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SENSITIVE FLUORESCENCE METHOD FOR THE DETERMINATION OF CHLOROPHYLL *a*/CHLOROPHYLL *b* RATIOS

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SUMMARY

Sensitive spectrofluorimetric methods were developed for the determination of chlorophyll *a*/chlorophyll *b* ratios. Room temperature measurements were made in diethyl ether. Fluorescence was excited at 453 nm, and chlorophyll *a*/chlorophyll *b* ratios were determined from the relative fluorescence amplitudes at 646 nm and 666 nm. The room temperature method is suitable for the determination of chlorophyll *a*/chlorophyll *b* ratios in the range 6–60. Greater sensitivity is obtained at liquid nitrogen temperature with ethyl alcohol as solvent. At 77° K, excitation was at 478 nm and fluorescence emission was measured at 658 nm and 678 nm. The low temperature method is accurate over the range of chlorophyll *a*/chlorophyll *b* ratios from 6 to 100, and may be extended with lower precision to ratios of up to 1000. For measurements in the range 3–6, fluorescence was excited at 465 nm.

INTRODUCTION

Chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) in plant extracts are usually estimated by spectrophotometric or colorimetric methods[1–5]. The absorbance of the extract is determined at the wavelengths corresponding to the red absorption maxima of the two pigments, and the amounts of chl *a* and chl *b* calculated with the aid of simultaneous equations. Such a method is satisfactory for the estimation of chl *a* and chl *b* in normal plant extracts where the ratio of chl *a*/chl *b* is approx. 3, but it is unsuitable for determining small amounts of chl *b* and for the estimation of chl *a*/chl *b* ratios in excess of about 6.

In the work described in this paper, sensitive fluorometric methods were developed for the accurate estimation of chl *a*/chl *b* ratios in extracts of whole leaves or isolated plastids, where the amount of chl *b* is low compared with that of chl *a*.

OGAWA AND SHIBATA[6] have described a method for estimating small amounts of chl *b* in plant extracts, based on the reaction of the formyl group of chl *b*. The fluorescence method has the advantage of much greater sensitivity and satisfactory results can be obtained with as little as 0.2 μg of chlorophyll.

Abbreviations: chl *a*, chlorophyll *a*; chl *b*, chlorophyll *b*.

MATERIALS AND METHODS

Purification of pigments

Chl *a* and chl *b* were extracted from spinach leaves (*Spinacia oleracea*) and purified by repeated chromatography on columns of powdered sucrose, as described by STRAIN[7]. Pheophytin *a* and pheophytin *b* were obtained by acidification of purified solutions of chl *a* and chl *b*, and further chromatography on sucrose columns. Protochlorophyllide was extracted from dark-grown bean plants (*Phaseolus vulgaris*) and purified by the procedure of SMITH AND BENITEZ[3].

The purity of pigments was checked by spectral scans in the wavelength range 350–750 nm on a Cary Model 14R recording spectrophotometer. The following absorbance ratios were obtained: for chl *a*, $A_{428\ nm}/A_{661\ nm} = 1.31$ and for chl *b*, $A_{453\ nm}/A_{642\ nm} = 2.84$.

Concentrations of chl *a* in ether were calculated from absorbance readings at 661 nm, using a specific absorption coefficient of 100.9 $l \cdot g^{-1} \cdot cm^{-1}$ as determined by COMAR AND ZSCHEILE[1]. Chl *b* concentrations in ether were determined at 642 nm, with a specific absorption coefficient of 62.0 $l \cdot g^{-1} \cdot cm^{-1}$ (ref. 3). Specific absorption coefficients were determined for chl *a* and chl *b* in ethanol at room temperature, in the following way. A concentrated solution of chl *a* in ether was diluted 100-fold into (a) ether and (b) ethanol, and spectra were recorded. The specific absorption coefficient of chl *a* in ethanol was calculated from the relative absorbance readings at the red maxima (661 nm in ether, 664 nm in ethanol), using the specific absorption coefficient of COMAR AND ZSCHEILE[1] for chl *a* in ether. A similar method was used for chl *b*. The values obtained were 83.4 $l \cdot g^{-1} \cdot cm^{-1}$ for chl *a* at 664 nm and 40.9 $l \cdot g^{-1} \cdot cm^{-1}$ for chl *b* at 648 nm.

Mixtures of chl *a* and chl *b* with widely varying ratios of the two pigments were made from solutions of the purified samples.

To investigate the influence of carotenoids in the determination of chl *a*/chl *b* ratios, an extract was made from spinach leaves, and this was mixed with varying quantities of chl *a*. Spinach leaves were ground with ethanol in a Servall "Omnimixer", immersed in ice, and the extract clarified by centrifugation at 10000 × *g* for 30 min. An aliquot of the extract was diluted 30-fold with 80% acetone and the contents of chl *a* and chl *b* determined spectrophotometrically, using the equations of ARNON[2].

For the determination of chl *a*/chl *b* ratios of plant tissue by the ethanol method, the tissue is ground in ethanol, the extract clarified by centrifugation and diluted to an absorbance of 0.1 at 436 nm. If the ether method is to be used, the tissue is ground in acetone and the pigments transferred to ether as described by COMAR AND ZSCHEILE[1]. The ether solution is dried over anhydrous Na_2SO_4 and diluted to an absorbance of 0.1 at 436 nm.

All solvents were of Analytical Reagent quality.

Fluorescence measurements

Fluorescence emission and excitation spectra were recorded on a fluorescence spectrometer incorporating automatic correction for photomultiplier and monochromator responses, and variation in energy output of the light source. Details of the spectrofluorimeter were published previously[8]. In the present work, the instrument was operated with an excitation bandwidth of ± 1.5 nm and a fluorescence emission

bandwidth of ± 1.0 nm. The scanning speed was 40 nm/min. Fluorescence measurements at the temperature of liquid nitrogen were carried out in a cylindrical glass tube, as described previously[8,9].

Fluorescence quantum efficiencies were determined relative to fluorescein as described previously[8].

RESULTS

Determination of chl a/chl b ratios at room temperature

Diethyl ether was chosen as the solvent for the estimation of chl *a*/chl *b* ratios at room temperature, because the fluorescence bands of chl *a* and chl *b*, both emission and excitation, are narrower and they overlap to a lesser extent in diethyl ether than in solvents such as acetone, methanol, ethanol or light petroleum.

Fluorescence emission and excitation spectra of chl *a* and chl *b* in ether at room temperature are shown in Fig. 1. Chl *a* and chl *b* show fluorescence emission maxima at 666 nm and 646 nm respectively and there is considerable overlap of the two emission spectra. Our determinations of the quantum yields of fluorescence in ether at room temperature gave 0.27 for chl *a* and 0.10 for chl *b*, in good agreement with previous determinations[10]. Thus chl *b* is considerably less fluorescent than chl *a*, which is a disadvantage when small amounts of chl *b* are being estimated in the presence of large amounts of chl *a*.

The fluorescence excitation maximum of chl *b* in ether is at 453 nm, compared with 428 nm for chl *a*. There is some overlap of the excitation spectra, but chl *a* is only poorly excited at the wavelength of maximum excitation of chl *b*. Absorption spectra were determined for our purified samples of chl *a* and chl *b*, and in the Soret band region, the absorption spectra corresponded closely to the fluorescence excitation spectra.

Our method for measuring chl *a*/chl *b* ratios in mixtures where the amount of chl *b* is low compared with chl *a* (chl *a*/chl *b* > 6) is based on the low excitation of

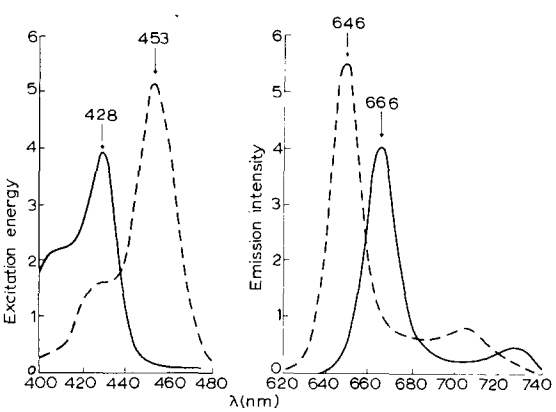
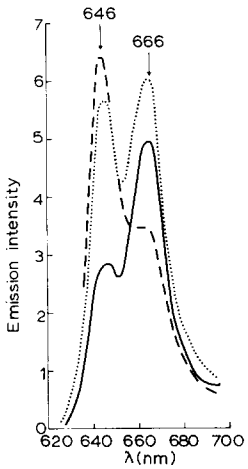


Fig. 1. Fluorescence excitation and emission spectra of chl *a* and chl *b* in diethyl ether at 20°. ———, chl *a*. Absorbance 0.10 cm^{-1} at 428 nm (0.76 μg chl *a*/ml). Emission for excitation at 428 nm, excitation for emission at 666 nm. – – – –, chl *b*. Absorbance 0.10 cm^{-1} at 453 nm (0.57 μg chl *b*/ml). Emission for excitation at 453 nm, excitation for emission at 646 nm. The electrical gain was × 3 to record the chl *b* spectra.

chl *a* at the wavelength where excitation of chl *b* is maximal. Known mixtures were prepared from the purified samples of chl *a* and chl *b* to give chl *a*/chl *b* ratios in the range 6–60. Fluorescence was excited at the excitation maximum of chl *b* (453 nm) and fluorescence emission spectra recorded. Representative spectra are shown in Fig. 2. The chl *a*/chl *b* ratio was determined from the relative fluorescence amplitudes



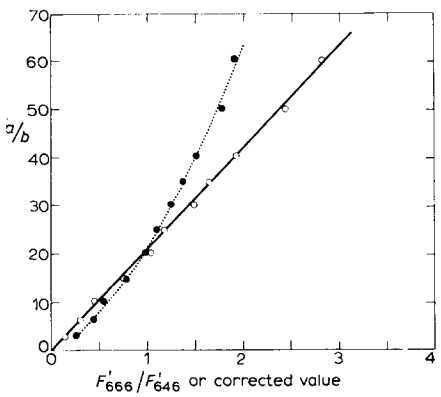


Fig. 2. Fluorescence emission spectra of prepared mixtures of chl *a* and chl *b* in diethyl ether at 20°. Excitation at 453 nm. ———, chl *a*/chl *b* ratio = 50. ·····, chl *a*/chl *b* ratio = 25; absorbance 0.1 cm^{-1} at 428 nm. – – –, chl *a*/chl *b* ratio = 10; absorbance 0.05 cm^{-1} at 428 nm.

Fig. 3. Calibration data for mixtures of chl *a* and chl *b* in diethyl ether at 20°. Chl *a*/chl *b* value as prepared from pure chlorophylls. Excitation at 453 nm to give values F'_{666} and F'_{646} from curves of Fig. 2. ●---●, ratio F'_{666}/F'_{646}; ○—○, corrected value (see text and Eqn. 8).

at 666 nm (the maximum of chl *a*) and 646 nm (the maximum for chl *b*). The broken line in Fig. 3 shows a plot of chl *a*/chl *b* ratio *versus* the ratio of the fluorescence amplitudes at 666 nm and 646 nm (F'_{666}/F'_{646}). The plot is non-linear because the fluorescence emission at 666 nm contains a small component due to chl *b* and the emission at 646 nm a component due to chl *a*. The contributions of chl *b* to the fluorescence emission at 666 nm and chl *a* to the emission at 646 nm were determined from fluorescence spectra of chl *a* and chl *b*.

For pure chl *a* and chl *b*, excited at a fixed wavelength and small bandwidth:

$$F_a = k_1 c_a \tag{1}$$

$$F_b = k_2 c_b \tag{2}$$

and

$$\frac{F_a}{F_b} = \frac{k_1 c_a}{k_2 c_b} \tag{3}$$

where F_a and F_b are the peak fluorescence amplitudes of chl *a* and chl *b*, c_a and c_b are their respective concentrations and k_1 and k_2 are constants. If there were no overlap of the fluorescence emission spectra of chl *a* and chl *b*, then excitation of a mixture of chl *a* and chl *b* at a single wavelength would give

$$F_a/F_b = k_1c_a/k_2c_b$$

and a direct linear relation would hold between the ratio of the fluorescence amplitudes and the chl *a*/chl *b* ratio.

In practice, however, there is overlap of the fluorescence emission spectra. If F'_a is the fluorescence emission amplitude of a mixture of chl *a* and chl *b* at the peak wavelength of chl *a*, and F'_b is the fluorescence amplitude at the peak wavelength of chl *b* then we may write:

$$F'_a = k_1c_a + K_bk_2c_b \tag{4}$$

$$F'_b = k_2c_b + K_ak_1c_a \tag{5}$$

where K_b is the ratio of the fluorescence emission amplitude of chl *b* at the peak wavelength of chl *a* compared with the peak wavelength of chl *b* and K_a is the ratio of the fluorescence emission amplitude of chl *a* at the peak wavelength of chl *b*, compared with the peak wavelength of chl *a*. From Eqns. 4 and 5,

$$\frac{F'_a}{F'_b} = \frac{k_1c_a/c_b + K_bk_2}{k_2 + K_ak_1c_a/c_b} \tag{6}$$

Eqn. 6 may be rearranged to give

$$c_a/c_b = \frac{k_2}{k_1}\cdot\frac{F'_a/F'_b - K_b}{1 - K_aF'_a/F'_b} \tag{7}$$

K_a and K_b are determined from the individual fluorescence emission spectra of pure chl *a* and chl *b*, respectively. Thus in ether at room temperature $K_b = F_{666}/F_{646}$ (for chl *b*) and $K_a = F_{646}/F_{666}$ (for chl *a*). The values obtained were 0.142 for K_b and 0.185 for K_a.

$$\frac{c_a}{c_b} = \frac{k_2}{k_1}\cdot\frac{F'_{666}/F'_{646} - 0.142}{1 - 0.185\ F'_{666}/F'_{646}} \tag{8}$$

A plot of c_a/c_b against $(F'_{666}/F'_{646} - 0.142)/(1 - 0.185\ F'_{666}/F'_{646})$ is shown by the solid line in Fig. 3. It is linear with a slope of 21.

The chl *a*/chl *b* ratio of an unknown mixture is determined from its F'_{666}/F'_{646} ratio, either by direct reading from the calibration graph (broken line in Fig. 3) or by use of Eqn. 8, with $k_2/k_1 = 21$.

Determination of chl a/chl b ratios in ethanol extracts at liquid nitrogen temperature

It is convenient to be able to determine chl *a*/chl *b* ratios directly on extracts of leaves, without transferring the pigments to diethyl ether. Polar solvents such as acetone, methanol or ethanol are used to extract the chlorophylls from plant tissue, but as mentioned earlier the fluorescence bands both excitation and emission are broader in these solvents than in diethyl ether. The overlap of the bands due to chl *a* and chl *b* are too great to permit accurate determination of chl *a*/chl *b* ratios, particularly at high ratios.

We have found, however, that sharp fluorescence bands are obtained for chl *a* and chl *b*, dissolved in ethanol, if the temperature is lowered to 77°K (Fig. 4). The overlap of the emission bands of chl *a* and chl *b* is considerably less at 77°K than at room temperature. This is due not only to the sharpening of the emission bands

at low temperature, but to the relative shift in the peak position of chl *a*. On lowering the temperature to 77° K from room temperature, the fluorescence emission peak of chl *a* shifts from 672 to 678 nm, whereas the emission peak of chl *b* remains at 658 nm.

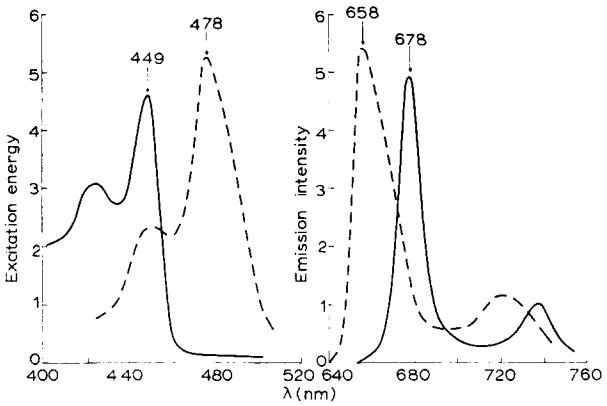


Fig. 4. Fluorescence excitation and emission spectra of chl *a* and chl *b* in ethanol at 77° K. ———, chl *a*; absorbance 0.05 at 449 nm at 77° K. Emission for excitation at 449 nm, excitation for emission at 678 nm. — — —, chl *b*; absorbance 0.05 at 478 nm at 77° K. Emission for excitation at 478 nm, excitation for emission at 658 nm. The electrical gain was × 3 to record the chl *b* spectra.

Measurements at 77° K give up to a 10-fold increase in sensitivity over measurements at room temperature. The excitation spectrum of chl *b* in ethanol at 77° K shows some splitting, with a peak at 478 nm and a shoulder at 482 nm. Chl *a* shows an excitation maximum at 449 nm, and very low excitation at 478 nm (Fig. 4). We determined the fluorescence quantum efficiencies of chl *a* and chl *b* in ethanol at liquid-nitrogen temperature, and obtained values of 0.265 and 0.10, respectively.

The method of determining chl *a*/chl *b* ratios in ethanol at 77° K was similar to that described for determining ratios in ether at room temperature. Excitation was at the maximum for chl *b* (478 nm), the fluorescence emission spectrum was recorded, and the fluorescence amplitudes read at 678 nm, the fluorescence emission maximum of chl *a* and 658 nm, the emission maximum of chl *b*. Representative spectra are shown in Fig. 5, and a calibration curve by the broken line in Fig. 6. The method is accurate for the determination of chl *a*/chl *b* ratios in the range from 6 to over 100, and may be extended with reduced accuracy to 1000/1.

In ethanol at 77° K Eqn. 7 reduces to

$$c_a/c_b = k'_2/k'_1(F'_{678}/F'_{658} - 0.20)/(1 - 0.079\ F'_{678}/F'_{658}) \quad (9)$$

A plot of c_a/c_b against $(F'_{687}/F'_{658} - 0.20)/(1 - 0.079\ F'_{678}/F'_{658})$ is a straight line with a slope of 32.5 (solid line in Fig. 6). As indicated for the ether method, the chl *a*/chl *b* ratio of an unknown mixture either may be read directly from the calibration curve or calculated from the F'_{678}/F'_{658} ratio by means of Eqn. 9, with $k'_2/k'_1 = 32.5$.

To test whether carotenoids would interfere with the determination of chl *a*/chl *b* ratios, known amounts of chl *a* were added to aliquots of a spinach leaf extract in ethanol and chl *a*/chl *b* ratios estimated. A leaf extract has a (chl *a* + chl *b*)/carotenoid

ratio of 6.3, and its contents of chl *a* and chl *b* were determined by spectrophotometry as described in MATERIALS AND METHODS. The solid rectangles in Fig. 6 show the relationship between chl *a*/chl *b* ratio and the F'_{678}/F'_{658} ratio, in the presence of carotenoids. There is excellent agreement between the calibration curves, in the presence and absence of carotenoids, showing that the carotenoids did not interfere with the determination of chl *a*/chl *b* ratios.

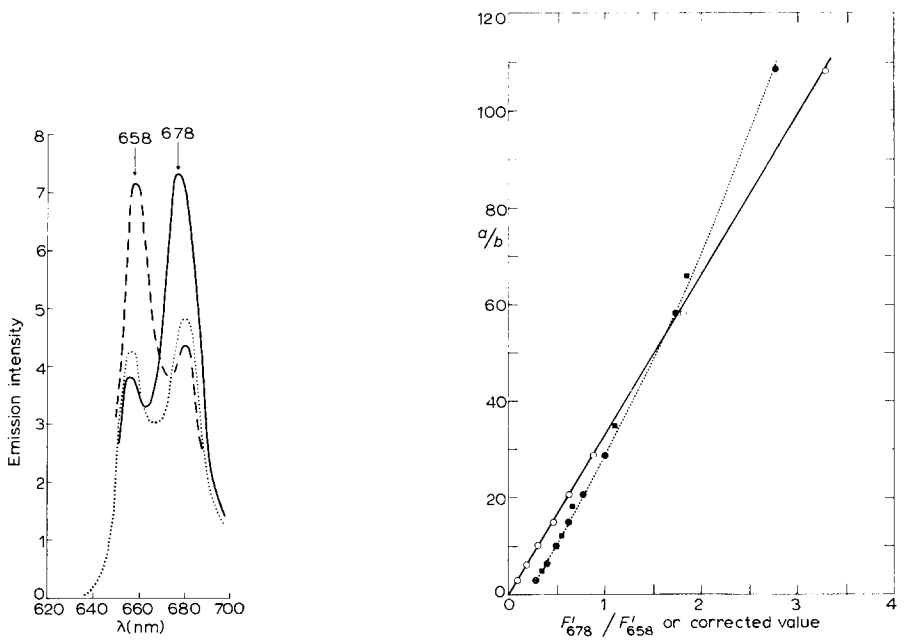


Fig. 5. Fluorescence emission spectra of prepared mixtures of chl *a* and chl *b* in ethanol at 77° K. ———, chl *a*/chl *b* ratio = 66; ·····, chl *a*/chl *b* ratio = 35; — — —, chl *a*/chl *b* ratio = 13.5. Absorbance <0.1 cm^{-1} at 429 nm at 20°. Excitation at 478 nm.

Fig. 6. Calibration data for mixtures of chl *a* and chl *b* in ethanol at 77° K. Chl *a*/chl *b* value as prepared from pure chlorophylls. Excitation at 478 nm to give values of F'_{678} and F'_{658} from curves of Fig. 5; ●---●, ratio F'_{678}/F'_{658}; ○—○, corrected value. (See text and Eqn. 9.) Points marked (■) determined in the presence of carotenoids (see text).

We measured the fluorescence emission and excitation spectra of pheophytin *a* and pheophytin *b* to determine whether small amounts of these pigments would interfere with our methods for estimating chl *a*/chl *b* ratio. The wavelengths of the excitation and emission maxima of pheophytin *a* and pheophytin *b* in ether at room temperature and ethanol at 77° K are shown in Table I. Although the emission bands of the pheophytin *a* and pheophytin *b* overlap the corresponding bands of chl *a* and chl *b*, the excitation maxima are displaced considerably to shorter wavelengths. Excitation of pheophytin *a* or pheophytin *b* at the excitation wavelength maximum of chl *b* is negligible, and small amounts of either pigment do not interfere with our determination of chl *a*/chl *b*.

During the early stages of greening of dark-grown plants, some protochlorophyll(ide) is present but it did not interfere with the determination of chl *a*/chl *b* ratios.

In ether at room temperature, protochlorophyll(ide) shows an excitation maximum at 432 nm and an emission maximum at 623 nm. In ethanol at 77° K, the excitation and emission maxima are at 442 and 630 nm (Table I). The excitation spectra for protochlorophyll(ide) in the Soret band region are very sharp and there is poor excitation of protochlorophyll(ide) fluorescence at the wavelength of maximum excitation for chl *b*.

Our methods of determining chl *a*/chl *b* ratios either in ether or ethanol, were designed to measure ratios which could not be determined satisfactorily by spectrophotometry. The spectrophotometric method is suitable for estimating chl *a*/chl *b* of

TABLE I

FLUORESCENCE EXCITATION AND EMISSION MAXIMA

The pigments were purified as described in MATERIALS AND METHODS. Fluorescence emission and excitation spectra were measured in diethyl ether at 20° and ethanol at 77° K.

Pigment	λ_{max} (*nm*)			
	Ether at 20° C		*Ethanol at 77° K*	
	Excitation	*Emission*	*Excitation*	*Emission*
Chl *a*	428	666	449	678
Chl *b*	453	646	478	658
Pheophytin *a*	408	672	415	670
Pheophytin *b*	434	658	440	655
Protochlorophyll(ide)	432	623	442	630

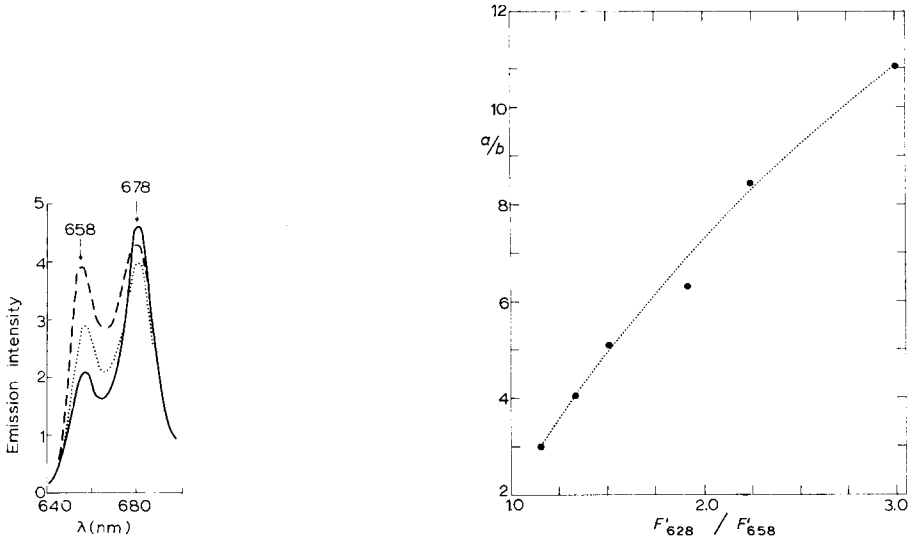


Fig. 7. Fluorescence emission spectra for mixtures of chl *a* and chl *b* in ethanol at 77° K. ———, chl *a*/chl *b* ratio = 8.5; · · · · · ·, chl *a*/chl *b* ratio = 5.1; — — —, chl *a*/chl *b* ratio = 3.0. Absorbance <0.1 cm^{-1} at 429 nm at 20°. Excitation at 465 nm.

Fig. 8. Calibration data for mixtures of chl *a* and chl *b* in ethanol at 77° K. Chl *a*/chl *b* value in the range 3–11 as prepared from pure chlorophylls. Excitation at 465 nm, to give values of F'_{678} and F'_{658} from curves of Fig. 7.

up to 6. On the other hand, the accuracy of the spectrofluorimetric methods declines as the chl *a*/chl *b* ratio falls below 6, because the fluorescence emission from chl *b* makes a large contribution to the fluorescence amplitude at the wavelength of the chl *a* peak. To reduce the contribution of chl *b*, mixtures with relatively low ratios of chl *a*/chl *b* were excited at a wavelength which was intermediate between the excitation maxima of chl *a* and chl *b*. In ethanol at 77° K, the excitation wavelength was 465 nm. Representative emission spectra are shown in Fig. 7. A calibration curve in ethanol at 77° K in which chl *a*/chl *b* ratio is plotted against F'_{678}/F'_{658} is shown in Fig. 8.

DISCUSSION

The present methods for measuring chl *a*/chl *b* ratios were developed for the analyses of mixtures where the proportion of chl *b* is low compared with chl *a* (chl *a*/chl *b* > 6). The methods have proved invaluable for our work on the formation of chl *b* during chloroplast development[11], and for studies on a pea mutant deficient in chl *b*[12]. Previously, spectrofluorimetry of leaf extracts at liquid nitrogen temperature was used to demonstrate the absence of chl *b* from a barley mutant[13].

In the commonly used spectrophotometric methods for determining chl *a*/chl *b* ratios, absorbance readings are made at two wavelengths. Such methods are not suitable for the accurate estimation of chl *a*/chl *b* ratios in excess of 6, because any error in the wavelength setting at the wavelength position of the chl *b* maximum (645 nm in 80% acetone) introduces very appreciable errors in the estimated chl *a*/chl *b* ratios.

Spectrofluorimetry is an extremely sensitive method for the determination of chlorophylls, particularly if measurements are made at 77° K. In the present work chl *a*/chl *b* ratios were determined on as little as 0.5–1 μg of chlorophyll, but satisfactory results can be obtained with 0.2 μg of chlorophyll. The ethanol method at liquid-nitrogen temperature is suitable for measuring chl *a*/chl *b* ratios of up to 100 with an accuracy of better than ± 5%. The ether method at room temperature covers the range up to a chl *a*/chl *b* ratio of 60, with an accuracy of ± 5%.

The present methods are also suitable for determining absolute amounts of chl *a* and chl *b*, if the spectrofluorimeter is calibrated with known amounts of chl *a* and chl *b*. Fluorescein is a convenient standard for periodic standardization of the instrument[8,10].

The accuracy of the methods described in this paper is dependent on the resolution of the emission and excitation monochromators. For our measurements we used an excitation half bandwidth of ± 1.5 nm and an emission half bandwidth of ± 1.0 nm. The methods, however, are suitable for use with spectrofluorimeters of lower resolution, but the accuracy of the chl *a*/chl *b* ratios will not be as high as attained here. The fluorescence spectra recorded by our instrument are automatically corrected for variations in the photomultiplier and monochromator responses, but the methods are applicable to uncorrected spectrofluorimeters.

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