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ARTICLE

Spectrophotometric determinations of chloroplastidic pigments in acetone, ethanol and dimethylsulphoxide

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ABSTRACT: (Spectrophotometric determinations of chloroplastidic pigments in acetone, ethanol and dimethylsulphoxide). The concentration of pigment indicates a range of properties in the physiological processes of plants. A large number of procedures have been developed to measure leaf pigment content. Some of these procedures involve leaf maceration and centrifugation, while others require incubation of a leaf sample in solvent for a few minutes or hours, which makes the process laborious. The choice of the best procedure may vary according to the plant species and type of solvent used. This study compared the efficiency of three commonly used solvents (dimethylsulphoxide, ethanol and acetone) in the extraction of chlorophylls and carotenoids of five plant species and one lichen species. The results indicated that dimethylsulphoxide was more efficient at chlorophyll extraction in all of the studied species. The ethanol and acetone extracts quickly lost molecular stability. Acetone extract may also cause phaeophytinisation and reduce the chlorophyll content.
Keywords: Chlorophyll extraction procedures, dichroic and tetrachroic equation, validation, chlorophyll, carotenoids

RESUMO: (Determinações espectrofotométricas de pigmentos cloroplastídicos em acetona, etanol e dimetilsulfóxido). A concentração dos pigmentos foliares é indicativa de uma série de propriedades e processos fisiológicos. Diversas metodologias têm sido desenvolvidas para a determinação da concentração dos pigmentos foliares. Destas metodologias, algumas requerem maceração e centrifugação, enquanto outras requerem incubação das amostras foliares no solvente por alguns minutos ou horas, tornando o processo laborioso. A escolha do melhor método pode variar de espécie para espécie e do tipo de solvente utilizado. Neste sentido, testou-se a eficácia de três solventes mais comumente utilizados (dimetilsulfóxido, etanol e acetona) para a extração de clorofilas e carotenóides em cinco espécies de plantas e um líquen. Os resultados indicam que o dimetilsulfóxido possui maior eficiência na extração de clorofilas em todas as espécies estudadas. Os extratos etanólicos e cetônicos rapidamente perderam sua estabilidade. No extrato cetônico pode ocorrer, ainda, a feofitinização e a diminuição do conteúdo de clorofilas.
Palavras-chave: métodos de extração de clorofilas, equações dicróicas e tetracróicas, validação, clorofila, carotenóides.

INTRODUCTION

The determination of leaf pigment content is an important analytical tool in the field of plant physiology. It can be used to classify plant material, establish appropriate treatments and to understand the interactions between plants and environmental factors. Given this importance, reliable assays for chlorophylls (Chls) are required.

The amount of solar radiation absorbed by a leaf is largely dependent on the foliar concentrations of its photosynthetic pigments. Therefore, low Chl concentration can limit photosynthetic potential and primary production (DaMatta *et al.* 2008). In addition, a variety of environmental stresses, such as water shortage, temperature and light extremes, UV-B radiation and mineral deficiencies, reduce chlorophyll levels (Campos *et al.* 2012, Pompelli *et al.* 2010a, Pompelli *et al.* 2010c). This reduction may be related to the photobleaching of chlorophyll (Campos *et al.* 2012). Therefore, the Chl level is an accurate indicator of plant vigor and is routinely measured in physiological research.

In higher plants, Chl *a* and Chl *b* are the most important forms of chlorophyll and are essential for the oxygenic conversion of light energy into the stored chemical energy that powers the biosphere (Richardson et al 2002). Chl *c* and Chl *d* are other common forms of chlorophylls, but are only found in the photosynthetic members of the Chromista and Dinoflagellates (Ceulemans & Saugier 1993). In all the above mentioned cases, carotenoids (Car) are accessory pigments that protect the photosystem when excitation energy is high. To accurately determine Car by measuring A470, one needs to know the exact level of Chl *b*, which, in contrast to Chl *a*, absorbs considerable energy at 470 nm wavelength (Lichtenthaler & Buschmann 2001). If Chl *b* is underestimated, the level of total Car will be overestimated.

The accuracy of any Chl extraction method will vary according to the solvent's nature, polarity (López *et al.* 2004), temperature and incubation time (Ritchie 2008, Tait & Hik 2003). Methods of extraction and conserva-

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tion of chlorophyll have to be adjusted for different plant species due to variations in Chl levels (Porra 2002). Absolute ethanol or acetone are often used in basic research for Chl and Car extraction from leaves (Lichtenthaler & Buschmann 2001). To increase the extraction yield, methanol/water mixtures or dimethylsulfoxide (DMSO) are commonly used (Tait & Hik 2003).

Under field conditions, the ideal solvent would be non-toxic, stable and capable of complete and unbiased extraction of whole tissues across a range of species and environmental conditions. DMSO has been widely used to extract Chl from higher plants (Barbieri Junior *et al.* 2010, Cruz *et al.* 2007, Santos *et al.* 2007, Tait & Hik 2003), lichens (Calatayud *et al.* 1994, Castle *et al.* 2011), fungi (Castle *et al.* 2011), aquatic plants (Castle *et al.* 2011) and algae (Burnison 1980). In a study using lichens, Calatayud *et al.* (1994) found that DMSO extracted more than 98% of chlorophyll, while acetone extracted less than 60%. DMSO extracts are highly stable, with losses smaller than 1% for Chl *a* after 48 hours at 20 ºC. However, the level of Chl begins to decline immediately in extracts with acetone (Hiscox & Israelstam 1979).

This study selected the two most representative families of the Caatinga ecosystem, Fabaceae and Euphorbiaceae, because of their economic and environmental potential. *Jatropha curcas* and *J. mollissima* (Euphorbiaceae) are drought tolerant and have perennial behavior. They are economically important for their use in biodiesel, and their seeds contain between 25 to 40% of oil (Pompelli *et al.* 2010b). *Jatropha* plants have the potential to be used in soil erosion control, mainly in arid environments (Reubens *et al.* 2011). *Prosopis juliflora* (Fabaceae*)* is used in food supplementation for livestock and is an economical alternative for forage and wood production and firewood (Andrade *et al.* 2009). *Calotropis procera* (Asclepiadaceae) is used in traditional medicine to treat ulcers and tumors, and for spleen, liver and abdomen disorders (Akinloye *et al.* 2002, Kanojiya & Madhusudanan 2012). *C. procera* contains various active substances, such as non-enzymatic and enzymatic proteins and cardiac glycosides that can be toxic to animals (Akinloye *et al.* 2002, Kanojiya & Madhusudanan 2012). *Anadenanthera colubrina* (Fabaceae) is a leguminous tree found in primary and secondary forests; in Brazil it is naturally found in the semi-deciduous, tropical wet forests. This species is commonly used to restore degraded soils. Due to the high resistance and durability of its heavy and compact wood, it is used in civil and naval construction as well as for railway sleepers and in carpentry. *Cladonia verticillaris* is an endemic species of the Brazilian coast and occurs in the lowlands of northeast Brazil (Mota-Filho *et al.* 2007). It is a symbiosis of green algae with filamentous fungus and contains some compounds with biological activity. The species is sensitive to airborne pollution, so it is commonly used to biomonitor the environment in northeast Brazil (Mota-Filho *et al.* 2007).

The objective of this study was to determine the efficiency of extracting chloroplastidic pigments from the plant species *Jatropha curcas* L., *Jatropha mollissima* (Pohl) Baill, *Prosopis juliflora* (Sw) DC, *Anadenanthera colubrina* (Vell.) Brenan and *Calotropis procera* (Aiton) W.T. Aiton leaves and *Cladonia verticillaris* (Raddi) Fr. thalli. The study measured the concentrations of chlorophyll and total carotenoids (*i.e.*, xanthophylls and carotenoids) in line with published spectrophotometry procedures (Lichtenthaler & Buschmann 2001, Ritchie 2008).

MATERIAL AND METHODS

Plant species were collected from Serra Talhada, Pernambuco, Brazil (7º54'S; 38º18'W; 420 m.asl.) The lichen was collected from Saloá, Pernambuco, Brazil (8º57'S; 36º43'W; 954 m.asl.). Fully expanded leaves from five plant species or thalli from *C. verticillaris* were collected. The mid-laminar areas, excluding the central vein, from five blade fragments were collected and used in the analysis. Chlorophyll *a* and *b* contents were analyzed using spectrophotometry after extraction with DMSO, ethanol or acetone.

For the Chl and Car extraction from higher plants using DMSO, glass tubes containing 5 mL of DMSO were preheated to 65 ºC in a water bath for 24, 48, 72 and 96 hours. Once the extraction time was complete, the samples were removed from the water bath, and 3 mL of extraction solvent were transferred to glass cuvettes and characterized using a dual-beam spectrophotometer at A_{665}, A_{649}, A_{480} (equations 1-3), as described by Wellburn (1994).

For the Chl and Car extraction using ethanol, the samples were incubated with 10 mL of ethanol at 25 ºC for 24, 48, 72 and 96 hours. A total of 3 mL was transferred to glass cuvettes and characterized using a dual-beam spectrophotometer at $A_{664.2}$, $A_{648.6}$, A_{470} (equations 4-6), as described by Lichtenthaler (1987).

For the Chl extraction using acetone, the samples were ground with 2 mL of 80% acetone in combination with 0.1% sand and/or 0.1% $CaCO_3$ to prevent chlorophyllase activities. After grinding, the samples were filtered, and the final volume (25 mL) was transferred to graduate tubes and characterized using a dual-beam spectrophotometer at $A_{663.2}$, $A_{646.8}$, A_{470} (equations 7-9), as described by Lichtenthaler (1987).

For all measurements, an unspecific 750 nm absorbance was recorded to correct for turbidity and contaminating colored compounds. Chls *a* and *b* and carotenoids do not absorb at this wavelength. If turbidity was present, the tubes were centrifuged in the dark at 1,120 g force for 10 minutes before analysis (Lichtenthaler & Buschmann 2001).

To measure the Chl of *C. verticillaris*, two calculation procedures were carried out: standard dichroic equations (Wellburn 1994), and alternative quadrichroic equations (Equations 10-13 for acetone and 14-17 for ethanol) (Ritchie 2008). Readings were taken immediately after preparation of the extract (acetone only) and after 24, 48, 72 and 96 h with DMSO and ethanol. The concentration

of the extract using these equations was then converted to leaf Chl content (g.kg^{-1} DW). Spurious negative values of Chl were deleted as recommended by Ritchie (2008).All measurements were carried out with a spectrophotometer (Biochrom Libra S22 UV/Vis Spectrophotometer, Biochrom, Cambridge, UK) using standard scanning settings and a 1 nm bandwidth and 1 nm sampling interval.

The spectrophotometer was calibrated to zero absorbance using a blank of pure DMSO, 95% ethanol or 80% acetone. The normal lighting in the laboratory under such conditions was approximately 2 µmol.m^2.s^{-1} (400-700 nm PAR) (Li-Cor quantum photometer LI-189).

The collected data were analyzed with a mixed-model ANOVA, and the means were compared using an SNK test with the statistical software package SigmaPlot 11.0 (Systat Software Inc., Chicago, USA). The correlations were carried out with the statistical software Statistica version 7.0 (StatSoft, Tulsa, OK, USA). The results were considered to be significant when $P \leq 0.05$. All measurements were replicated five times.

$$Eq\ (1) \quad Chl\ "a" = (12.19\ A_{665}) - (3.45\ A_{649})$$
$$Eq\ (2) \quad Chl\ "b" = (21.99\ A_{649}) - (5.32\ A_{665})$$
$$Eq\ (3) \quad Car = [(1000\ A_{480}) - (2.14\ Chl\ "a") - (70.16\ Chl\ "b")] / 220$$

$$Eq\ (4) \quad Chl\ "a" = (13.36\ A_{664.2}) - (5.19\ A_{648.6})$$
$$Eq\ (5) \quad Chl\ "b" = (27.43\ A_{648.6}) - (8.12\ A_{664.2})$$
$$Eq\ (6) \quad Car = [(1000\ A_{470}) - (2.13\ Chl\ "a") - (97.64\ Chl\ "b")] / 209$$

$$Eq\ (7) \quad Chl\ "a" = (12.25\ A_{663.2}) - (2.79\ A_{646.8})$$
$$Eq\ (8) \quad Chl\ "b" = (21.50\ A_{646.8}) - (5.10\ A_{663.2})$$
$$Eq\ (9) \quad Car = [(1000\ A_{470}) - (1.82\ Chl\ "a") - (85.02\ Chl\ "b")] / 198$$

$$Eq\ (10) \quad Chl\ "a" = (-0.3319\ A_{630}) - (1.7485\ A_{647}) + (11.9442\ A_{664}) - (1.4306\ A_{691})$$
$$Eq\ (11) \quad Chl\ "b" = (-1.2825\ A_{630}) + (19.8839\ A_{647}) - (4.8860\ A_{664}) - (2.3416\ A_{691})$$
$$Eq\ (12) \quad Chl\ "c" = (23.5902\ A_{630}) - (7.8516\ A_{647}) - (1.5214\ A_{664}) - (1.7443\ A_{691})$$
$$Eq\ (13) \quad Chl\ "d" = (-0.5881\ A_{630}) + (0.0902\ A_{647}) - (0.1564\ A_{664}) + (11.0473\ A_{691})$$

$$Eq\ (14) \quad Chl\ "a" = (0.0604\ A_{632}) - (4.5224\ A_{649}) + (13.2969\ A_{665}) - (1.7453\ A_{696})$$
$$Eq\ (15) \quad Chl\ "b" = (-4.1982\ A_{632}) + (25.7205\ A_{649}) - (7.4096\ A_{665}) - (2.7418\ A_{696})$$
$$Eq\ (16) \quad Chl\ "c" = (28.4593\ A_{632}) - (9.9944\ A_{649}) - (1.9344\ A_{665}) - (1.8093\ A_{696})$$
$$Eq\ (17) \quad Chl\ "d" = (-0.2007\ A_{632}) + (0.0848\ A_{649}) - (0.1909\ A_{665}) + (12.1302\ A_{696})$$

RESULTS AND DISCUSSION

The results of chlorophyll and carotenoid concentrations in higher plants are presented in Table 1 and Table 2. Regardless of the plant species, DMSO extracted much larger concentrations of chlorophylls when compared with 95% ethanol or 80% acetone. However, leaf exposure time to DMSO was highly positive and significantly correlated with Chl extraction (correlation coefficients 0.801, 0.256, 0.858, 0.451, 0.555 for *A. colubrina*, *C. procera*, *J. curcas*, *J. molissima* and *P. juliflora,* respectively; $p \leq 0.05$). When 95% ethanol was used as extractor, the incubation time of the leaves was not always positively and significantly correlated with the extraction efficiency. These extracts sometimes lose their stability over time, which reduces the Chl yield (Table 1). These findings corroborate those of other researchers who reported the high extraction efficiency of DMSO (Barbieri Junior *et al.* 2010, Castle *et al.* 2011, Cruz *et al.* 2007). DMSO was considered to be the best chlorophyll extractor because it provided the best stability for extracts (Tait & Hik 2003). The amount of time that leaves are exposed to the extractor is important. In leaves from plants with rootstocks of *Vitis* sp. (Santos *et al.* 2007) or *Cynodon spp.* (Barbieri Junior *et al.* 2010), 48 hours in DMSO was sufficient to obtain the best chlorophyll extraction efficiency. However, in *Bixa orelana* (Cruz *et al.* 2007), at least 72 hours in DMSO was needed to extract chlorophylls. Changes in Chl content should be measured using a stable reference. For example, a decrease in Chl per dry weight, which was found using 95% ethanol, may only be due to tissue dehydration and the dilution of extracts (Lichtenthaler & Buschmann 2001). In this instance, 95% ethanol was not considered to be a good chlorophyll extractor. Barbieri Junior et al. (2010) and Wintermans & DeMots (1965) found similar results.

In this study, none of the acetone combinations were considered good extractors of chlorophylls (Table 1). Acetone has been reported to be a poor extractor of chlorophyll from many vascular plants and some algae, particularly green algae (Ritchie 2008, and references therein). In many practical situations, acetone is not an ideal solvent for extraction, although it is a useful solvent for assays of chlorophylls. Aqueous acidic acetone extracts (*i.e.* not buffered) were shown to contain a phaeophytinising factor that increases chlorophyll degradation (Brown & Hooker 1977). The phaeophytinisation of chlorophyll, which substituted Mg^{+2} in chlorophyll by H^+, is the direct result of the acidification (Kong *et al.* 1999). In this sense, the pH increase promoted by $CaCO_3$ addition may partially inhibit Chl breakdown into phaeophytin, which may raise the concentration of extracted Chl (Table 1 and 2; see more details in Porra *et al.* (1989) and Ritchie (2008).

In natural assemblages of lichen (*e.g.*, *Cladonia verticillaris*) or algae, any significant Chl *b* that is present will also interfere with the measurement of Chl *c* and/or *d*. Formulae with only two wavelengths do not

Table 1. Concentration of total chlorophylls (Chl) and carotenoids (g.kg^{-1} DW) in leaves of *Jatropha curcas*, *J. mollissima*, *Prosopis juliflora, Anadenanthera colubrina* and *Calotropis procera* as a function of 12 extraction methods by dichroic equations. Different letters represent statistical significance among the extraction methods.

Extraction method	Chl *a*		Chl *b*		Total Chl		Carotenoids	
Jatropha curcas								
DMSO 24 h (1)	6.78 ± 0.10	c	1.68 ± 0.40	d	8.46 ± 0.66	b	0.69 ± 0.14	f
DMSO 48 h (2)	8.57 ± 0.13	a	2.14 ± 0.16	cd	10.71 ± 0.58	ab	1.14 ± 0.07	cd
DMSO 72 h (3)	8.26 ± 0.10	ab	2.91 ± 0.07	ab	11.17 ± 0.15	a	1.49 ± 0.01	bcd
DMSO 96 h (4)	8.61 ± 0.42	a	3.22 ± 0.30	a	12.03 ± 0.45	a	1.41 ± 0.08	cd
Ethanol 95% 24 h (5)	2.61 ± 0.16	e	0.82 ± 0.08	e	3.44 ± 0.24	c	0.74 ± 0.04	f
Ethanol 95% 48 h (6)	5.80 ± 0.12	d	2.86 ± 0.13	ab	8.18 ± 0.17	b	1.76 ± 0.04	b
Ethanol 95% 72 h (7)	7.66 ± 0.13	b	2.39 ± 0.05	bc	10.90 ± 0.29	a	1.31 ± 0.03	de
Ethanol 95% 96 h (8)	8.04 ± 0.16	ab	3.03 ± 0.05	a	10.69 ± 0.18	a	2.04 ± 0.02	a
Acetone 80% + $CaCO_3$ + sand 0.1% (9)	5.76 ± 0.16	d	2.27 ± 0.06	cd	7.98 ± 0.19	b	1.56 ± 0.08	bcd
Acetone 80% + $CaCO_3$ 0.1% (10)	6.24 ± 0.16	cd	2.26 ± 0.09	cd	8.50 ± 0.24	b	1.69 ± 0.04	bc
Acetone 80% + sand 0.1% (11)	5.99 ± 0.24	cd	2.26 ± 0.06	cd	8.25 ± 0.29	b	1.61 ± 0.06	bcd
Acetone 80% (12)	5.90 ± 0.26	cd	2.08 ± 0.12	cd	7.98 ± 0.37	b	1.57 ± 0.11	bcd
Jatropha mollissima								
DMSO 24 h (1)	3.05 ± 0.24	a	0.88 ± 0.13	a	3.94 ± 0.36	a	0.61 ± 0.07	ab
DMSO 48 h (2)	2.89 ± 0.26	a	0.86 ± 0.11	a	3.75 ± 0.37	a	0.62 ± 0.06	ab
DMSO 72 h (3)	3.53 ± 0.21	a	1.13 ± 0.13	a	4.66 ± 0.34	a	0.79 ± 0.06	ab
DMSO 96 h (4)	3.59 ± 0.32	a	1.28 ± 0.17	a	4.87 ± 0.49	a	0.74 ± 0.07	ab
Ethanol 95% 24 h (5)	1.01 ± 0.48	a	0.46 ± 0.23	a	1.47 ± 0.71	a	0.21 ± 0.11	b
Ethanol 95% 48 h (6)	1.94 ± 0.43	a	0.90 ± 0.28	a	2.84 ± 0.70	a	0.45 ± 0.14	ab
Ethanol 95% 72 h (7)	2.71 ± 0.42	a	1.29 ± 0.22	a	4.33 ± 1.91	a	0.67 ± 0.29	ab
Ethanol 95% 96 h (8)	2.94 ± 1.29	a	1.39 ± 0.62	a	4.00 ± 0.64	a	0.76 ± 0.14	ab
Acetone 80% + $CaCO_3$ + sand 0.1% (9)	2.66 ± 0.26	a	1.12 ± 0.13	a	3.78 ± 0.39	a	0.81 ± 0.10	ab
Acetone 80% + $CaCO_3$ 0.1% (10)	2.95 ± 0.38	a	1.34 ± 0.17	a	4.66 ± 0.49	a	0.92 ± 0.10	a
Acetone 80% + sand 0.1% (11)	3.31 ± 0.32	a	1.55 ± 0.16	a	4.45 ± 0.50	a	0.93 ± 0.08	a
Acetone 80% (12)	3.39 ± 0.34	a	1.36 ± 0.19	a	4.21 ± 0.58	a	0.91 ± 0.10	a
Prosopis juliflora								
DMSO 24 h (1)	1.39 ± 0.12	ab	0.40 ± 0.21	a	1.79 ± 0.20	ab	0.13 ± 0.06	c
DMSO 48 h (2)	2.51 ± 0.36	a	0.67 ± 0.13	a	3.15 ± 0.39	a	0.38 ± 0.03	b
DMSO 72 h (3)	2.48 ± 0.26	a	0.76 ± 0.15	a	3.41 ± 0.42	a	0.47 ± 0.04	ab
DMSO 96 h (4)	2.64 ± 0.27	a	0.93 ± 0.20	a	3.44 ± 0.52	a	0.55 ± 0.04	ab
Ethanol 95% 24 h (5)	0.02 ± 0.00	c	0.01 ± 0.00	a	0.02 ± 0.01	b	0.01 ± 0.00	c
Ethanol 95% 48 h (6)	0.27 ± 0.11	c	0.08 ± 0.04	a	0.35 ± 0.15	b	0.04 ± 0.02	c
Ethanol 95% 72 h (7)	0.44 ± 0.32	bc	0.15 ± 0.12	a	0.59 ± 0.44	b	0.08 ± 0.06	c
Ethanol 95% 96 h (8)	0.59 ± 0.31	bc	0.84 ± 0.63	a	1.43 ± 0.71	ab	0.08 ± 0.09	c
Acetone 80% + $CaCO_3$ + sand 0.1% (9)	2.10 ± 0.18	a	0.54 ± 0.05	a	2.77 ± 0.25	a	0.61 ± 0.04	ab
Acetone 80% + $CaCO_3$ 0.1% (10)	2.17 ± 0.27	a	0.62 ± 0.15	a	2.86 ± 0.37	a	0.62 ± 0.07	ab
Acetone 80% + sand 0.1% (11)	2.18 ± 0.28	a	0.66 ± 0.08	a	2.72 ± 0.33	a	0.75 ± 0.09	a
Acetone 80% (12)	2.28 ± 0.37	a	0.70 ± 0.10	a	2.90 ± 0.52	a	0.75 ± 0.09	a
Calotropis procera								
DMSO 24 h (1)	3.36 ± 0.41	bcd	2.49 ± 0.17	a	5.90 ± 0.41	ab	nd	
DMSO 48 h (2)	4.22 ± 0.12	abc	2.76 ± 0.20	a	6.74 ± 0.59	a	nd	
DMSO 72 h (3)	5.28 ± 0.98	ab	1.61 ± 3.71	a	6.98 ± 0.28	a	0.65 ± 0.24	cd
DMSO 96 h (4)	5.77 ± 1.16	a	0.55 ± 0.51	a	6.32 ± 0 .76	ab	2.42 ± 0.69	b
Ethanol 95% 24 h (5)	0.60 ±0.08	e	0.43 ± 0.24	a	0.91 ± 0.07	e	0.27 ± 0.14	cd
Ethanol 95% 48 h (6)	1.01 ± 0.50	de	0.34 ± 0.17	a	1.38 ± 0.69	e	0.31 ± 0.14	cd
Ethanol 95% 72 h (7)	1.20 ± 0.58	de	0.31 ± 0.06	a	2.34 ± 0.55	de	0.34 ± 0.14	cd
Ethanol 95% 96 h (8)	2.59 ± 0.56	cde	nd		2.62 ± 0.50	cde	5.05 ± 1.28	a
Acetone 80% + $CaCO_3$ + sand 0.1% (9)	2.87 ± 0.16	cde	2.17 ± 0.91	a	5.59 ± 1.19	ab	0.75 ± 0.31	cd
Acetone 80% + $CaCO_3$ 0.1% (10)	2.91 ± 0.18	cde	1.80 ± 0.59	a	4.77 ± 0.98	abc	0.97 ± 0.29	cd
Acetone 80% + sand 0.1% (11)	3.43 ± 0.29	bcd	1.21 ± 0.09	a	3.99 ± 0.28	bcd	1.28 ± 0.04	c
Acetone 80% (12)	3.48 ± 0.33	bcd	1.52 ± 0.08	a	4.92 ± 0.43	abc	1.34 ± 0.09	c
Anadenanthera colubrina								
DMSO 24 h (1)	1.13 ± 0.09	cd	0.34 ± 0.03	e	1.47 ± 0.11	c	0.59 ± 0.05	bc
DMSO 48 h (2)	1.51 ± 0.09	bc	0.57 ± 0.05	cde	2.08 ± 0.17	c	0.68 ± 0.06	bc
DMSO 72 h (3)	1.55 ± 0.12	bc	0.57 ± 0.05	cde	2.07 ± 0.13	c	0.68 ± 0.05	bc
DMSO 96 h (4)	2.05 ± 0.14	ab	0.63 ± 0.03	cde	2.65 ± 0.17	b	0.70 ± 0.03	bc
Ethanol 95% 24 h (5)	0.62 ± 0.41	d	0.41 ± 0.26	de	1.51 ± 0.25	c	0.52 ± 0.07	bc
Ethanol 95% 48 h (6)	1.16 ± 0.15	cd	0.45 ± 0.01	cde	1.89 ± 0.95	c	0.66 ± 0.12	bc
Ethanol 95% 72 h (7)	2.26 ± 0.07	ab	0.86 ± 0.07	cd	3.12 ± 0.13	b	1.15 ± 0.05	a
Ethanol 95% 96 h (8)	2.32 ± 0.09	ab	0.80 ± 0.04	cde	3.12 ± 0.13	b	1.32 ± 0.02	a
Acetone 80% + $CaCO_3$ + sand 0.1% (9)	1.93 ± 0.09	ab	0.87 ± 0.06	cd	2.80 ± 0.15	b	1.19 ± 0.03	a
Acetone 80% + $CaCO_3$ 0.1% (10)	1.90 ± 0.03	ab	0.92 ± 0.05	c	2.83 ± 0.07	b	0.98 ± 0.04	ab
Acetone 80% + sand 0.1% (11)	2.15 ± 0.15	ab	1.77 ± 0.30	b	3.32 ± 0.18	b	0.32 ± 0.44	c
Acetone 80% (12)	2.66 ± 0.13	a	2.77 ± 0.07	a	5.42 ± 0.19	a	nd	

Data are expressed as means ± SE, n = 5.

accurately calculate the Chl *c* or Chl *d* in extracts, and the proportions of Chl *c* are not known or have an inherently limited accuracy, particularly if only small amounts of Chl *c* or Chl *d* are present (Jeffrey & Humphrey 1975). This study showed that the dichroic formulae all gave incorrectly high estimates of Chl *a, b,* and *c* (Table 2), and therefore cannot be used to accurately estimate Chl *a* in a Chl extract containing large amounts of Chl *c* or *d*. Dichroic equations do not enable the measurement of Chl *c* and Chl *d* (values presented in Table 2 as not determined).

The other circumstances in which misleading errors can be made in estimates of Chl *a* include cases in which a dichroic formula is used to assay Chl *a* in a mixture containing large amounts of Chl *d* (Ritchie 2008). When choosing the best solvent for the extraction of pigments in leaf samples, there was very little Chl b and c Chl (Table 2) and a preponderance of Chl (Jeffrey and Humphrey 1975). The presence of Chl *d* in a Chl extract of *C. verticcilaris* (Table 2) resulted in the dichroic algorithms, providing false spurious values of Chl *b*, particularly where algorithms for ethanol or acetone were used. In agreement with Ritchie (2008), the dichroic formulae are simpler than the quadrichroic formulae but should only be used when a Chl extract has no significant Chl *c* or Chl *d* content (Ritchie 2008). Spectrophotometric assays of Chl *c* can be problematic because the red peak intensity for Chl *c* is much lower than for equimolar amounts of Chl *a* (Ritchie 2008). Furthermore, Chl *c* compounds normally represent less than 10% of the total Chl of algal cells and hence are difficult to assay in Chl mixtures. Thus, spectrophotometric determinations of Chl *c* compounds should be carefully carried out. Chl extracts contain large amounts of Chl *d* and will result in a severe overestimation of Chl *c* in a sample, whereas substantial amounts of Chl *a* will mask the presence of Chl *c*, resulting in a false low value (Ritchie 2008). Therefore, if the quadrichroic formulae indicate that substantial amounts of Chl *d* are present, the dichroic algorithms should not be used (Ritchie 2008). It is worth noting that quadrichroic equations detected the Chl *c* and Chl *d* present in lichens in a distinct manner from the dichroic equations. However, Ritchie (2008) did not examine DMSO. For this reason, this study calculated DMSO Chl values using the quadrichroic equation presented for acetone because there is a precedent for this in the literature.

In *C. verticillaris*, DMSO was more efficient at extracting pigments than 95% ethanol or 80% acetone (Table 2). The concentration of chlorophyll *a* and *b* in the buffered acetone extract was higher when compared to the acetone extract without $CaCO_3$ (Table 2). However, the presence of fumarprotocetraric acid may have overcome the capacity of the $CaCO_3$ to prevent phaeophytinisation completely (Brown & Hooker 1977). Moreover, it may have prevented the conversion of chlorophyll to phaeophytin (Brown & Hooker 1977), thus lowering the Chl concentrations in ethanol or acetone extracts. We assumed that phaeophytinisation did not occur in DMSO extracts (Table 2) because extractions were carried out at 65 ºC, where all chlorophyllase are denatured (Ritchie 2006). A similar pattern has been previously described in *Xanthoria parietina* (Pisani *et al.* 2011), *Cladonia pleurota* (Backor & Fahselt 2004) and *Xanthoparmelia mexicana* (Kong *et al.* 1999). Hesitancy in using alternative solvents to acetone can be partially attributed to the problem of chlorophyllase degradation of chlorophylls (Porra 2002). Chlorophyllase hydrolyses the phytol chain from phytollated chlorophylls to produce the corresponding chlorophyllides (Matile *et al.* 1999). Furthermore, the use of DMSO can facilitate Chl extraction because maceration, filtration or centrifugation can be omitted from the extraction process. Taken together, these results indicate that DMSO can be recommended for the extraction of Chl, as described in this study.

Table 2. Comparison of concentrations of chlorophylls (Chl) and total carotenoids (mg.kg^{-1} DW) in thalli of *Cladonia verticillaris* as a function of 12 extraction methods method calculated by dichroic and quadrichroic equations. Different lower letter represent statistical significance as a function of 12 extraction methods, and upper letters represent statistical significance among dichroic and quadridroic equation used to measure chlorophylls and carotenoids at $P \geq 0.05$ Newman-Keuls' test.

	Chl *a*		Chl *b*		Chl *c*		Chl *d*		Total Chl		Carotenoids	
Method	**Dich**	**Quadr**	**Dich**	**Quadr**	**Dich**	**Quadr**	**Dich**	**Quadr**	**Dich**	**Quadr**	**Dich**	**Quadr**
(1)	220.90 Ba	318.42 Aab	84.21 Aa	63.61 Bb	nd	4.62 ab	nd	24.28 a	299.69 Ba	409.80 Aab	31.78 Bc	77.13 Aab
(2)	174.32 Bb	328.65 Aa	68.63 Aa	67.75 Aa	nd	5.52 a	nd	24.21 a	250.88 Ba	422.37 Aa	28.48 Bc	82.13 Aa
(3)	122.83 Bc	304.60 Abc	56.86 Aa	63.30 Ab	nd	4.98 ab	nd	24.85 a	198.59 Ba	399.12 Aab	27.96 Bc	78.98 Aab
(4)	142.13 Bc	294.61 Ac	55.99 Aa	62.03 Ab	nd	4.03 b	nd	23.75 a	198.98 Ba	385.90 Ab	24.49 Bc	75.47 Ab
(5)	0.95 Be	5.30 Ad	5.34 Aa	0.84 Bc	nd	1.71 cd	nd	0.44 b	7.11 Ab	8.10 Ac	nd	4.36 c
(6)	1.66 Be	6.52 Ad	nd	0.47 c	nd	nd cd	nd	0.41 b	1.68 Bb	9.43 Ac	nd	4.43 c
(7)	1.77 Be	5.24 Ad	nd	0.33 c	nd	nd cd	nd	0.27 b	1.77 Be	6.11 Ac	1.27 Ac	3.28 Ac
(8)	1.88 Be	5.48 Ad	nd	0.31 c	nd	0.70 cd	nd	0.48 b	1.88 Be	5.77 Ac	2.33 Ac	3.03 Ac
(9)	61.18 Ad	13.00 Bd	nd	3.76 c	nd	0.38 d	nd	1.39 b	61.18 Ad	19.18 Bc	340.15 Aa	1.09 Bc
(10)	16.47 Ae	12.48 Ad	nd	3.44 c	nd	0.60 cd	nd	1.33 b	16.47 Ae	15.96 Ac	165.69 Ab	1.26 Bc
(11)	13.01 Ae	8.22 Ad	nd	0.43 c	nd	2.19 c	nd	0.95 b	13.01 Ae	9.01 Ac	146.37 Ab	1.14 Bc
(12)	5.95 Af	8.06 Ad	nd	0.43 c	nd	2.02 c	nd	0.77 b	5.95 Af	7.77 Ac	137.78 Ab	1.15 Bc

Data are expressed as means ± SE, n = 5.

CONCLUSIONS

There is a trade-off between choosing the best solvent for efficient quantitative extraction of chlorophylls and using the solvent that is most suited to spectrophotometric assays. Although flammable and less toxic than acetone and DMSO, ethanol is suitable for use in a teaching laboratory.

Ethanol has many advantages (it is practical, safe and inexpensive) as a solvent for chlorophyll extraction and assays. However, this study showed that it always underestimates the concentration of pigments in leaf extracts, and its use should be carefully evaluated.

Acetone can be recommended, but is very volatile, highly flammable, causes headaches, is narcotic in high concentrations and is a skin irritant; it is therefore an impractical solvent for use outside the research laboratory.

The best of the three solvents used in this study, appears to be dimethylsulphoxide, because it does not require maceration, centrifugation or filtration. Dimethylsulphoxide is solid at temperatures below 18 ºC and re-crystallizes slowly, but is good for dealing with delicate tissues, such as those found in lichens. Care should be taken when it is heated during extraction.

The choice of solvent for extracting pigments from leaf samples must take several factors into account: toxicity, cost, the number of extractions and efficiency.

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