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The Spectral Determination of Chlorophylls *a* and *b*, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution*

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Summary

Specific absorption (α) coefficients for individual carotenoids and chlorophylls *a* and *b*, as well as the $E^{1\%}_{1cm}$ values for combined carotenoids, have been (re)estimated using 6 solvents (80% acetone, chloroform, diethyl ether, dimethyl formamide, dimethyl sulphoxide, and methanol) using 2 different types of spectrophotometer (0.1–0.5 nm and 1–4 nm band pass resolution). From these values, 2 sets of equations to calculate concentrations of chlorophyll *a* (C_a), chlorophyll *b* (C_b) and total carotenoids (C_{x+c}) in $\mu g\,mL^{-1}$ for the different instrument types were freshly derived or confirmed from earlier publications. These were then tested with 3 different types of spectrophotometers (the two variable types plus 2 nm fixed resolution diode array) using equal aliquots of a mixed extract in the 6 different solvents. These showed that the concentrations and ratios derived by the 2 sets of equations were comparable when used with their own type of spectrophotometer but less so if the inappropriate equations were used. Measurements taken with the diode array spectrophotometer, however, did not give accurate concentrations or ratios of chlorophylls and carotenoids.

Key words: 80% Acetone, Chloroform, Diethyl-ether, Dimethyl-formamide, Dimethyl-sulphoxide, Methanol, Xanthophylls.

Introduction

The first practical demonstration of chromatography and confirmation that there were two forms of chlorophyll was by Mikhail Tswett, at a meeting of Warsaw Society for Natural Science in 1903, when he separated leaf pigments using a chalk column although Day (1897) had previously separated hydrocarbons in petroleum using finely-divided clay and limestone. However, Tswett (1906) is normal awarded all the credit having had the advantage of using attractive green, orange and yellow-coloured pigments from which he derived the word ‹chromatography› or colour-writing. In fact, over 40 years earlier, spectrophotometric measurements by Stokes (1864) had already suggested that green plants probably contained more than one type of chlorophyll as well as different yellow pigments. Meanwhile, Sorby (1873) had started to partition chlorophylls between immissible solvents such as carbon disulphide or benzene and aqueous alcohol and was the first to see the blue-green colour of chlorophyll *a* which he called ‹blue chlorophyll› when he extracted *Fucus* or *Laminaria*. These algae, he found lacked the ‹grass-green› of chlorophyll *b* which he called ‹yellow chlorophyll› but did contain a third type of chlorophyll which he called ‹chlorofuscine›, now known as the chlorophylls c_1 and c_2. Sorby (1873) also partially separated many orange-yellow compounds which he called ‹xanthophylls›, realized that chlorophyll *a* was formed before chlorophyll *b* during the regreening of etiolated leaves, described the marked differences in pigment composition between sun

* Dedicated to Prof. H. K. Lichtenthaler on the occasion of his 60th birthday.

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and shade leaves, appreciated the role of light in photo-oxidation, and started the study of chemical taxonomy which he called ‹comparative chromatology›.

Well over a hundred years later from some of these events, it is still frequent practice to measure the amounts of individual chlorophylls (and total carotenoids) spectrophotometrically in mixed extracts rather than separate them by HPLC which is both costly in time and materials and often difficult to correct for measurements at a single wavelength and for losses during the extract manipulations. The much-quoted equations of Arnon (1949) to determine individual levels of chlorophyll *a* and *b* in 80 % (v/v) acetone in water are still used by many researchers despite the fact that they are inaccurate and that particular solvent mix has many disadvantages. Arnon's equations were originally derived from the specific absorption (or extinction) coefficients (α) of Mackinney (1941) and always underestimate the ratio of chlorophyll *a* to *b* (Chl *a/b*). There are many reasons for this inaccuracy including the poor resolution of the spectrophotometers of the 1940s but the main problem is the solvent itself, 80 % acetone in water. Most importantly, it does not extract all the major pigments completely (Lichtenthaler, 1987). Some of the less polar chlorophyll *a* and β-carotene are always left behind especially in fibrous and other difficult plant tissues. Nevertheless, efforts to improve the 80 % acetone equations have been made (Vernon, 1960; Ziegler and Egle, 1965; Lichtenthaler and Wellburn, 1983; Inskeep and Bloom, 1985; Lichtenthaler, 1987; Porra, Thompson, and Kriedemann, 1989; Barnes et al., 1992), including a graph to recorrect old Arnon (1949) – derived Chl *a/b* ratios (Porra et al., 1989). Even so, the basic problems with the extractant mix remain (i.e. incomplete extraction and variable evaporation of acetone during maceration, centrifugation, filtration, and spectrophotometric reading). Changes in acetone to water ratios are important because the specific absorption coefficients of chlorophyll *a* and *b* vary with acetone content (i.e. those for 79 % are slightly different from those of 80 %) but to a lesser extent with pH or mineral content. For example, accurate equations derived with 80 % acetone in distilled water (Lichtenthaler, 1987) are only slightly different to those for 80 % acetone in buffered water (Porra et al., 1989).

The use of alternative solvents to 80 % acetone for spectrophotometric determinations of pigments has been prompted for a variety of reasons. Firstly, accurate α coefficients can only be determined in a reliable reference solution. By convention, this has devolved to diethyl-ether but a better case could have been made for methanol. As Lichtenthaler (1987) has shown, α coefficients derived in ultra-pure or water-free or water-saturated diethyl-ether are different and, moreover, diethyl-ether evaporates readily during volumetric and spectrophotometric operations despite the greatest of care. Nevertheless, there is general agreement that the α coefficients of chlorophyll *a* and *b* derived by Smith and Benitez (1955) for diethyl-ether (ultra-pure) are accurate and all other α coefficients in other solvents should be derived relative to these values in the manner fully described by Porra et al. (1989).

Second, each type of plant tissue often suits one type of extractant better than another. Some of these are inefficient (e.g. 80 % acetone), some are highly efficient but still require maceration or grinding and centrifugation or filtration, and some will allow immersion of the intact tissue in water-missible solvents when the pigments will eventually come out, often after shaking. Dimethyl-formamide and dimethyl-sulphoxide are examples of this last type of solvent and simultaneous equations for determination of chlorophylls in both have gradually been refined (dimethyl-foramide – Moran and Porath, 1980; Moran, 1982; Inskeep and Bloom, 1985; Porra et al., 1989; dimethyl-sulphoxide – Barnes et al., 1992) although, with the exception of Porra et al. (1989), none of these have used the approved procedure described in the paragraph above. The others used comparisons to 80 % acetone and commercial sources of chlorophyll *a* and *b* which are still contaminated and partially degraded rather than freshly-separated chlorophyll *a* and chlorophyll *b* from an approved procedure. Of the two solvents, dimethyl-formamide appears to have advantages over dimethyl-sulphoxide for most plant tissues. The latter is solid up to 18 °C and recrystallizes slowly but has advantages for delicate tissues such as lichens although care has to be taken when heating during extraction (Barnes et al., 1992).

Sometimes, however, maceration or grinding and filtration or centrifugation has to be used for tough plant material (e.g. certain conifer needles). In the past, the procedure of Quail, Gallager and Wellburn (1976) using a 1 : 3 mixture (v/v) of methanol : chloroform has been found to remove all trace of ‹green-ness› from macerated plant tissues and, upon centrifugation, all the pigments pass into the lower chloroform layer which can then be washed and the concentrations of chlorophyll *a* and *b* determined by spectrophotometry (Wolfenden et al., 1988). The simultaneous equations used were derived by cross-comparison of α coefficients in chloroform with those in ultra-pure diethyl-ether in the approved manner (see above). These equations, like those of Lichtenthaler and Wellburn (1983) and Lichtenthaler (1987), also have an additional feature in that with one extra spectrophotometric determination they also permit the determination of total carotenoid content (C_{x+c}) in the same solution as the chlorophylls using $E^{1\%}_{1cm}$ values in a procedure described fully by Lichtenthaler (1987).

Finally, a renewed warning about the different types of spectrophotometers which may be used. Some modern microprocessor-controlled spectrophotometers have a spectral resolution of 0.1 – 0.5 nm over the visible spectrum and these were the type of instrument used by Lichtenthaler (1987) and Porra et al. (1989) to derive their equations. Earlier instruments capable of 1 – 4 nm resolution were used up to and including the determinations of Lichtenthaler and Wellburn (1983). However, it is often the case that routine laboratory instruments more similar to the latter type are still used. Recently, instrument manufacturers have tended to emphasize speed of scanning over resolution. Often this means the use of diode-array spectrophotometers and for technical reasons the resolution of such machines is still 2 nm. All of this has implications for spectral measurements of mixed solutions containing both chlorophyll *a* and *b* plus total carotenoids because all spectral peaks are much sharper at smaller resolutions. This means that, although the peak heights at wavelength (λ) maxima of chlorophylls *a* or *b* will be similar on different types of instrument, because all α coefficients are

back-corrected to those of Smith and Benetiez (1955) in diethyl ether, the value of chlorophyll *b* under the λ_{max} of chlorophyll *a* and, more significantly, that of chlorophyll *a* under the λ_{max} of chlorophyll *b* will be higher using a spectrophotometer with a resolution of 1–4 nm than one with 0.1–0.5 nm. In other words, the equations of Lichtenthaler (1987) and Porra et al. (1989) cannot be used to achieve greater accuracy than some of the earlier equations (e.g. Lichtenthaler and Wellburn, 1983) on an instrument which has a resolution of 1–4 nm. On the contrary, by using these later equations on such machines, further inaccuracy is possibly introduced. If doubt remains, valid equations should be recalculated for each new instrument according to the methods described by Lichtenthaler (1987) and Porra et al. (1989).

The same principles apply to the determination of the total carotenoids, the $E^{1\%}_{1cm}$ values remain the same but the spread of the Soret bands of the chlorophylls, especially chlorophyll *b*, into the 470–480 nm measurement region in 1–4 nm instruments requires use of the total carotenoid equations derived for 1–4 nm resolution instruments rather than those calculated from 0.1–0.5 nm machines.

This introduction sets the scene for this paper which describes the parallel use of three spectrophotometers of the types described (0.1–0.5 nm variable, 1–4 nm variable and diode-array 2 nm fixed resolution) to (re)determine α values for chlorophyll *a* and chlorophyll *b*, as well as appropriate $E^{1\%}_{1cm}$ values for total carotenoids, using a range of 6 solvents. From these it was possible to (re)derive appropriate simultaneous equations for different types of spectrophotometers adding equations to those for dimethyl-formamide and dimethyl sulphoxide for the determination of total carotenoids (C_{x+c}) where none existed before.

Material and Methods

Purification of chlorophylls and carotenoids

A bulk extract of total lipids from macerated tree mallow (*Lavatera olbia* L.) leaves was made in semi-darkness using the methanol : chloroform (1 : 3 v/v) procedure of Quail et al. (1976). The washed chloroform layer was then taken to dryness under reduced pressure, a small volume of *n*-hexane was added and the extract was stored under O_2-free N_2 at −20 °C until required. Immediately before the spectrophotometric measurements, aliquots of this extract (0.75 mL) were chromatographed as lines on pre-coated silica gel 20 × 20 cm thin-layer chromatography (TLC) plates (Type 60 without indicator, Merck) using 20 % (v/v) ethyl acetate in diethyl-ether. The various pigments separated rapidly (< 20 min) with this solvent mix and, once removed from the tank, the TLC plate dried quickly. Specific bands were then ploughed up with a small screwdriver (3 mm blade width), not a spatula, while the TLC plate was flat on the bench. The fragments of the silica gel were then collected by banging the edge of the TLC plate vertically onto glazed paper, before eluting them with acetone through a cone of Whatman No. 4 filter paper while crushing the fragments of gel with the screwdriver blade. This procedure avoids the disadvantage of pre-coated plates where the fragments are apt to fly in all directions yet retains the great advantage of reproducibility. Equal aliquots of the acetone extracts were then pipetted into 10 or 20 mL volumetric flasks, taken to dryness under a stream of O_2-free N_2, and then made up to volume with the various solvents (i.e. 80 % acetone in distilled water, dimethyl-formamide, and dimethyl-sulphoxide, Analar-grade, BDH/Merck; chloroform, diethyl-ether and methanol, Aristar-grade) before spectrophotometry using quartz glass cells (10 mm path length, 3 mL volume). All these procedures were carried out in semi-darkness.

Spectrophotometry

Four different spectrophotometers were used in this study, all of which were checked with a holmium filter before use and, where necessary, recalibrated. The high resolution instrument was a Uvikon 941 Plus (Kontron [U.K.] Ltd.) capable of 0.2 nm resolution over the range 350–550 nm or 0.5 nm resolution from 550–750 nm in scan mode, and 0.1 nm in fixed λ mode. This latter mode was used to pin-point the λ maxima once a scan had been done. One of the two low resolution spectrophotometers was the Pye Unicam SP8000 that had been used previously by Lichtenthaler and Wellburn (1983) and the other was a Pye Unicam SP30 with very similar optics (i.e. variable 1–4 nm resolution from 400–700 nm). The diode array spectrophotometer was a Hewlett Packard HP8452A with a fixed resolution of 2 nm.

Spectra of purified chlorophyll *a* and *b*, β-carotene, lutein, violaxanthin, neoxanthin, and the combined carotenoids were determined on the Uvikon 941 Plus and compared to those of chlorophylls *a* and *b*, and combined carotenoids on the Pye Unicam SP8000 using all 6 solvents. Peak maxima were then established (as above) and the specific absorption coefficients (α), or the $E^{1\%}_{1cm}$ values for the combined carotenoids at either 470 or 480 nm, were recalculated and compared to those already published (Lichtenthaler and Wellburn, 1983; Lichtenthaler, 1987; Porra et al., 1989; Barnes et al., 1992). Appropriate simultaneous equations were then calculated and then checked repeatedly using 50 µL aliquots of the tree mallow lipid extract made up to 20 mL in the various solvents using the Uvikon 941 Plus, the Pye Unicam SP30, and the Hewlett Pakkard HP8452A.

Results

All the λ_{max} of the chlorophylls at high resolution in diethyl-ether, methanol and 80 % acetone were found to be identical to those already published by Lichtenthaler (1987) but those for chloroform, dimethyl-formamide and dimethyl-sulphoxide are shown in Table 1. The long wavelength λ_{max} are slightly higher than those quoted by Barnes et al. (1992) for dimethyl-sulphoxide but the same as those for dimethyl-formamide given by Porra et al. (1989). Table 2 also gives the absorption maxima of the 4 principal carotenoids

Table 1: Absorption maxima (λ, nm) of the two chlorophylls in chloroform, dimethyl-formamide, and dimethyl-sulphoxide.

Pigment	Chloroform	Dimethyl-formamide	Dimethyl-sulphoxide
Chlorophyll *a*	**665.6**	**663.8**	**665.1**
	618.1	616.2	617.6
	583.2	581.2	584.1
	431.5	**431.5**	**432.6**
	415.5	412.4	414.4
Chlorophyll *b*	**647.6**	**646.8**	**649.1**
	598.1	600.1	602.0
	460.3	**459.5**	**461.0**
	435.2	435.2	437.0

Major peaks in bold typeface.

Table 2: Absorption maxima (λ, nm) of the major primary carotenoids in chloroform, dimethyl-formamide, and dimethyl-sulphoxide.

Carotenoid	Chloroform	Dimethyl-formamide	Dimethyl-sulphoxide
β-Carotene	490.4	484.6	495.8
	479.8	**459.4**	**477.8**
Lutein	484.0	484.6	491.0
	456.0	**455.6**	**461.0**
	431.6	sh	sh
Violaxanthin	479.8	479.4	484.6
	449.8	**450.6**	**454.8**
	424.8	425.8	429.6
Neoxanthin	474.8	474.4	481.8
	445.8	**445.4**	**453.4**
	421.0	421.8	sh

Major peaks in bold, sh = shoulder but not a peak.

in chloroform, dimethyl-formamide and dimethyl-sulphoxide. Comparison of Table 2 with Table 5 of Lichtenthaler (1987) for diethyl-ether, 80% acetone and methanol reveals that the λ_{max} of all 4 carotenoids in the first group of solvents (i.e. those in Table 2) are shifted by over 10 nm to longer wavelengths than those of the second group (Table 5 in Lichtenthaler, 1987).

The specific absorption coefficients (α) for chlorophylls *a* and *b* in chloroform, dimethyl-formamide and dimethyl-sulphoxide at the long wavelength λ_{max} of each, as well as at 470 and 480 nm, for both high and low resolution spectrophotometers are shown in Table 3. The equivalent values for diethyl-ether, 80% acetone and methanol were also determined (not shown) but were found to be virtually identical to those for high resolution instruments given by Lichtenthaler (1987, Table 2) or for diethyl-ether and methanol in low resolution spectrophotometers by Lichtenthaler and Wellburn (1983). The low resolution α values given for chloroform in Table 3 are the same as those previously used by Wolfenden et al. (1988) to calculate their equations while the α coefficients given for dimethyl-formamide at longer wavelengths (Table 3) are very similar to those quoted by Porra et al. (1989). However, those for chlorophyll *a* in dimethyl-sulphoxide are slightly higher than those given by Barnes et al. (1992) while the equivalent values for chlorophyll *b* over both resolution ranges in Table 3 straddle those given in the same paper.

Appropriate equations to calculate the concentrations (in $\mu g\,mL^{-1}$) of chlorophyll *a* (C_a), chlorophyll *b* (C_b) and total carotenoids (C_{x+c}) in all 6 solvents shown in Table 4 were either derived from the data in Table 3 or accepted from Lichtenthaler and Wellburn (1983), Lichtenthaler (1987) and Porra et al. (1989). The $E^{1\%}_{1cm}$ values for combined carotenoids in diethyl-ether, 80% acetone and methanol at 470 nm given by Lichtenthaler (1987, Table 4) were accepted and fresh $E^{1\%}_{1cm}$ values for combined carotenoids in chloroform, dimethyl-formamide and dimethyl-sulphoxide at 480 nm (2020, 2450, and 2200 respectively) were derived by cross comparison with those in diethyl-ether at 470 and 480 nm (2050 and 1604 respectively).

Table 5 shows a typical set of results from reading various solvents containing the same amounts of pigments in high and low resolution spectrophotometers (Uvikon 941 and Pye Unicam SP30). The order of reading was: 1 – Uvikon 941 Plus, 2 – Hewlett-Packard HP8452A and 3 – Pye Unicam SP30 but the calculated data from the diode array spectrophotometer (HP8452A) were so poor by comparison to the other 2 that they have been omitted for clarity. Rarely did they achieve values that were within 10% of those obtained from other types of instrument. By comparison, the other two data sets for high and low resolution instruments using the appropriate equations (Table 5) are well within 10% of each other and often better than 5%. If the inappropriate equations are used (also shown in Table 5) the gap widens again. This is most marked when the Chl *a*/*b* or especially when the total chlorophyll/carotenoid $(a+b)/(x+c)$ ratios are compared. In the latter case, these are all on the low side because the tree mallow leaves were gathered in mid-winter from a dark corner of the Institute quadrangle.

Table 3: Specific absorption coefficients (α) for chlorophylls *a* and *b* in chloroform, dimethyl-formamide and dimethyl-sulphoxide determined with spectrophotometers with high and low resolving capabilities.

Chloroform				Dimethyl-formamide				Dimethyl-sulphoxide			
0.1–0.5 nm		1–4 nm		0.1–0.5 nm		1–4 nm		0.1–0.5 nm		1–4 nm	
(nm)	α	(nm)	α	(nm)	α	(nm)	α	(nm)	α	(nm)	α
				Chlorophyll *a*							
665.6	90.42	666	94.57	**663.8**	89.78	664	90.41	**665.1**	86.73	665	88.08
646.8	18.74	648	26.37	646.8	20.37	647	19.87	649.1	22.51	649	21.33
480	1.33	480	1.42	480	1.12	480	0.89	480	1.29	480	2.14
470	1.66	470	2.84	470	1.36	470	1.23	470	1.54	470	3.11
				Chlorophyll *b*							
665.6	8.26	666	6.95	663.8	10.89	664	11.68	665.1	12.53	665	13.94
646.8	47.47	648	62.98	**646.8**	48.11	647	50.59	**649.1**	43.16	649	48.83
480	23.93	480	46.09	480	34.07	480	52.02	480	53.78	480	70.16
470	73.45	470	142.1	470	78.92	470	105.2	470	84.97	470	118.4

Table 4: Equations to determine concentrations of chlorophyll *a* (C_a) and *b* (C_b) as well as total carotenoids (C_{x+c}) in μg ml^{-1} in different solvents with spectrophotometers of different resolution capabilities. The equations for 80 % acetone diethyl-ether and methanol for 0.1–0.5 nm and 1–4 nm instruments are those of Lichtenthaler (1987) and Lichtenthaler and Wellburn (1983) respectively. The calculations of C_a and C_b in the equations for dimethyl-formamide for use with 0.1–0.5 nm machines are those of Porra et al. (1989).

Solvent	Spectrophotometer resolution range	
	0.1–0.5 nm	1–4 nm
80 % Acetone	$C_a = 12.25A_{663.2} - 2.79A_{646.8}$ $C_b = 21.5A_{646.8} - 5.1A_{663.2}$ $C_{x+c} = (1000A_{470} - 1.82C_a - 85.02C_b)/198$	$C_a = 12.21A_{663} - 2.81A_{646}$ $C_b = 20.13A_{646} - 5.03A_{663}$ $C_{x+c} = (1000A_{470} - 3.27C_a - 104C_b)/198$
Chloroform	$C_a = 11.47A_{665.6} - 2A_{647.6}$ $C_b = 21.85A_{647.6} - 4.53A_{665.6}$ $C_{x+c} = (1000A_{480} - 1.33C_a - 23.93C_b)/202$	$C_a = 10.91A_{666} - 1.2A_{648}$ $C_b = 16.38A_{648} - 4.57A_{666}$ $C_{x+c} = (1000A_{480} - 1.42C_a - 46.09C_b)/202$
Diethyl-ether	$C_a = 10.05A_{660.6} - 0.97A_{642.2}$ $C_b = 16.36A_{642.2} - 2.43A_{660.6}$ $C_{x+c} = (1000A_{470} - 1.43C_a - 35.87C_b)/205$	$C_a = 10.05A_{662} - 0.77A_{644}$ $C_b = 16.37A_{644} - 3.14A_{662}$ $C_{x+c} = (1000A_{470} - 1.28C_a - 56.7C_b)/205$
Dimethyl-formamide	$C_a = 12A_{663.8} - 3.11A_{646.8}$ $C_b = 20.78A_{646.8} - 4.88A_{663.8}$ $C_{x+c} = (1000A_{480} - 1.12C_a - 34.07C_b)/245$	$C_a = 11.65A_{664} - 2.69A_{647}$ $C_b = 20.81A_{647} - 4.53A_{664}$ $C_{x+c} = (1000A_{480} - 0.89C_a - 52.02C_b)/245$
Dimethyl-sulphoxide	$C_a = 12.47A_{665.1} - 3.62A_{649.1}$ $C_b = 25.06A_{649.1} - 6.5A_{665.1}$ $C_{x+c} = (1000A_{480} - 1.29C_a - 53.78C_b)/220$	$C_a = 12.19A_{665} - 3.45A_{649}$ $C_b = 21.99A_{649} - 5.32A_{665}$ $C_{x+c} = (1000A_{480} - 2.14C_a - 70.16C_b)/220$
Methanol	$C_a = 16.72A_{665.2} - 9.16A_{652.4}$ $C_b = 34.09A_{652.4} - 15.28A_{665.2}$ $C_{x+c} = (1000A_{470} - 1.63C_a - 104.96C_b)/221$	$C_a = 15.65A_{666} - 7.34A_{653}$ $C_b = 27.05A_{653} - 11.21A_{666}$ $C_{x+c} = (1000A_{470} - 2.86C_a - 129.2C_b)/221$

Table 5: Optical densities, amounts (μg ml^{-1}), and ratios of chlorophylls and carotenoids in different solvents using similar extracts from tree mallow (collected in mid-winter) in high (**in bold**) and low resolution spectrophotometers. The values shown in italics below each were achieved using the opposite (i.e. inappropriate) equations.

Solvent	A_{66X}	A_{64Y}	A_{47Z0}	Chl *a*	Chl *b*	*a*/*b* Ratio	*Total* $x + c$	$\frac{(a+b)}{(x+c)}$
80 % Acetone	**0.432**	**0.195**	**0.513**	**94.96**	**39.79**	**2.39**	**33.86**	**3.98**
				94.53	***33.05***	***2.70***	***31.85***	***4.07***
	0.445	0.195	0.530	97.71	33.74	2.90	34.20	3.84
				98.08	*38.89*	*2.55*	*35.94*	*3.81*
Chloroform	**0.434**	**0.173**	**0.449**	**92.64**	**36.28**	**2.55**	**39.55**	**3.26**
				90.55	***17.01***	***5.32***	***39.94***	***2.69***
	0.430	0.210	0.480	88.79	29.49	3.01	40.17	2.94
				90.24	*52.81*	*1.71*	*40.67*	*3.52*
Diethyl-ether	**0.462**	**0.175**	**0.440**	**89.47**	**34.81**	**2.57**	**36.04**	**3.43**
				90.17	***28.28***	***3.19***	***34.04***	***3.48***
	0.452	0.177	0.455	88.13	29.56	2.98	35.54	3.30
				87.42	*35.95*	*2.43*	*37.49*	*3.29*
Dimethyl-formamide	**0.441**	**0.183**	**0.503**	**94.46**	**33.01**	**2.86**	**36.04**	**3.54**
				92.91	***36.21***	***2.56***	***33.04***	***3.91***
	0.460	0.186	0.570	97.23	35.32	2.75	38.97	3.43
				98.89	*31.99*	*3.09*	*41.63*	*3.14*
Dimethyl-sulphoxide	**0.403**	**0.175**	**0.510**	**87.84**	**35.32**	**2.49**	**37.21**	**3.31**
				86.18	***34.09***	***2.53***	***34.66***	***3.47***
	0.423	0.180	0.550	90.71	34.16	2.66	38.23	3.27
				92.46	*35.23*	*2.63*	*40.85*	*3.31*
Methanol	**0.415**	**0.235**	**0.550**	**95.72**	**33.40**	**2.87**	**33.21**	**3.89**
				95.40	***34.09***	***2.80***	***28.61***	***4.53***
	0.430	0.250	0.660	97.89	38.84	2.52	35.75	3.82
				97.99	*39.04*	*2.51*	*40.46*	*3.39*

These extracts were also interesting in that they showed traces of a third chlorophyll which co-chromatographs on TLC with chlorophyll a_{GG} (Wellburn, 1970, 1976).

Discussion

This study, like many others beforehand, demonstrates that accurate equations to determine concentrations of chlorophylls *a* and *b*, as well as total carotenoids, in mixed extracts can be derived and used with confidence with a range of spectrophotometers. If, however, a large number of measurements of this type are to be made regularly then there is no real substitute for deriving the precise α coefficients for that instrument and working out the individual equations in the manner described by Lichtenthaler (1987) and Porra et al. (1989). The important point is that there must be fixed and agreed starting comparison values. Both Lichtenthaler (1987) and Porra et al. (1989), when using high resolution instruments, returned to the α coefficients of chlorophyll *a* and *b* given Smith and Benitez (1955) for diethyl-ether which were derived using a spectrophotometer with a poorer specification. Even then they produced slightly different equations because the former took a value of 101 for chlorophyll *a* while the latter used 100.9 from the same source. The problem with the α values quoted by Barnes et al. (1992) for dimethyl-sulphoxide and 80 % acetone, apart from the fact that the quoted values for chlorophyll *b* in 80 % acetone are inverted, was that they had no fixed starting point (e.g. diethyl-ether). What they have are two sets of equations for dimethyl-sulphoxide and 80 % acetone that give corresponding concentrations and ratios to each other which match their conditions but, given the other problems of 80 % acetone (see introduction), it may be difficult to reconcile these with values obtained with other solvents.

The α coefficients given for chlorophyll *a* and *b* in dimethyl-formamide given in Table 3 differ slightly from those quoted

by Porra et al. (1989) and would produce the following equations for high resolution instruments:

$$C_a = 11.74\,A_{663.8} - 2.66\,A_{646.8}$$
$$C_b = 21.91\,A_{646.8} - 4.53\,A_{663.8}$$

However, as these are so similar to those of Porra et al. (1989), those of the latter researchers were retained in Table 4. Similarly, the equations for 80% acetone for a low resolution spectrophotometer given by Lichtenthaler and Wellburn (1983) were used. However, due to an error, these do not match the quoted α coefficients in the same paper. This study was able to show that the α coefficients given in Lichtenthaler and Wellburn (1983) for 80% acetone were incorrect. They should have read 86.90 at 663 nm and 21.71 at 646 nm for chlorophyll *a* and 12.13 at 663 nm and 52.71 at 646 nm for chlorophyll *b*. The α values at 470 nm for 80% acetone, however, remain unchanged.

Spectra of combined and individual carotenoids in chloroform, dimethyl-formamide and dimethyl sulphoxide, showing a spectral shift of over 10 nm to longer wavelengths, also prompted the use of 480 nm rather than 470 nm as the C_{x+c}-measuring wavelength for these solvents. This has the double advantage of being further away from the Soret band of chlorophyll *b* and avoids measuring total carotenoids in the trough between the 2 main absorption bands of the carotenoids. This procedure therefore improves on those equations for chloroform previously given by Wolfenden et al. (1988).

This study has also demonstrated that diode array spectrophotometers, with their fixed 2 nm band pass across the spectrum, are not suitable for use with equations that have been derived by spectrophotometers with variable resolution across the spectrum. Measurements at 470–480 nm, as compared to those from 640–670 nm, were generally lower in the diode array instrument. One of the problems appears to be linked both to differences in dynamic response of the photodiodes compared to photomultipliers across the whole spectrum and that the wider band widths at longer wavelengths in variable band pass instruments permit proportionately more light to fall on the detecting system. Moreover, it is quite disconcerting to find that such an instrument always rounds up an input fixed wavelength value before measurement. This means, for example, that measurements using dimethyl-sulphoxide are made at 666 and 650 nm instead of 665 and 649 nm. It is conceivable that a third set of α coefficients and simultaneous equations could be derived for use with diode array instruments given that currently over 50% of spectrophotometers now sold are of the fixed 2 nm band pass type (Kontron (UK) Ltd., personal communication). However, most laboratories also have at least one of the other type which can be used immediately so the effort may not be worthwhile.

In summary, a range of relatively robust equations are now available for use with 6 different solvents for both high and low resolution spectrophotometers. A computer program written in Microsoft Quickbasic™ using these equations, which can be scanned using optical character recognition into any PC as an ASCII file, is provided in the Appendix.

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Appendix

```
REM **Program to calculate total carotenoids and chlorophylls a & b in different solvents**
REM **Written by Alan R.Wellburn in 1994 and based upon equations derived by**
REM **Lichtenthaler and Wellburn (1983), Lichtenthaler (1987), Porra et al (1989)**
REM **and Wellburn (1994); variable resolution version:**
REM **Variables A-C hold the spectral data - D is the dilution**
REM **E&F the amounts of chlorophyll a & b - H is the a/b Ratio**
REM **G&I are the amounts of total chlorophylls & carotenoids**
start:
   GOSUB title
PRINT "      Spectrophotometer Resolution Range (nm)"
PRINT
PRINT "              0.1 - 0.5       1 - 4"
PRINT
PRINT "80% Acetone.....1 .....7":PRINT
PRINT "Chloroform.....2 .....8":PRINT
PRINT "Diethyl-ether.....3 .....9":PRINT
PRINT "Dimethyl-formamide...4 .....10":PRINT
PRINT "Dimethyl-sulphoxide. 5 .....11":PRINT
PRINT "Methanol.....6 .....12": PRINT
INPUT "CHOOSE..... (1-12)";X: PRINT : PRINT
IF X < 1 OR X > 12 THEN  GOTO start
ON INT (X) GOTO Acet1,Chloro1,Dieth1,Dimethfor1,Dimethsul1,Meth1,
Acet2,Chloro2,Dieth2,Dimethfor2,Dimethsul2,Meth2
GOSUB results
INPUT "RUN AGAIN? (Y/N) ";A$
IF LEFT$ (A$,1) = "y" GOTO start: RETURN
IF LEFT$ (A$,1) = "n" THEN WIDTH 80:END

Acet1:
   INPUT "O.D.   663.2 ";A: PRINT:INPUT "O.D.   646.8 ";B: GOSUB carots1
   E = (A * 12.25 - B * 2.79) * D
   F = (B * 21.5 - A * 5.1) * D
   I = (((1000 * C) - (1.82 * (E/D)) - (85.02 * (F/D))) / 198) * D: GOTO calc
Chloro1:
   INPUT "O.D.   665.6 ";A: PRINT: INPUT "O.D.   647.6 ";B: GOSUB carots2
   E = (A * 11.47 - B * 2) * D
   F = (B * 21.85 - A * 4.53) * D
   I = (((1000 * C) - (1.33 * (E/ D)) - (23.93 * (F/D)))/202) * D: GOTO calc
Dieth1:
   INPUT "O.D.   660.6 ";A : PRINT: INPUT "O.D.   642.2 ";B: GOSUB carots1
   E = (A * 10.05 - B * .97) * D
   F = (B * 16.36 - A * 2.43) * D
   I = (((1000*C) - (1.43 * (E/D)) - (35.87* (F/D)))/205) * D: GOTO calc
Dimethfor1:
   INPUT "O.D.   663.8 ";A: PRINT:INPUT "O.D.   646.8 ";B: GOSUB carots2
   E = (A * 12 - B * 3.11) * D
   F = (B * 20.78 - A * 4.88) * D
   I = (((1000 * C) - (1.12 * (E/D)) - (34.07 * (F/D))) / 245) * D: GOTO calc
Dimethsul1:
   INPUT "O.D.   665.1 ";A: PRINT:INPUT "O.D.   649.1 ";B: GOSUB carots2
   E = (A * 12.47 - B * 3.62) * D
   F = (B * 25.06 - A * 6.5) * D
   I = (((1000 * C) - (1.29 * (E / D)) - (53.78*(F/D)))/220)*D: GOTO calc
Meth1:
   INPUT "O.D.   665.2 ";A: PRINT:INPUT "O.D.   652.4 ";B: GOSUB carots1
   E = (A * 16.72 - B * 9.16) * D
   F = (B * 34.09 - A * 15.28) * D
   I = (((1000 * C) - (1.63 * (E / D)) - (104.96* (F/D))) / 221) * D: GOTO calc
Acet2:
   INPUT "O.D.   663 ";A: PRINT:INPUT "O.D.   646 ";B: GOSUB carots1
   E = (A * 12.21 - B * 2.81) * D
   F = (B * 20.13 - A * 5.03) * D
   I = (((1000 * C) - (3.27 * (E /D)) - (104 * (F / D))) / 198) * D: GOTO calc
Chloro2:
   INPUT "O.D.   666 ";A: PRINT:INPUT "O.D.   648 ";B: GOSUB carots2
   E = (A * 10.91 - B * 1.2) * D
   F = (B * 16.38 - A * 4.57) * D
   I = (((1000 * C) - (1.42 * (E/D)) - (46.09 * (F/D))) / 202) * D: GOTO calc
Dieth2:
   INPUT "O.D.   662 ";A: PRINT:INPUT "O.D.   644 ";B: GOSUB carots1
   E = (A * 10.05 - B * .77) * D
   F = (B *16.37 - A * 3.14) * D
   I =  (((1000 * C) - (1.28 *  (E/D)) - (56.7* (F/D))) / 205) * D: GOTO calc
Dimethfor2:
   INPUT  "O.D.   664 ";A: PRINT:INPUT  "O.D.   647 ";B: GOSUB carots2
   E = (A * 11.65 - B * 2.69) * D
   F = (B * 20.81 - A * 4.53) * D
   I = (((1000 * C) - (0.89 * (E/D)) - (52.02 * (F/D))) / 245) * D: GOTO calc
Dimethsul2:
   INPUT "O.D.   665 ";A: PRINT:INPUT "O.D.   649 ";B: GOSUB carots2
   E = (A * 12.19 - B * 3.45) * D
   F = (B * 21.99 - A * 5.32) * D
   I = (((1000 *  C) - (2.14 * (E /  D))  -  (70.16 * (F / D)))/220)  *  D:GOTO calc
Meth2:
   INPUT  "O.D.   666 ";A: PRINT:INPUT  "O.D.   653 ";B: GOSUB carots1
   E = (A *  15.65 - B * 7.34) * D
   F = (B * 27.05 - A * 11.21) * D
   I = (((1000 * C) - (2.86 * (E / D)) - (129.2 * (F/D))) / 221) * D: GOTO calc
calc:
   G = E + F:H = E/F: GOSUB results: GOSUB printout: WIDTH 80:END
title:
   CLS
   PRINT "This program calculates micrograms per ml of chlorophyll and carotenoids "
   PRINT "using spectral data from spectrophotometers with different ranges of resolution"
   PRINT:PRINT "Data required are spectral measurements at the specified"
   PRINT "wavelengths and the total volume of solution": PRINT:RETURN
results:
   PRINT "Pigment amounts in micrograms per ml":PRINT
   PRINT "Chlorophyll a       ="; INT (E * 1000 + .5) / 1000
   PRINT "Chlorophyll b       ="; INT (F * 1000 + .5) / 1000
   PRINT "Total Chlorophylls  ="; INT (G * 1000 + .5) / 1000
   PRINT "Chlorophyll a/b Ratio ="; INT (H * 1000 + .5) / 1000
   PRINT "Total Carotenoids   ="; INT (I * 1000 + .5) / 1000
   PRINT "a+b/x+c Ratio       ="; INT (((E +  F) / I) * 1000 + .5) / 1000
   PRINT:PRINT  "66X =  ";A; "64Y = ";B;"  4Z0 = ";C;" VOL. = ";D;"mls": PRINT:RETURN
carots1:
   PRINT: INPUT "O.D. 470 = ";C: PRINT
   INPUT "TOTAL VOLUME = ";D: PRINT:RETURN
carots2:
   PRINT: INPUT "O.D. 480 = ";C: PRINT
   INPUT "TOTAL VOLUME = ";D: PRINT:RETURN
printout:
   INPUT "PRINT RESULTS ? (y/n) ";B$
   IF LEFT$ (B$,1) = "n" THEN GOTO again
   INPUT "Name Or No. Of Expt = ";C$
   LPRINT C$: PRINT: GOSUB hardcopy
again:
   INPUT "RUN AGAIN? (y/n) ";A$
   IF LEFT$ (A$,1) = "y" THEN GOTO start: RETURN
   IF LEFT$ (A$,1) = "n" THEN WIDTH 80:END
hardcopy:
   LPRINT "Pigment amounts in micrograms per ml":PRINT
   LPRINT "Chlorophyll a       = "; INT (E * 1000 + .5) / 1000
   LPRINT "Chlorophyll b       = "; INT (F * 1000 + .5) / 1000
   LPRINT "Total Chlorophylls  = "; INT (G * 1000 + .5) / 1000
   LPRINT "Chlorophyll a/b Ratio = "; INT (H * 1000 + .5) / 1000
   LPRINT "Total Carotenoids   = "; INT (I * 1000 + .5) / 1000
   LPRINT "a+b/x+c Ratio       = "; INT (((E + F)/ I) * 1000 + .5) / 1000:LPRINT
   LPRINT  "66X =  ";A;"  64Y =  ";B;"  4Z0 = ";C;"  VOL. = ";D;" mls":LPRINT: RETURN
```