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Influence of the degree of solvent impurity on the spectrophotometric determination of chlorophylls in 80 % aqueous acetone and dimethyl formamide. Application to non-abrasive extraction of leaves of *Citrus aurantium*

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Abstract

Chlorophyll (Chl) *a* and Chl *b* absorbances were determined in extracts from leaves of *Citrus aurantium* L. using the solvents diethyl ether (100 %), buffered 80 % aqueous acetone, and N,N′-dimethyl formamide (DMF) (99.83 %). The respective standard deviations (σ) and coefficients of variability (CV) were used to establish the errors obtained when using impure solvents. Buffered 80 % aqueous acetone gave an error of 2.1 % for Chl *a* and 7.1 % for Chl *b*, while DMF gave errors of 3.5 and 6.7 %, respectively. For current Chl determination we propose a non-abrasive method, which extracts 15 leaf discs (diameter 6 mm) with 10 cm^3 of buffered 80 % aqueous acetone or DMF in hermetically sealed flasks during 24 h in darkness at 4 °C.

Introduction

The Chl content and Chl *a/b* ratio has often been used to characterize the physiological state of plants and various stress effects. Since the late forties, spectrophotometric methods are used to measure the amounts of individual Chls in different solvents (Smith and Benitez 1955, Vernon 1960, Šesták 1971, Inskeep and Bloom 1985, Šesták and Zima 1987, Lichtenthaler 1987, Wellburn 1994).

The principal practical problems of such measurements in plant extracts are: (*a*) a particular type of plant tissue will often suit one type of extractant better than another (Shinano *et al.* 1996); (*b*) the passage of water from the fresh material into the extractant may give rise to impurities in the extractant and lead to irregularities in the spectral properties of these pigments.

Diethylether and buffered 80 % aqueous acetone may be inefficient extractants,

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while others may be highly efficient but involve maceration or grinding, centrifugation, filtration, or transfer. Others, such as DMF allow immersion of the intact tissue and can then simply be shaken off (Moran 1982, Porra *et al.* 1989). In this work we studied the influence of extractant impurities in the quantification of Chls by means of equations proposed by García and Nicolás (1998) and a non-abrasive method for extracting Chls from fresh plant material.

Materials and methods

Reagents and products: Diethylether, buffered 80 % aqueous acetone, and DMF were analysis reagents supplied by *Sigma-Aldrich*. The aqueous acetone contained sodium phosphate, pH 7.8 as regulator to minimize the conversion of Chls into phaeophytins. The buffer components were from *Merck*.

Plant material: Mature leaves of sour orange (*Citrus aurantium* L.) were collected as proposed by Carpena *et al.* (1972) and placed in a coolbox for transfer to the laboratory, where they were washed in non-ionic detergent, rinsed with distilled water, and dried with filter paper. The plant material was then submitted to one of the following treatments: (*1*) with the central nerve removed, the leaves were chopped finely and then triturated; (*2*) from each leaf, 5 discs of 6 mm diameter were excised. The first treatment provided a crude extract of Chls in acetone which was used to study the influence of solvent purity on the quantification of Chls obtained by non-abrasive methods, while the material of the second treatment was used to study the choice of solvent and the best conditions for determining Chls by this method.

Preparation of extracts of known purity: The acetonic crude Chl extract was transferred to diethyl ether, dried by successive steps on beds of sodium sulphate, and concentrated to the pigments's solubility limit using a vacuum rotary evaporator at low temperature and dim light. The extract was kept at -25 °C until use, when the concentration was adjusted so that 5 mm^3 diluted in 3 cm^3 of solvent had absorbances of 0.150-0.160 at wavelengths between 660 and 663 nm (A_a). Then three repetitions in 5 cm^3 capacity *Teflon*-sealed glass vials were prepared in a nitrogen atmosphere, and 3 cm^3 of diethyl ether, buffered 80 % aqueous acetone, or DMF were injected into each vial by means of a syringe. Then 5 mm^3 of the ethereal Chls solution was injected into each vial, giving solvent purities of 100 % for diethyl ether and 99.83 % for buffered 80 % aqueous acetone and DMF (*i.e.*, 0.17 % contamination with diethyl ether). The vials were left to rest for 60 min at 4 °C in darkness before the respective absorbances were measured using a cell of 1 cm^3 capacity and path length of 1 cm. This measurement was made in triplicate and successively for each series of vials containing the same solvent.

Non-abrasive method for pigment extraction: This method is affected by impurities in the solvents that depend on manufacturer (diethylether U.S.P., diethyl ether plus 2-3 % methanol; Mackinney 1941) and also on the pass to extractants of water from plant material. 10 to 20 discs were used to study different ratios of discs/volume of solvent during pigment extraction. For this, 36 vials (4 repetitions per solvent for three disc/solvent volume ratios) of 10 cm^3 capacity were prepared as mentioned above. 10, 15, or 20 discs were then placed in each set of the 12 previously tared vials. After weighing, 10 cm^3 of diethyl ether, buffered 80 % aqueous acetone, or DMF (4 vials each) were added. The vials were closed hermetically, as before. To refer the concentrations of Chls to leaf dry matter, a large number of discs were dried to constant mass in a stove at 70 °C.

The vials were placed in a refrigerator (4 °C) for 24, 48, 72, and 96 h. After each time of incubation, a volume ($\approx$ 1 cm^3) was extracted to determine the absorbance at the wavelengths of maximum Chl *a* and Chl *b* absorption for each solvent. The same volume was then returned to the vial so as not to greatly alter the discs/cm^3 solvent ratio. The measurements were made using a *Hitachi 3200* spectrophotometer, 0.5 nm bandwidth adjusted to 0 absorbancy at 700 nm. The samples were assessed twice in rapid succession to minimize variations in the responses of the spectrophotometer.

After determining the absorbances, the respective concentrations [kg m^{-3}] of Chl *a* and Chl *b* were calculated using the following equations proposed by García and Nicolás (1998) and usable over a wide range of concentrations corresponding to absorbances of 0.800-0.150 for Chl *a* and 0.600-0.050 for Chl *b*:

Diethyl ether, λ_{max}: Chl *a* 660.0 nm, Chl *b* 641.5 nm
$[\text{Chl } a] = 10.10 \pm 0.40\ A_a - 1.04 \pm 0.01\ A_b$
$[\text{Chl } b] = 16.40 \pm 0.53\ A_b - 2.21 \pm 0.003\ A_a$

80 % acetone, λ_{max}: Chl *a* 662.5 nm, Chl *b* 646.0 nm
$[\text{Chl } a] = 12.50 \pm 0.60\ A_a - 3.76 \pm 0.011\ A_b$
$[\text{Chl } b] = 20.80 \pm 0.84\ A_b - 4.53 \pm 0.06\ A_a$

DMF, λ_{max}: Chl *a* 663.0 nm, Chl *b* 646.5 nm
$[\text{Chl } a] = 12.50 \pm 0.66\ A_a - 4.44 \pm 0.02\ A_b$
$[\text{Chl } b] = 21.80 \pm 1.05\ A_b - 4.75 \pm 0.09\ A_a$

where A_a is the absorbance near 660 nm and A_b that near 640 nm.

Results

Influence of impurities in the solvents (Tables 1 and 2): The standard deviations (σ) and coefficients of variability (CV) were low for all three solvents and both Chls. The lowest CV was found for Chl *a* in buffered 80 % aqueous acetone and the highest for Chl *b* in DMF, and the lowest σ was found for Chl *b* in diethyl ether and the highest for Chl *a* in DMF. These values confirm the possibility to use the method for preparing Chl solutions and determine the possible effect of 0.17 % contamination with diethyl ether.

The per cent errors obtained when the proposed equations are applied (Table 2) were for diethyl ether within the interval of confidence determined by García and Nicolás (1998). The increases in error by 2.1 to 7.1 % were due to impurities in the solvents.

Table 1. Mean absorbances (A), standard deviations (σ), coefficients of variability (CV), and maximum and minimum chlorophyll (Chl) *a* and *b* concentrations [kg m^{-3}] for the three solvents: pure diethyl ether, 80 % acetone, and DMF, the last two solvents containing diethyl ether. Eight repetitions, two measurements per repetition.

Solvent	Parameters	Chl *a*	Chl *b*
Diethyl ether	mean A	1.59×10^{-1} (λ_{max} 660.0 nm)	5.41×10^{-2} (λ_{max} 641.5 nm)
	σ	3.08×10^{-3}	1.10×10^{-3}
	CV [%]	1.94	2.04
	$[Chl]_{max}$	1.62	0.57
	$[Chl]_{min}$	1.49	0.51
80 % acetone	mean A	1.47×10^{-1} (λ_{max} 662.5 nm)	6.17×10^{-2} (λ_{max} 646.0 nm)
	σ	1.64×10^{-3}	1.41×10^{-3}
	CV [%]	1.12	2.29
	$[Chl]_{max}$	1.69	0.68
	$[Chl]_{min}$	1.52	0.56
DMF	mean A	1.62×10^{-1} (λ_{max} 663.0 nm)	6.93×10^{-2} (λ_{max} 646.5 nm)
	σ	3.67×10^{-3}	2.54×10^{-3}
	CV [%]	2.27	3.66
	$[Chl]_{max}$	1.83	0.81
	$[Chl]_{min}$	1.62	0.67

Table 2. Per cent error in the determination of chlorophyll (Chl) concentrations using the proposed equations for extracts in 80 % acetone and DMF containing diethyl ether.

Solvent	% error Chl *a*	Dif. to diethyl ether	Chl *b*	Dif. to diethyl ether
Diethyl ether	8.0	0.0	10.5	0.0
Acetone	10.1	+2.1	17.6	+7.1
DMF	11.5	+3.5	17.3	+6.7

Non-abrasive extraction: 10 to 20 discs were an appropriate amount to obtain absorbances of the extract for Chl *a* (A_a) from 0.30 to 0.85 and for Chl *b* from 0.15 to 0.50, when using 80 % acetone or DMF (Table 3). For extraction in diethyl ether, the absorbances were always lower than the above limits, but they were proportional to the number of discs and did not differ much during prolonged extraction. Generally, diethyl ether is not proper for extraction of Chls from raw plant material. In buffered 80 % acetone, absorbances were proportional to the number of discs and increased with the duration of extraction. The largest increment in absorbances was found between 24 and 48 h. DMF was the most suitable extractant when the non-abrasive method (extraction by immersion) was used. For all the incubation times considered, absorbances were proportional to the quantity of plant material when 10 or 15 discs were used for extraction. Thus 15 discs immersed in DMF for 48 h are the best combination for non-abrasive extraction (Table 4).

Table 3. Mean absorbances in absorption maxima of chlorophylls (Chl) *a* and *b* at different times of extraction for different quantities of discs (6 mm diameter) immersed in different solvents.

Solvent	Time [h]	Number of discs 10 Chl *a*	 Chl *b*	 15 Chl *a*	 Chl *b*	 20 Chl *a*	 Chl *b*
Diethyl ether	24	0.055	0.023	0.080	0.035	0.113	0.049
	48	0.058	0.022	0.080	0.031	0.130	0.058
	72	0.058	0.019	0.079	0.027	0.141	0.064
	96	0.058	0.019	0.078	0.024	0.143	0.063
80 % acetone	24	0.267	0.109	0.447	0.168	0.537	0.206
	48	0.381	0.146	0.598	0.233	0.722	0.280
	72	0.389	0.156	0.649	0.247	0.773	0.299
	96	0.450	0.172	0.702	0-270	0.779	0.342
DMF	24	0.410	0.147	0.641	0.234	0.775	0.290
	48	0.506	0.180	0.777	0.281	0.985	0.367
	72	0.530	0.189	0.819	0.297	1.031	0.388
	96	0.529	0.190	0.835	0.304	1.018	0.366

Table 4. Absorbances (A) and chlorophyll (Chl) concentrations [kg m^{-3}, kg m^{-2}] of *C. aurantium* samples composed of 15 discs immersed for 48 h into various solvents.

Solvent	Chl *a* mean A	 [kg m^{-3}]	 [kg m^{-2}]	Chl *b* mean A	 [kg m^{-3}]	 [kg m^{-2}]	Chl *a/b*
Diethyl ether	0.080	0.77	1.8	0.031	0.33	0.7	2.33
Acetone	0.597	6.59	15.7	0.233	2.14	5.1	3.08
DMF	0.777	8.46	20.1	0.281	2.42	5.8	3.50

Discussion

As regards the effect of impurities on the absorption maxima and ε values, considerable differences between the values for Chl *a* and Chl *b* were already obtained in the spectrophotometric investigations of Mackinney (1941). His ε value for λ_{max} of Chl *a* was 3.6 % greater in U.S.P. ether (containing 2-3 % ethanol) than in anhydrous diethyl ether, while that for Chl *b* was 2.6 % lower. Vernon (1960) obtained similar errors arising from the differing composition of solvent for 100, 90, and 80 % acetone. Lichtenthaler and Wellburn (1983) confirmed the difference between these parameters in pure and 80 % acetone. Lichtenthaler (1987) studied spectral properties and ε of Chls in different solvents: diethyl ether (water free, pure, water-saturated), acetone (100 and 80 % in water), and methanol (100 and 90 % in water). He found significant differences in λ_{max} and ε. Similar differences were also significant in our study where the solvents contained impurities (<2 %) and the Chls were extracted either by abrasive or non-abrasive methods. In both cases, water from plant material dilutes the solvent, more in abrasive than in non-abrasive methods, and the amount of water increases with the ratio of plant material mass/extractant volume. The determined errors of +2.1 to +7.1 % are within the range reported by Mackinney (1941), Vernon (1960), Lichtenthaler (1987), and Porra *et al.* (1989). Also the importance of extractant quality in non-abrasive methods (Table 3) was confirmed. DMF was found preferable for non-abrasive extraction methods; the ratio of Chl *a/b* was highest in these extracts (Table 4). Of course, extracts obtained in this way can be used only for general determination of Chl content, not for a detailed analysis of Chls and their degradation products. During long incubation in solvents, various degradative changes in pigments take place.

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