value of 835.1 for the [8-propyl-12-ethyl-17^3-farnesyl]-homologue of BChl *e*.

assembled in Table 3. Many homologues of BChls *c, d* and *e* exist (See Chapter 1, Scheer and Chapter 15, Freegard et al.); for example, Airs et al., (2001) have isolated thirty BChl *e* homologues using HPLC from a *Chlorobium phaeobacteroides* strain with Mr values between 763 and 887. The ε_{mM} value for BChl *e* (see Table 3) was calculated by Borrego et al., (1999) using an Mr value of 835.1 for the [8-propyl-12-ethyl-17^3-farnesyl]-homologue of BChl *e*. Borrego et al. (1999) also calculated the ε_{mM} values for BChls *c* and *d* (see Table 3) from the α coefficients of Stanier and Smith (1960): these original α coefficients can be determined with Eq. (5) using 809 and 792 for the Mr values of BChls *c* and *d*, respectively.

IX. Spectrofluorimetric Assays for Chlorophylls *a* and *b*

A. Advantages and Disadvantages of Spectrofluorimetric Assays

Plant Chls fluoresce strongly when irradiated at their Soret absorption maxima (Chapter 1, Scheer) enabling spectrofluorimetric methods to assay Chls in the pmol/ml range while spectrophotometry is reliable only in the nmol/ml range and only then if the true Chl a/b^T ratio does not exceed ~15 thus obscuring a clear Chl *b* Q_y peak.

Unfortunately, the constants for determining the concentrations of Chl *a* and *b* mixtures by spectrofluorimetry are not as universally applicable as spectrophotometric extinction coefficients (see below): they vary significantly between individual spectrofluorimeters which require frequent calibration. A single Chl can be assayed with a simple fluorimeter from a calibration curve of fluorescence amplitudes (intensities) constructed with Chl solutions of known concentrations but the assay of Chl mixtures requires a sophisticated spectrofluorimeter with automatic correction for photomultiplier responses, for both monochromator characteristics delivering actinic light and receiving emitted fluorescence, and for wavelength-dependent variation in energy output of the actinic light source.

An advantage of spectrofluorimetry for assaying Chl *a* and *b* mixtures is that selective excitation of the minor Chl *b* component by irradiation with 453nm light (the Chl *b* Soret maximum in diethylether) helps negate the disadvantage imposed by the generally lower concentration of Chl *b* because Chl *a* absorption at 453nm relative to Chl *b* is much reduced and thus the fluorescence yield of Chl *a* relative to Chl *b* is lowered. Boardman and Thorne (1971) showed, with 453nm irradiation, that Chl *b* in a diethylether solution (Chl $a/b \equiv 1.3$) produced more intense fluorescence (F_b) at 646nm than Chl *a* (F_a) at 666nm and, because F_a and F_b are related to the molar concentrations of Chls *a* and *b,* respectively, Chl *a* and *b* could thus be accurately determined at room temperature by fluorescence emission even with large excesses of Chl *a* present (Boardman and Thorne, 1971; see also equations in Section IX B).

B. Equations for the Spectrofluorimetric Assay of Chlorophyll a and b Mixtures in Diethylether

For individual solutions of Chl *a* and Chl *b* in diethylether excited with 453nm light at room temperature, Boardman and Thorne (1971) found the following relationships between Chl concentration and fluorescence emission:

$$F_a = k_1[\text{Chl } a] \quad (6)$$

$$F_b = k_2[\text{Chl } b] \quad (7)$$

Because of the overlap in the fluorescence emission spectra of Chls *a* and *b,* Eqs. (6) and (7) do not apply to mixed solutions and must be replaced by Eqs. (8) and (9):

$$F'_a = k_1[\text{Chl } a] + K_b \bullet k_2[\text{Chl } b] \quad (8)$$

$$F'_b = k_2[\text{Chl } b] + K_a \bullet k_1[\text{Chl } a] \quad (9)$$

where K_b is the ratio of the fluorescence emission amplitude of Chl *b* at 666 nm (the Chl *a* emission peak) relative to its amplitude at 646 nm (the Chl *b* emission peak), while K_a is the amplitude of Chl *a* at 646 nm relative to its amplitude at 666 nm. Both K_a (0.185) and K_b (0.142) and also k_1 and k_2 can be calculated directly from the emission spectra of pure solutions of Chls *a* and *b*. Then spectrofluorimetric simultaneous equations can be written to solve for (Chl *a*) and (Chl *b*) as was done for spectrophotometric methods (see Section IV).

C. Determination of Chlorophyll a/b Ratios by Spectrofluorimetry

K_a and K_b were determined in diethylether as 0.185 and 0.142, respectively. Thus Eq. (10), which was calculated from Eqs. (8) and (9) becomes Eq. (11):

$$\text{Chl } a/b = (k_2/k_1)\bullet\{(F'_a/F'_b - K_b) /(1 - K_a F'_a/F'_b)\} \quad (10)$$

$$\text{Chl } a/b = (k_2/k_1)\bullet \{(F'^{666}/F'^{646} - 0.142) /(1 - 0.185\bullet F'^{666}/F'^{646})\}. \quad (11)$$

Equation (10) is sometimes abbreviated as shown in Eq. (12):

$$\text{Chl } a/b = (\mathbf{m})\bullet\{\mathbf{R}\} \quad (12)$$

where **m** equals (k_2/k_1) and **R**, known as the corrected fluorescence ratio, is the term included in **{ }** in Eqs. (10) and (11). The constants k_1, k_2, K_a and K_b, and hence **m** and **R**, are all dependent on the solvent used, the emission wavelengths, and also on instrument parameters such as band widths. Consequently, these constants must be newly determined for each spectrofluorimeter used and for each solvent used.

Boardman and Thorne (1971) plotted Chl *a/b* against **R** for a series of standards with Chl *a/b* ratios of 6–60 and showed that the slope **m** (i.e. k_2/k_1) was 21, so that Eq. (11) becomes Eq. (13) when using their particular instrument and diethylether as solvent.

$$\text{Chl } a/b = 21\bullet\{(F'^{666}/F'^{646} - 0.142) /(1 - 0.185\bullet F'^{666}/F'^{646})\} \quad (13)$$

To obtain the Chl *a/b* ratio, the fluorescence emission values of the Chl mixture at 646 nm (F'^{646}) and at 666 nm (F'^{666}) were entered into Eq. (13).

D. Determination of Chlorophyll a/b Ratios in Water-miscible Solvents

Talbot and Sauer (1997) showed that the spectrofluorimetric method of Boardman and Thorne (1971) is also valid for water-miscible solvents such as aq 80% acetone, DMF and DMSO which are commonly used Chl extractants. Talbot and Sauer (1997) plotted **R** against Chl *a/b* ratio ranges from 10–125 in DMSO and from 10–220 in diethylether, aq 80% acetone, and DMF (see Table 4). The coefficients K_a and K_b and ratio k_2/k_1 results of Boardman and Thorne (1971) in diethylether (shown in brackets) differ from those of Talbot and Sauer (1997) who suggested that instrument differences were involved. This emphasizes the need for constant calibration, for determination of constants for individual spectrofluorimeters and for strict control of entry and exit slit widths for both excitation and emission monochromators.

Talbot and Sauer (1997) showed that sensitivity limits in determining Chl *a/b* ratios in various solvents increased not only with slope **m** and but also, not surprisingly, with increases in the wavelength difference (Δ) between the fluorescence emission peaks of Chls *a* and *b*. Solvents were rated as follows**:** diethylether (**m=39.9**: Δ=19 nm)> aq 80% acetone (**24.3**; 16 nm)> DMF (**18.5**; 16 nm)> DMSO (**11.0**; 12 nm).

Another method (Meister, 1992), using the optimal excitation wavelengths of both pigments, can extend this wide range of ratios to include downwards to normal biological Chl *a/b* ratios (~3.0–4.0), where the single Chl *b* excitation wavelength method is less accurate (Boardman and Thorne 1971; Talbot and Sauer, 1997).

E. Spectrofluorimetric Assays for Chlorophyll Intermediates and Catabolites

Fluorescence spectrometry, by selective excitation of minor Chl components in a mixture, is a very sensitive method for detection of cross-contaminants (e.g. in Chls *a* and *b* isolates); similarly, it can be used to assay Chl intermediates or catabolites using irradiation at their Soret maximum to overcome the disadvantage of their low concentrations relative to those of the major products. Spectrofluorimetric assays have been developed for the following combinations of fluorescent Chl derivatives: PChl(ide) and Chl(ides) *a* and *b* (see Rebeiz et al., 1975); Phe(ides) *a* and *b* and Chl(ides) *a* and *b* (Bazzaz and Rebeiz, 1979); and, Chl(ides) *a* and *b* (which are 3-monovinyl (MV) compounds) and their 3,8 divinyl (DV) forms (Wu et al., 1989). [DV]-Chls *a* and *b* occur only transiently in greening plants (Ioannides et al., 1994) but, in some microalgae of subtropical oceans, they play a major role in global photosynthesis (Chisholm et al., 1992; Goericke and Repeta, 1993). For details of the extraction of the above pigment groups in 90% acetone in 0.01N-NH_4OH, the separation of their phytylated and unphytylated forms, the required excitation and emission wavelengths in ammoniacal acetone, the derivation of the necessary equations, of correction procedures and of the multitude of constants required

Table 4. Calibration parameters for fluorimetric determination of Chls *a* and *b* in various solvents. Data supplied by Talbot and Sauer (1997)

Solvent:	Diethylether		Aq 80% acetone	DMF	DMSO
Excitation maxima	453 nm	(453)[a]	461 nm	462 nm	463 nm
Emission maxima					
Chl *a*	666 nm	(666)[a]	672 nm	672 nm	674 nm
Chl *b*	647 nm	(646)[a]	656 nm	656 nm	662 nm
K_a	0.0573	(0.185)[a]	0.237	0.234	0.433
K_b	0.132	(0.142)[a]	0.423	0.435	0.631
Slope ($m = k_2/k_1$)	39.9	(21.0)[a]	24.3	18.5	11.0

[a] The figures in parentheses are those obtained by Boardman and Thorne (1971)

for their assay, readers are referred to the original papers and their extensive appendices.

New and possibly simpler assays for these intermediates and catabolites are now available using new HPLC methods interfaced with various detection techniques (Chapter 8, Garrido and Zapata).

X. Concluding Remarks

In summary, the light absorption and fluorescence emission properties of Chls provide a vast number of reliable spectrophotometric and spectrofluorimetric assays for Chls and their derivatives after extraction from photosynthetic cells and tissues in a wide variety of solvents.

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