

# Formulae for Determination of Chlorophyllous Pigments Extracted with *N,N*-Dimethylformamide<sup>1</sup>

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## ABSTRACT

The extraction of chlorophylls in higher plant tissue using *N,N*-dimethylformamide expedites the process and enables the determination of small samples with low pigment level.

Absorption spectra of Chl *a*, Chl *b*, and Pchl and of their acidified derivatives, the phaeophytins, were recorded. Conversion of Chl *b* to its corresponding acidified product occurs much more slowly than that of Chl *a* and Pchl. When acidified, Pchl differs from Chl *a* and Chl *b* by the disappearance of the red band in the absorption spectrum. Specific extinction coefficients were determined and formulae for quantitative determination of pigments concentrations were developed. When concentrations of pigments are low, as in etiolated plant material, the absorption spectra of the chlorophylls can be distorted due to the presence of other substances simultaneously extracted; formulae for pigment determination under such circumstances were also derived.

There is a vast array of solvents used for the extraction and determination of the Chl, but most of them necessitate grinding and centrifuging of material with or without heating (2, 5, 9). Recently, it was shown that the use of the solvent DMF<sup>2</sup> renders the process simpler and faster, since the pigments can be extracted from intact tissues (11). A similar method which was described for green leaves, using ethanol, includes several repetitions of the extraction in relatively high volume (7). Another method was suggested, using dimethylsulfoxide. However, the tissue must be heated to 60°C (4).

In a previous paper (11), the SEC of the red band of Chl *a* in DMF was determined. The present work provides the SEC in the corresponding wavelength and formulae for quantitative determination of Chl *a*, *b*, and Pchl in DMF extracts, which were calculated from the absorption at the main bands in the red as described for other solvents (12). Formulae were also developed for determination of the Chl in etiolated plant material, where their low concentration makes their accurate determination difficult. The corresponding acidic derivatives (phaeo *a*, phaeo *b*, and protophaeophytin) studied earlier in other solvents (8, 18, 19) were similarly investigated.

## MATERIALS AND METHODS

For determination of the SEC solutions of pure Chl *a* and *b* (Sigma) were prepared as already described for Chl *a* (11); solu-

tions of pure Pchl were similarly prepared from DMF extracts of intact *Cyclanthera explodense* seed coats (17). The seeds were kindly supplied by Dr. C. Sundquist, from the Department of Botany, University of Göteborg. Additional Pchl extracts from the seed coats were also prepared in 80% acetone or with DMF by grinding and centrifuging methods (2) and diluted with diethylether or with DMF.<sup>3</sup>

The results are averages of eight to 12 repetitions. Various mixtures of the pigments were also prepared. Extracts of plant material were obtained from green leaves of *Emex spinosus*, *Malva silvestris*, *Sonchus oleraceus*, and *Spirodella oligorrhiza* and from etiolated and greening cotyledons of *Cucumis sativus*, by direct immersion in DMF (11). Approximate ratios of 1:100 and 1:10 (w/v) for green and etiolated material, respectively, were used. Differences in spectra were not attributable to differences in extracting methods. Spectrophotometric measurements were made by means of Varian Techtron model 635 UV-VIS scanning spectrophotometer, calibrated at 703 nm, using the 0.2 nm band width measuring beam and the 1 ml cuvette having a path length of 1 cm.

Conversion of the Chl to their acidic derivatives was carried out according to (15) modified as follows: the nonacidified solution was stirred in the cuvette with a sealed pasteur pipette which was first dipped in a 5 N HCl solution. This treatment was repeated twice, although conversion of the pigments was almost complete after the first time. Change in volume was negligible. The change in pH was from 6.8 to 6.6 and was enough to enhance the total disappearance of the specific absorption spectrum of the Chl and the replacement by a stable spectrum of the corresponding acidic derivatives. All the SEC given for the phaeophytins are not corrected for loss of Mg, so that they can be utilized for the calculating of the concentrations of their corresponding Chl (18).

## RESULTS

**Determination of SEC for the Chl:** The SEC were determined by the equation:

$$A_{\lambda} = \epsilon_{\lambda} c l \quad (1)$$

where  $A_{\lambda}$  is the absorbance (OD units) at a given wavelength,  $\epsilon_{\lambda}$  is the SEC of the solution at wavelength  $\lambda$ ,  $c$  is the concentration ( $\text{g/l}^{-1}$ ) and  $l$  is the beam-path (1 cm) in the measuring cuvette. For any  $\lambda_1, \lambda_2$  in an interval where relation (1) holds, we have:

$$\epsilon_{\lambda_2} = \frac{A_{\lambda_2}}{A_{\lambda_1}} \times \epsilon_{\lambda_1} \quad (2)$$

The absorbances at the maxima of the main bands in the red of Chl *b* in 80% acetone and of Pchl in diethylether as solvents were compared with the absorbances of the corresponding maxima of

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<sup>2</sup> Abbreviations: DMF, *N,N*-dimethylformamide; SEC, specific extinction coefficient; Phaeo, phaeophytin.

<sup>3</sup> In this work, the reading of absorption of Chl *a* at 664 nm is used. It was practically the same as that measured at 664.5 nm (11).

Table I. SEC  $\epsilon$  (l/g·cm) of Chl *a*, *b*, and Pchl at the Main Bands

Pigment	$\epsilon$ Wavelength (nm)											
	414	432	435	459	535	573	583	597	603	625	647	664
Chl <i>a</i>	75.6	93.7			3.8		9.1		10.6 <sup>a</sup>	14.1 <sup>b</sup>	20.2 <sup>b</sup>	83.9 <sup>c</sup>
Chl <i>b</i>				132.3				11.3	10.9 <sup>a</sup>	9.8 <sup>b</sup>	45.6 <sup>c</sup>	10.8 <sup>b</sup>
Pchl			266.4		7.2	13.6			11.1 <sup>a</sup>	35.7 <sup>c</sup>	1.6 <sup>b</sup>	0.5 <sup>b</sup>

<sup>a</sup> Isobestic point.<sup>b</sup> Not a maximum.<sup>c</sup> Maxima in the red.

each of the pigments in DMF solutions with the same concentration. The SEC were accordingly determined as was already done with Chl *a* in DMF (11), using equations (1) and (2) and the data already obtained for the SEC of Chl *b* (9) and Pchl (8). The location of the bands together with their SEC are shown in Table I.

**Equations for Chl *a*, Chl *b*, and Pchl Determination.** Using the data from Table I the following equations can be written for the absorbance at the maxima of the main bands in the red:

$$A_{664} = 83.9 C_a + 10.8 C_b + 0.5 C_p \quad (3)$$

$$A_{647} = 20.2 C_a + 45.6 C_b + 1.6 C_p \quad (4)$$

$$A_{625} = 14.1 C_a + 9.8 C_b + 35.7 C_p \quad (5)$$

Where  $A_\lambda$  is the absorption recorded at the individual wavelength, expressed in OD units,  $C_a$ ,  $C_b$  and  $C_p$  represent the concentrations of Chl *a*, *b*, and Pchl, respectively, in g/l.

Solving equations (3), (4), and (5) for  $C_a$ ,  $C_b$ , and  $C_p$ , in  $\mu\text{g/ml}$ , we obtain:

$$C_a = 12.65 A_{664} - 2.99 A_{647} - 0.04 A_{625} \quad (6)$$

$$C_b = -5.48 A_{664} + 23.44 A_{647} - 0.97 A_{625} \quad (7)$$

$$C_p = -3.49 A_{664} - 5.25 A_{647} + 28.3 A_{625} \quad (8)$$

Inasmuch as no Pchl is expected when a green tissue is extracted, other formulae to use in such conditions are derived using only the two SEC which correspond to the main bands of Chl *a* and *b*, as follows:

$$A_{664} = 83.9 C_a + 10.8 C_b \quad (9)$$

$$A_{647} = 20.2 C_a + 45.6 C_b \quad (10)$$

Solving equations (9) and (10) for  $C_a$  and  $C_b$ , we obtain:

$$C_a = 12.64 A_{664} - 2.99 A_{647} \quad (11)$$

$$C_b = -5.6 A_{664} + 23.26 A_{647} \quad (12)$$

hence

$$C_t = 7.04 A_{664} + 20.27 A_{647} \quad (13)$$

where  $C_t = C_a + C_b$  is the total Chl concentration, expressed in  $\mu\text{g/ml}$ .

**Determination of Low Concentration Chl.** When the concentration of the Chl is relatively low, as in etiolated plants, accurate band measurement is sometimes difficult since other materials present in the extract distort the spectrum. In such cases (Fig. 1), a base-line can be drawn as a reference line (e.g. ref. 14) for measuring the absorption. When determination of more than one pigment is required the situation is more complicated inasmuch as different baselines may be needed. This can be avoided if a reference line is drawn between two isobestic points on the spectrum lines (see Fig. 3; Appendix). For Chl *a*, *b*, and Pchl dissolved in DMF it was found that a reliable reference line can be drawn

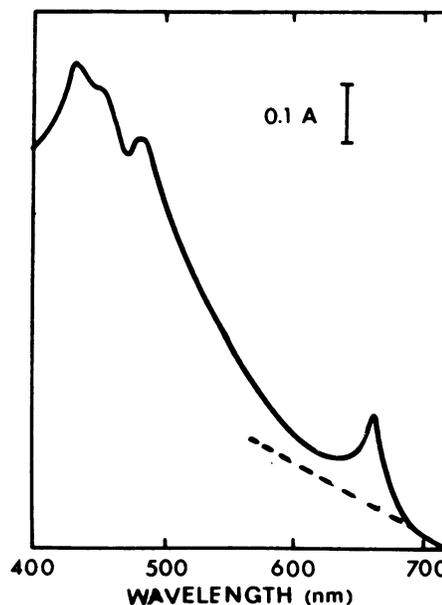


FIG. 1. Absorption spectrum of an extract of 72-h-old etiolated cucumber cotyledons. Relatively high absorbance by a substance whose main band is in the blue distorts the spectrum of the chlorophyllous pigments in the red region. Thus, a reference line is drawn (---) in an attempt to obtain corrected values for the main red bands.

between 603 nm, which is practically an isobestic point for Chl *a*, *b*, and Pchl (Table I), and 703 nm, the first wavelength beyond 603 nm where absorption of neither pigment is detected. However, determination of concentrations in this way (14) is a long and tedious process. The formulae (14) to (19) which were developed (see Appendix) enable the determination of the required concentrations under similar conditions using three or four absorption data only:

$$C_a = 12.81 A_{664} - 2.16 A_{647} + 1.44 A_{625} - 4.91 A_{603} \quad (14)$$

$$C_b = -4.93 A_{664} + 26.01 A_{647} + 3.74 A_{625} - 15.55 A_{603} \quad (15)$$

$$C_p = -2.52 A_{664} - 0.79 A_{647} + 36.55 A_{625} - 27.08 A_{603} \quad (16)$$

Where no detectable traces of Pchl are expected the following formulae, similarly obtained, are useful:

$$C_a = 12.92 A_{664} - 2.12 A_{647} - 3.85 A_{603} \quad (17)$$

$$C_b = -4.67 A_{664} + 26.09 A_{647} - 12.79 A_{603} \quad (18)$$

$$C_t = 8.24 A_{664} + 23.97 A_{647} - 16.64 A_{603} \quad (19)$$

The validity of formulae (14) to (19) was experimentally tested as follows: pure solutions of Chl *a*, *b*, and Pchl were prepared as well as mixtures of these solutions. Distortion of the spectra did

Table II. Ratios (expressed in %) between Results Obtained by Determination of the Concentrations of Chl using the Total Absorbance Formulae, as Opposed to the Reference Line Formulae

The concentrations ranged between 3 and 26 µg/ml; (a) and (c) are averages of five samples.

	Chl <i>a</i>	Formulae Used	Chl <i>b</i>	Formulae Used	Pchl	Formulae Used	Total Chl	Formulae Used
(a) Single pure pigment	99.9 ± 0.7	(17)/(11)	99.6 ± 0.3	(18)/(12)	102.9 ± 1.4	(16)/(8)		
(b) Pigment mixtures								
Chl <i>a</i> + Chl <i>b</i>	98.8	(17)/(11)	99.2	(18)/(12)		(16)/(8)	99.0	(19)/(13)
Chl <i>a</i> + Pchl	99.9	(14)/(6)			99.5	(16)/(8)	100.4	(16) + (14)/(6) + (8)
(c) Extracts from green leaves	99.9 ± 0.4	(17)/(11)	99.7 ± 5.1	(18)/(12)			100.0 ± 1.7	(19)/(13)

Table III. Concentrations of Chl (µg/ml) Both before and after Centrifugation of Cotyledon Extracts

The calculations were made by (T) total absorbance and (R) reference line formulae. Various short-term light pulses were given to the etiolated cucumber seedlings before extraction to ensure the presence of Chl *a* in addition to Pchl. The ratios between the results are reported in the text.

Extracts	Chl <i>a</i>		Chl <i>b</i>		Pchl		Total Chl	
	(T)	(R)	(T)	(R)	(T)	(R)	(T)	(R)
Before	16.7	14.9	5.0	-0.5	10.9	1.4	32.6	15.8
After	14.8	14.3	1.5	0.0	9.7	2.1	21.0	16.3
Before	8.3	6.8	5.4	0.7	11.3	3.2	25.1	10.7
After	6.5	6.2	1.0	0.1	4.9	3.3	12.3	9.6
Before	10.7	9.0	5.8	0.4	13.3	4.1	29.8	13.6
After	9.4	9.1	0.8	0.0	5.2	3.8	15.4	12.9
Before	19.8	18.5	3.7	-0.3	8.8	2.0	32.2	20.2
After	18.8	18.4	1.2	-0.2	4.6	2.2	24.6	20.3
Before	7.3	5.6	5.1	-0.3	23.2	13.7	35.6	18.9
After	5.2	5.3	-0.6	-0.6	12.8	12.8	21.4	17.4
Before	7.2	4.7	7.8	0.6	27.7	14.2	42.6	18.9
After	5.6	4.9	1.7	-0.3	17.8	14.3	25.2	18.8
Before	1.8	0.6	3.6	-0.2	19.9	13.4	25.4	13.8
After	0.7	0.5	0.3	-0.4	14.1	13.1	15.1	13.2

not exist in these preparations. In addition, green leaves were extracted. Distortion of the spectra in the extracts solutions was negligible due to the high concentration of the Chl. The concentrations of the Chl were then determined according to the formulae (6) to (8) and (11) to (13)—afterwards referred to as “total absorbance” formulae—and then from formulae (14) to (19) which will be referred to as “reference line” formulae. The results obtained from both formulae systems were consistent for all three pigments (Table II).

The reference line system was then applied to cloudy extracts obtained from etiolated 5-day-old *Cucumis* cotyledons. Some of the seedlings were treated earlier with short-term light pulses so that small amounts of Chl *a* would be present in the extracts in addition to Pchl. The results obtained, using the reference line formulae, were compared to those obtained using the total absorption formulae and the same calculations were repeated following centrifugation of the extracts. It was obvious that while the differences between the results obtained before centrifugation and those obtained after it were significant when the total absorbance

system was used, they were practically negligible when the reference line system was applied (Table III).

When ratios (“before” versus “after” centrifugation) were calculated for the specific pigments in 32 extracts, the results were as follows: for total Chl the value ( $\pm$  SD) was  $1.02 \pm 0.07$  when the reference line formulae were used, while it was  $1.53 \pm 0.10$  for total absorbance. For Chl *a* the corresponding values were  $1.04 \pm 0.10$  against  $1.25 \pm 0.36$  and for Pchl they were  $1.01 \pm 0.15$  against  $1.69 \pm 0.54$ . For this ratio, Chl *b* was not calculated since normally under such conditions Chl *b* does not exist in detectable amounts, as was actually found when the reference line formula was used (Table III) and which is consistent with many previous works (e.g. refs. 1 and 16). However, results obtained for etiolated cloudy extracts using the total absorption formulae failed to show the absence of Chl *b*, unless centrifugation was applied (Table III).

**Acidification of Chl.** In transforming Chl by acidification to their corresponding phaeophytins (Fig. 2, a-c) it was found that the transformation of Chl *b* to Phaeo *b* was much slower than that

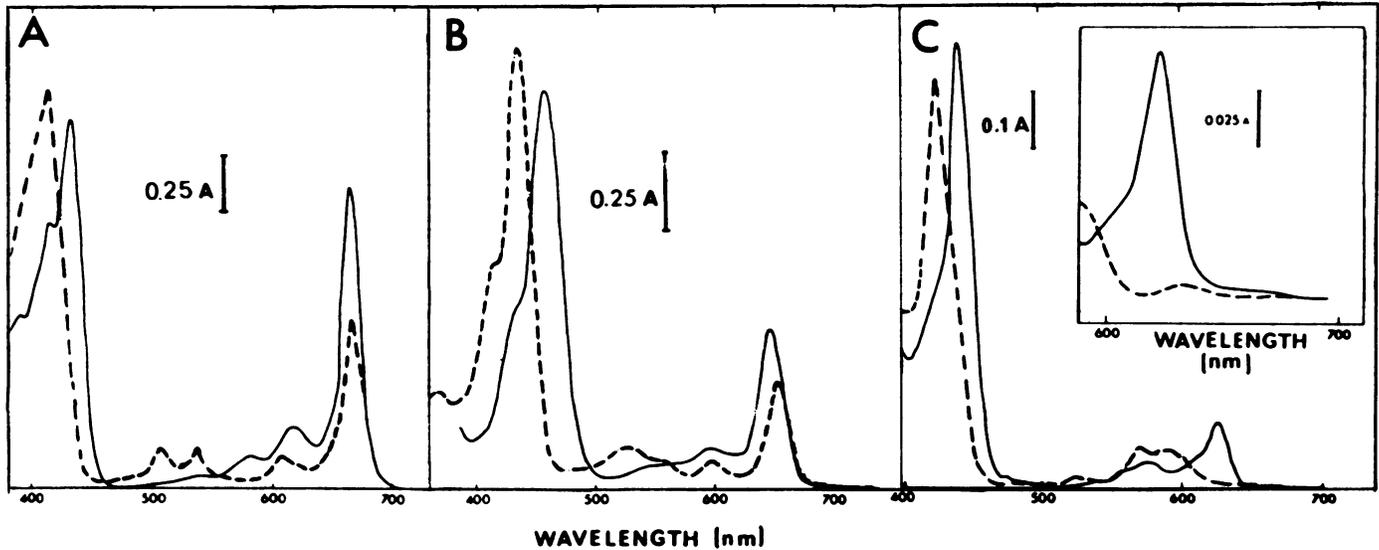


FIG. 2. Absorption spectra of (A) Chl *a* and Phaeo *a*; (B) Chl *b* and Phaeo *b*; and (C) Pchl and protophaeophytin in DMF solutions. For total disappearance of the red band of Pchl on acidification of the solution see (C) insert. (—), Chl; (---), Phaeo.

Table IV. The Kinetics of the Acidification of Chl *a*, Chl *b*, and Pchl

Pigment	Final HCl Concentration	Wavelength where Δ <i>A</i> was Measured	Time Needed to Achieve	
			50% Δ <i>A</i>	90% Δ <i>A</i>
		nm	S	
Chl <i>a</i>	0.02 N	664	10	10-15
	0.10 N		<5	<5
Chl <i>b</i>	0.02 N	647	50	300
	0.10 N		10	45
Pchl	0.02 N	625	<5	10-15
	0.10 N		<5	10

of the Pchl or that of Chl *a* (Table IV). On acidification, the red bands of Chl *a* and *b* were reduced by about 50% and their maxima only slightly shifted (Fig. 2, a and b), while Pchl acidification resulted in the practically complete disappearance of the red band (Fig. 2c, insert). However, when 80% acetone was the solvent instead of DMF, the red band, though significantly reduced, still existed as already reported (8).

**Determination of SEC for the Phaeophytins.** The SEC of the main bands of the acidified solutions (Fig. 2a, b, c) were determined by comparing the absorptions at the maxima of the main bands, as already described for the Chl (see earlier under "Results"). The bands in the red region were used when Chl *a* and Phaeo *a* or Chl *b* and Phaeo *b* were concerned and the bands in the blue region were taken for Pchl and Protophaeophytin. The location of the main bands together with their SEC are shown in Table V.

**Equations for Phaeo *a* and Phaeo *b* Determination.** Since Protophaeophytin in DMF solution has no significant absorption band in the red (Fig. 2c, insert), the absorption spectra in this region is useful for the determination of Phaeo *a* and *b* only. Using the data obtained (Table V), the following equations can be written for the absorbance at the maxima of the main bands in the red:

$$A_{666} = 48.2 C'a + 9.3 C'b \quad (20)$$

$$A_{654} = 21.1 C'a + 30.8 C'b \quad (21)$$

where *C'a* and *C'b* are the concentrations of phaeo *a* and phaeo *b*, respectively, in g/l.

Solving equations (20) and (21) for *C'a*, *C'b* in μg/ml we obtain:

$$C'a = 23.91 A_{666} - 7.22 A_{654} \quad (22)$$

$$C'b = -16.38 A_{666} + 37.41 A_{654} \quad (23)$$

$$C't = 7.53 A_{666} + 30.19 A_{654} \quad (24)$$

where *C't* = *C'a* + *C'b* is the total phaeophytin concentration in the DMF solution.

When formulae (22) to (24) were used to determine pigments concentration in acidified DMF solutions, the results were comparable to those obtained prior to acidification, thus showing the validity (Table VI).

## DISCUSSION

The use of DMF as a solvent for easy extraction and determination of pigments in low concentrations (11) invites further exploration. In this work, the main absorption bands of Chl *a*, *b*, and Pchl as well as those of Phaeo *a*, *b*, and Protophaeophytin in DMF were located and their SEC were determined (Fig. 2, Tables I and V). Formulae were developed for the calculation of the concentrations of Chl *a*, *b*, and Pchl (formulae [6] to [8], [11] to [13]) and of Phaeo *a* and *b* (formulae [22] to [24]) in the DMF solutions in the same way as formulae were worked out for other solvents (12). The use of DMF enables the detection of pigments in extracts from plant material having a low level of pigment concentration, but when such extracts are concerned, it often occurs that the Chl spectrum is distorted due to absorbance by other materials extracted along with it (Fig. 1). To cope with this problem, it is possible to scan and record the absorption spectrum and draw a relevant base-line for reference (e.g. ref. 14). However, because this can be a somewhat long and tedious procedure, which becomes even more complicated if more than one pigment is involved, reference line formulae [14] to [19] were developed, employing a common isobestic point for Chl *a*, *b*, and Pchl (Table I), which render the calculations faster, right from three or four absorbance data (see Appendix). The validity of the reference line formulae was experimentally tested (Table II) and it seems that their accuracy makes them preferable for the determination of Chl in etiolated plant material, where the level of the pigments concentration is low and small quantities of other substances co-

Table V. SEC  $\epsilon$  (1/g·cm) of Phaeo *a*, Phaeo *b* and Protophaeophytin at the Main Bands

Pigment	$\epsilon$ Wavelength (nm)														
	412-413	415	420	437	508	524	526	535-536	568	589	600	608	640	654	666
Phaeo <i>a</i>	111.4				11.5			10.9				10.2		21.1 <sup>a</sup>	48.2 <sup>b</sup>
Phaeo <i>b</i>		67.8 <sup>c</sup>		136.2			39.5				8.6			30.8 <sup>b</sup>	9.3 <sup>a</sup>
Protophaeophytin			246.7			11.8			23.2	19.7			3.2 <sup>b</sup>		

<sup>a</sup> Not a maximum.<sup>b</sup> Maxima in the red.<sup>c</sup> A shoulder.

Table VI. Ratios (expressed in %) between Results Obtained by Determination of the Concentrations of Chlorophyllous Pigments Using before Acidification the Formulae (11), (12), (13), and after Acidification the Formulae (22), (23), (24)

The concentrations ranged between 2 to 32  $\mu\text{g/ml}$ ; each result is an average of 3 to 5 samples.

	Chl <i>a</i>	Formulae Used	Chl <i>b</i>	Formulae Used	Total Chl	Formulae Used
(a) Single pure pigments	100.6 $\pm$ 4.0	(22)/(11)	100.2 $\pm$ 1.1	(23)/(12)		
(b) Pigment mixtures	102.4 $\pm$ 4.3	(22)/(11)	102.6 $\pm$ 3.8	(23)/(12)	101.2 $\pm$ 1.9	(24)/(13)
(c) Extract from green leaves	100.9 $\pm$ 1.4	(22)/(11)	108.4 $\pm$ 4.0	(23)/(12)	102.0 $\pm$ 1.4	(24)/(13)

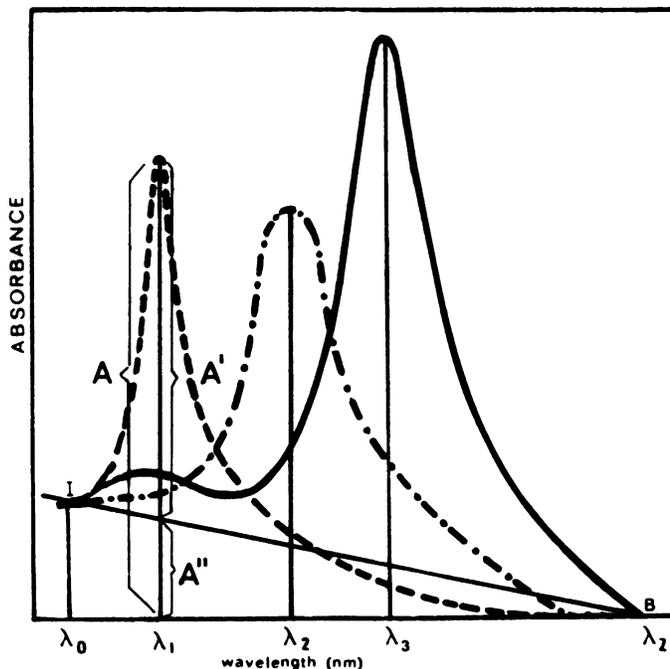


FIG. 3. Different main absorption bands  $\lambda_1$ ,  $\lambda_2$ ,  $\lambda_3$  are shown for pigment solutions in a given solvent all having the same concentrations. These three hypothetical absorption spectra have a common value at  $\lambda = \lambda_0$ , where their spectrum lines show a common isobestic point, *I*. Point *B* is the nearest point where no absorption by any of the pigments can be detected. The straight line *IB* serves as a reference line for the three pigments, while *A*, *A'*, *A''* represent the absorbance, the partial absorbance, and the reference line absorbance, respectively, of the pigment whose maximum is at  $\lambda_1$ . See text for further explanations.

extracted with the Chl interfere with absorbance. This was shown by comparing the results calculated with the two sets of formulae, both before and after clearing the extract by centrifugation. Discrepancies of 30 to 50% were observed between these results using total absorbance formulae ([6] to [8], [11] to [13]). When reference line formulae were applied, these differences were reduced to less

Table VII. Partial SEC (1/g·cm) for DMF Solutions of Chl *a*, Chl *b*, and Pchl in the Corresponding Maxima at the Red Region

Wavelength	Pigment		
	Chl <i>a</i>	Chl <i>b</i>	Pchl
<i>nm</i>			
664	79.8	6.5	-3.8
647	14.3	39.5	-4.6
625	5.8	1.3	27.0

than 5% (Table III and the related text).

When acidified, Chl *a* and *b* retain their bands in the red region, although the maxima are reduced by about one half (Fig. 2, a and b), while Pchl practically loses its red band (Fig. 2c, insert). Another difference observed with the kinetics of the acidification was that the process in DMF is much slower for Chl *b* than for Chl *a* or Pchl (Table IV). This observation is in accordance with similar studies done using acetone solutions (6, 10, 13, 18).

The formulae (22) to (24) obtained for the determination of Phaeo *a* and *b* concentrations were experimentally tested and gave the same results as for the Chl *a* and *b* solutions from which they were obtained by acidification and whose concentrations were determined using formulae (6) to (8) as shown (Table IV).

The spectrophotometric determination of Chl *b* in extracts from green leaves is problematic, because of the overlapping of the main absorption bands of Chl *a* and *b* (3), the higher ratio of Chl *a*: Chl *b* in green plants, and the lower specific absorbance of Chl *b* in the red (12). When a mixture of both Chl *a* and *b* or Phaeo *a* and *b* are determined, small changes in the setting of the spectrophotometer profoundly affect the reading between 666 and 635 nm, where the main bands in the red of Chl *b* and Phaeo *b* are located (ref. 18 and unpublished data). This may account for discrepancies sometimes observed between the results when plant material is extracted. However, total concentration of Chl *a* + Chl *b* calculated using formula (13) agreed with total concentration of Phaeo *a* + Phaeo *b* obtained using formula (24), as shown (Table IV).

The problem is further aggravated when the ratio of Chl *a* to Chl *b* increases, as it does when etiolated plant material is involved, in which usually no Chl *b* can be detected (1, 16). Under such

conditions, Chl *b* is practically determined by estimating very small differences between relatively large values (12), which would undoubtedly lead to inconsistent results due to small changes in background absorbance. However, after comparing the results obtained for Chl *b* before and after centrifugation (Table III) it seems apparent that by employing reference line formulae, especially in the case of low Chl concentrations, more reliable results can be achieved.

### APPENDIX

Figure 3 represents the absorption spectra of three different solutions of three different pigments all having the same concentration. The main bands (absorption maxima) are located at  $\lambda_1$ ,  $\lambda_2$ ,  $\lambda_3$ , with absorbances  $A_1$ ,  $A_2$ ,  $A_3$ , respectively. At wavelength  $\lambda_0$  the three solutions have equal absorbance  $A_0$ , and so define a common point  $I_0$  to the three spectrum lines, an isobestic point. Let  $\lambda_z$  be the closest wavelength to  $\lambda_0$  where no absorbance exists in all three solutions. Thus, the point *B* denoting  $\lambda_z$  lies on the three spectrum lines; hence also an isobestic point. We call the line *IB* a *common reference line* for the three pigments. Given a wavelength  $\lambda$  and one of the pigments, say with its maxima at  $\lambda_1$ , we define the *partial absorbance  $A'$  of pigment 1 (broken line) at  $\lambda$*  by:  $A'_\lambda = A_\lambda - A''_\lambda$ , where  $A_\lambda$  is the absorbance of pigment 1 at  $\lambda$  and  $A''_\lambda$  is the value of the reference line at  $\lambda$ .

The *partial SEC  $\epsilon'$*  (of 1 at  $\lambda$ ) is then obtained using formula (1):

$$\epsilon' = \epsilon \times \frac{A_\lambda}{A'_\lambda} \quad (25)$$

$A'_\lambda$  is given by the formula

$$A'_\lambda = A_\lambda - A_0 \times \frac{\lambda_z - \lambda}{\lambda_z - \lambda_0} \quad (26)$$

We now apply these formulae when the three pigments are Chl *a*, *b*, Pchl, and the solvent is DMF to obtain the corresponding partial SEC. By Table I we have:  $\lambda_0 = 603$  nm,  $\lambda_1 = 664$  nm,  $\lambda_2 = 647$  nm,  $\lambda_3 = 625$  nm.  $\lambda_z$  is located at 703 nm. Using (26) we obtain the equations:

$$A'_{664} = A_{664} - 0.39 A_{603} \quad (27)$$

$$A'_{647} = A_{647} - 0.56 A_{603} \quad (28)$$

$$A'_{625} = A_{625} - 0.78 A_{603} \quad (29)$$

Substituting those values in equation (25) the following values are obtained for the partial SEC  $\epsilon'$  at  $\lambda_1$ ,  $\lambda_2$ ,  $\lambda_3$  (Table VII).

Using these data the following equations for the  $A'$  of solutions containing the three pigments can be written for the same wavelength:

$$A'_{664} = 79.8 Ca + 6.5 Cb - 3.8 Cp \quad (30)$$

$$A'_{647} = 14.3 Ca + 39.5 Cb - 4.6 Cp \quad (31)$$

$$A'_{625} = 5.8 Ca + 1.3 Cb + 27.0 Cp \quad (32)$$

where *Ca*, *Cb* and *Cp* represent the concentrations of Chl *a*, Chl *b* and Pchl in mg/ml. The system of equations (30), (31) and (32) is solved for the individual pigments concentrations in  $\mu\text{g/ml}$  in terms of the  $\epsilon'$  at the indicated wavelengths (Table VII) giving:

$$Ca = 12.81 A'_{664} - 2.16 A'_{647} + 1.44 A_{625} \quad (33)$$

$$Cb = -4.93 A'_{664} + 26.01 A'_{647} + 3.74 A'_{625} \quad (34)$$

$$Cp = -2.52 A'_{664} - 0.79 A'_{647} + 36.55 A'_{625} \quad (35)$$

On substituting  $A'_\lambda$  from equations (27), (28), and (29), in equations (33), (34), and (35), the following systems of formulae were obtained for the determination of the concentrations of Chl *a*, *b*, and Pchl (see earlier under "Results").

$$Ca = 12.81 A_{664} - 2.16 A_{647} + 1.44 A_{625} - 4.91 A_{603} \quad (14)$$

$$Cb = -4.93 A_{664} + 26.01 A_{647} + 3.74 A_{625} - 15.55 A_{603} \quad (15)$$

$$Cp = -2.52 A_{664} - 0.79 A_{647} + 36.55 A_{625} - 27.08 A_{603} \quad (16)$$

when only Chl *a* and *b* are present in the same way the following formulae were obtained:

$$Ca = 12.91 A_{644} - 2.12 A_{647} - 3.85 A_{603} \quad (17)$$

$$Cb = -4.67 A_{644} + 26.09 A_{647} - 12.79 A_{603} \quad (18)$$

$$Ct = 8.24 A_{644} + 23.97 A_{647} - 16.64 A_{603} \quad (19)$$

where  $Ct = Ca + Cb$  is the total Chl concentration, expressed in  $\mu\text{g/ml}$ .

Assume now that a mixture contains some other pigment whose main bands are relatively remote. Their net contribution to the absorption spectrum of the mixture is then described approximately by a straight line. This implies a linear rise of the spectrum, which does not practically affect the value of  $A'_{664}$ ,  $A'_{647}$ , and  $A'_{625}$ . Hence formula (14) to (19) are expected to produce more reliable values under these circumstances, as shown in Table III (see also Table II and the foregoing discussion).

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