



Aqueous-acetone extraction improves the drawbacks of using dimethylsulfoxide as solvent for photometric pigment quantification in *Quercus ilex* leaves

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Abstract

Aim of study: We evaluated the use of dimethylsulfoxide (DMSO) for the photometric determination of chlorophyll (Chl_a and Chl_b) and carotenoids in *Quercus ilex* L. leaves by comparative analysis using aqueous-acetone extraction.

Area of study: a *Q. ilex* dehesa in Las Majadas del Tietar, Cáceres, Spain

Material and methods: *Q. ilex* leaves were sampled during two vegetative periods. Field SPAD-502 Chlorophyll measurements and photometric chlorophyll determinations were performed. Two procedures were used: extraction of intact foliar discs at 65°C with DMSO and fine foliar powder with cold aqueous-acetone.

Main results: DMSO produced Chl_b overestimation and different fitting performance for SPAD vs pigment calibrations ($R^2=0.64$, RMSE=0.20, $p<0.0001$ for Chl_a ($\mu\text{g}/\text{cm}^2$); $R^2=0.33$, RMSE=0.23, $p<0.0001$ for Chl_b ($\mu\text{g}/\text{cm}^2$) and $R^2=0.50$, RMSE=0.23, $p<0.0001$ for carotenoids ($\mu\text{g}/\text{cm}^2$)). Aqueous-acetone provided more accurate predictions ($R^2=0.90$, RMSE=0.16, $p<0.0001$ for Chl_a and $R^2=0.91$, RMSE=0.16, $p<0.0001$ for Chl_b, $R^2=0.90$, RMSE=0.02, $p<0.0001$ for carotenoids) and mean ratio Chl_a/Chl_b=3.6 inside the range for sun exposed leaves.

Research highlights: Oxidizing conditions and polyphenol concentrations in *Q. ilex* leaves generated brown colorations in the DMSO extraction procedure, interfering with the photometric measurements in the red-orange region. Aqueous-acetone extraction was free from interference. DMSO should be avoided for pigment determination in *Q. ilex* leaves or when comparing different tree species.

Additional keywords: chlorophyll; carotenoids; DMSO; SPAD-502; phenolic compounds.

Abbreviations used: Chl_a (chlorophyll a); Chl_b (chlorophyll b); Chl(-s) (chlorophyll(-s)); C_{x+c} (carotenoids); DMSO (dimethylsulfoxide); GA (gallic acid), RMSE (root mean squared error).

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Supplementary material (Tables S1-S3 and Figs. S1-S2) accompanies the communication on FS's website

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Introduction

Laboratory analysis of pigments in tree leaves has traditionally been conducted by extraction methods using several water-soluble organic solvents followed by spectrometric determination based on the maxima absorption spectra of Chlorophylls (Chls) and carotenoids in the red and blue spectral regions (Minocha *et al.*, 2009). Quantification is routinely made by applying various equations developed for different solvents and spectrophotometer resolutions (Barnes *et*

al., 1992; Wellburn, 1994). The solvent DMSO was introduced for pigment extractions as it avoids the need for grinding and centrifuging foliar samples required by other solvents, allowing a large amount of samples to be analysed and a higher stability of the extracted Chls with time (Hiscox & Israelstam, 1979). The Chl_b peak in the red region is much less intense than the Chl_a and is superposed by the later in fresh leaves extracts. For this reason, Chl_b quantification is very sensitive to extract turbidity or high pigments concentration (Lichtenthaler & Buschmann, 2001).

We investigated the drawbacks in the use of DMSO as solvent for pigment quantification in *Quercus ilex* L. leaves used for SPAD calibrations. An alternative extraction method with aqueous-acetone was studied.

Material and methods

Study site and sampling

Nine sampling campaigns were conducted in five mature *Q. ilex* trees along two vegetative periods. Field SPAD (SPAD-502, Konica Minolta) measurements were obtained from 12 leaves per tree (6 current and 6 from the previous year). Immediately, each leaf was individually packed in black polyethylene bags and transported in cooled containers to the laboratory. Leaves were frozen at -20°C within a maximum of 3 h after sampling until required.

Pigment analysis

DMSO extraction. One (two for new sprouted leaves) 6 mm diameter leaf disc was extracted with 5 mL of DMSO PRS (Panreac Quimica S.A.U.) in a 10 mL glass reagent tube wrapped in aluminium foil at 65°C for 4 h (to ensure complete disc decolouration) and centrifuged (3000 rpm). Pigment concentrations were measured spectrophotometrically immediately after extraction (Beckman DU 530 UV/Vis) at 665.1, 649.1 and 480 nm and quantified using Wellburn's equations (1994).

Aqueous-acetone extraction. The solvent (80% acetone) was prepared immediately before analysis using acetone Chromasolv® HPLC 99.8% (Sigma-Aldrich) and deionized water, both previously stored overnight at $3-4^{\circ}\text{C}$. Three foliar discs per leaf (5 mm diameter) were ground in a 2 mL eppendorf tube with 1 mL of aqueous-acetone (Technica Millmix 20). The grinding time was adapted to the grade of foliar cutinisation and leaf thickness that increases during the leaf aging process. Samples were transferred to a 10 mL reagent glass followed by washing the Eppendorf tube three times with 1 mL aqueous-acetone and completing to a final volume of 8 mL. Pigments were extracted for 2 h in a cooled isolated black box and centrifuged at 3000 rpm (Tables S1 and S2 [suppl]). Absorbance was immediately measured at 663.2, 646.8 and 470 nm and pigment concentration calculated by Wellburn's equations (1994).

A fourth measurement at 750 nm was performed in both methods as an extract turbidity control. Comparative extractions in the same leaf with DMSO and aqueous-acetone were performed in the current and

one year old leaves. For this comparison, the extraction-surface/solvent-volume ratio was equally maintained in both methods. Half of each leaf side was extracted with one of the two solvents. Ten leaves per solvent type and age were analysed.

Results and discussion

Pigment concentrations analysed in the current year leaves using DMSO solvent were in the range of 70-190 $\mu\text{g}/\text{cm}^2$ and exhibited low $\text{Chl}_a/\text{Chl}_b$ ratios (0.8 to 2.3). The SPAD regression performed with the DMSO method produced a significant correlation with the Chl_a concentrations analysed ($R^2=0.64$, $\text{RMSE}=0.20$, $p<0.0001$) (Fig. 1A). However the same regression with Chl_b concentrations was much weaker ($R^2=0.33$; $\text{RMSE}=0.23$; $p<0.0001$) (Fig. 1B). Gonsiorkiewicz Rigon *et al.* (2012) found a similar dispersion only for Chl_b calibration by DMSO extractions using a ClorofiLOG®-1030. We measured high and variable absorptions readings at 750 nm that should be close to zero in all the DMSO extracts. A strong significant relation between the absorption at 750 nm and Chl_b was found ($R^2=0.83$; $\text{RMSE}=0.007$; $p<0.0001$) (Fig. 1D). The ratio between $\text{Chl}_a/\text{Chl}_b$ decreased significantly as absorption at 750 nm increased ($R^2=0.57$; $\text{RMSE}=0.01$; $p<0.0001$) (Fig. 1E). Likewise, a relationship between absorption at 750 nm and extract weight was observed ($R^2=0.38$; $\text{RMSE}=0.02$; $p<0.0001$) (Fig. 1F). DMSO seemed to co-extract compounds that absorb especially in the 649.1 nm peak used for Chl_b quantification, increasing 2-3 fold its value and narrowing the $\text{Chl}_a/\text{Chl}_b$ ratio.

Extraction with DMSO increased significantly (20%) the Chl_a concentration in the younger leaves with lower pigment concentration and in 5% in the previous year leaves compared with the aqueous-acetone (Table 1). The differences were much greater and significant, in the case of Chl_b concentrations with an increase of 115% and 110% in the DMSO extracts in current and previous year leaves respectively compared with the aqueous-acetone.

Minocha *et al.* (2009) detected difficulties in using DMSO for pigment quantification in tree leaves when compared with other solvents due to brown colorations. They attributed the colour interference to the tannins and phenolic compounds co-extracted by this solvent. We detected the same brown colorations only in the DMSO extractions (Figs. S1 and S2 [suppl]). However phenolics and tannins should be present in both extracts, since DMSO and aqueous-acetone are able to dissolve them (Dai & Mumper, 2010; Alves *et al.*, 2013) and their absorption maxima in the UV was distant from the orange-red spectral region (Fig. 2A).

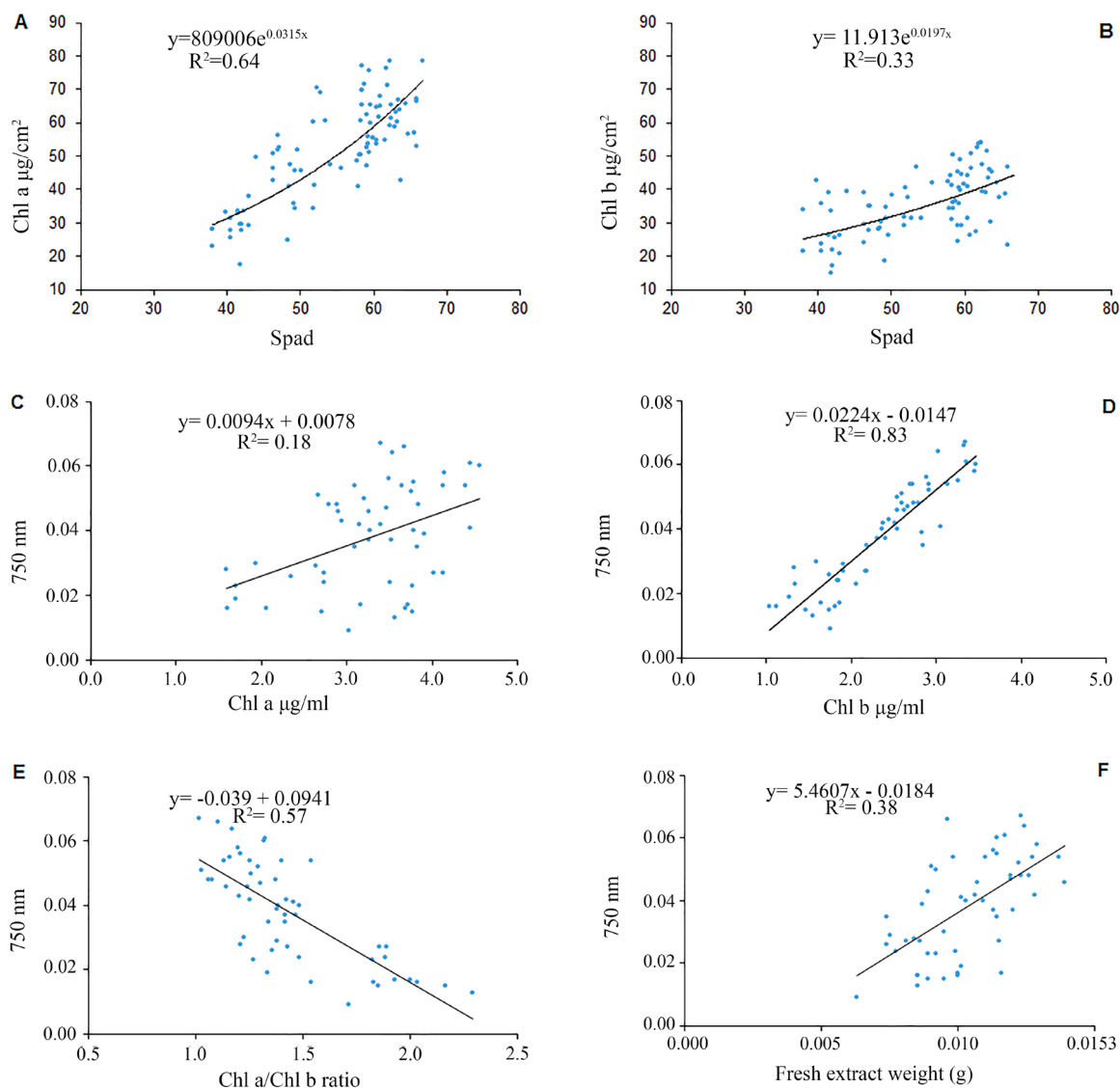


Figure 1. SPAD values versus chlorophyll a (A) and b (B) concentrations in $\mu\text{g}/\text{cm}^2$. Chlorophyll a (C), chlorophyll b (D) concentrations ($\mu\text{g}/\text{mL}$), $\text{Chl}_a/\text{Chl}_b$ ratio (E) and fresh extraction weight (F) vs absorption readings at 750 nm. All extractions for pigment quantification performed with the DMSO procedure (N=82).

Phenolic compounds are easily oxidized and degraded at high temperatures and during long extraction times (Dai & Mumper, 2010) forming stable polymerized brown coloured anthocyanin-tannin complexes (Giusti & Wrolstad, 2001). The main spectral differences between the fresh pigment extracts obtained with DMSO and acetone from the same *Q. ilex* leaf are located in the 500-650 nm region (Fig. 2A). Brownish DMSO extracts showed higher absorbance in this region overlapping the absorption at 649.1 nm and leading to a Chl_b overestimation. Subtraction of absorbance at 750 nm (Table 1) did not achieve reliable $\text{Chl}_a/\text{Chl}_b$ ratios because the absorbance addition effect

in the red region was not constant and higher as in the IR region (Fig. 2A). The oxidation process increased notably three days after extraction only in the DMSO extracts (Fig. 2A). Under the stronger oxidizing effect of DMSO (Clark *et al.*, 2008), enhanced by the higher temperature and longer extraction time of this procedure, the co-extracted phenolic compounds are easily oxidized producing the brown colorations that interfere during the photometric measurements.

In the alternative extraction with aqueous-acetone, absorption at 750 nm was negligible. $\text{Chl}_a/\text{Chl}_b$ ratios obtained for current and previous year leaves were in the range of 2.5-4.4

Table 1. Average \pm standard deviation of pigment concentration in current and previous year leaves. Same leaf extracted with acetone and DMSO simultaneously. N=10 for each age class and solvent type. Results from ANOVA test, different letters in the same column indicate significant differences ($\alpha < 0.01$).

Type of leaves	Solvent	Absorption 750 nm	Chl _a /Chl _b	Chl _a ($\mu\text{g}/\text{cm}^2$)	Chl _b ($\mu\text{g}/\text{cm}^2$)	carotenoids ($\mu\text{g}/\text{cm}^2$)	Chl _{a+b} ($\mu\text{g}/\text{cm}^2$)
Current year	DMSO	0.065 \pm 0.008a	1.2 \pm 0.1a	77.3 \pm 6.3a	62.4 \pm 4.4a	20.7 \pm 3.0a	139.7 \pm 8.6a
	DMSO corrected ^[1]	0.000	1.6 \pm 0.2	67.2 \pm 6.1	41.2 \pm 4.0	20.7 \pm 3.0	108.4 \pm 7.7
	80 % acetone	0.002 \pm 0.001b	3.3 \pm 0.3b	64.5 \pm 9.5b	19.8 \pm 4.0b	19.1 \pm 2.8a	84.3 \pm 13b
Previous year	DMSO	0.065 \pm 0.012a	1.5 \pm 0.1a	100.7 \pm 10.1a	68.5 \pm 6.8a	29.2 \pm 3.2a	169.2 \pm 15.1a
	DMSO corrected ^[1]	0.000	1.9 \pm 0.1	90.6 \pm 10.1	47.4 \pm 4.2	29.2 \pm 3.2	137.9 \pm 13.6
	80 % acetone	0.002 \pm 0.001b	2.9 \pm 0.2b	95.7 \pm 12.3a	32.6 \pm 4.9b	29.6 \pm 3.0a	128.4 \pm 17.0b

^[1] Absorbance at 665.1, 649.1 and 480 nm subtracted by the 750 nm absorbance readings

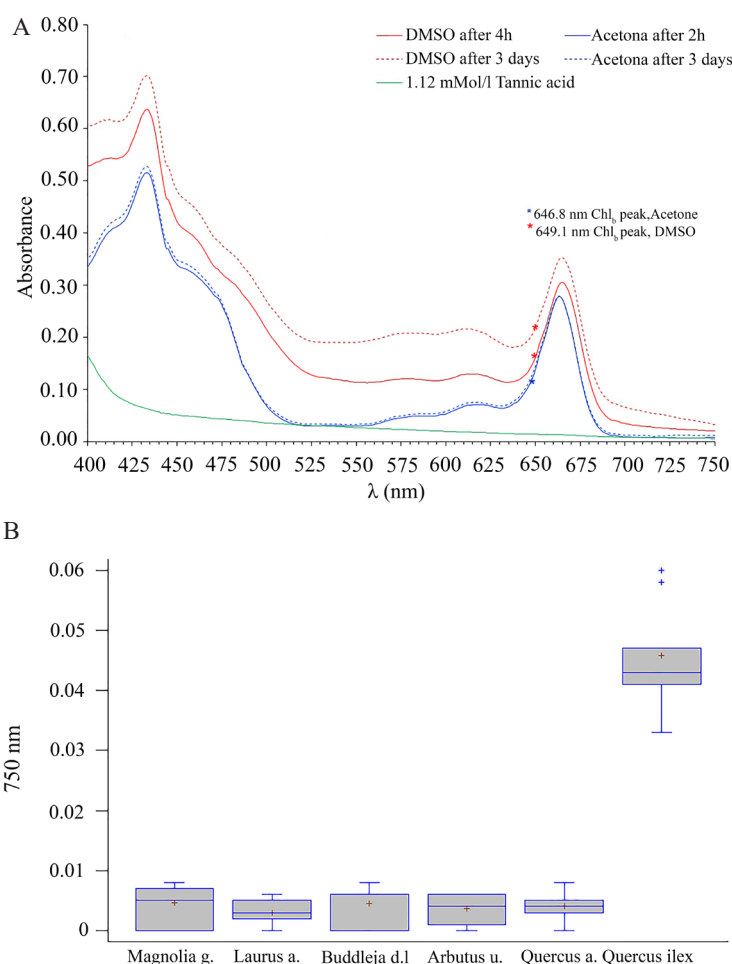


Figure 2. (A) VIS spectra of two pigment extracts of the same *Quercus ilex* leaf performed by the two extraction procedures: 4 h extraction of a 6 mm foliar disc in 5 mL DMSO at 65°C (continuous red line); 2 h extraction of a 6 mm foliar disc in 5 mL of 80% acetone at 3°C (continuous blue line). Spectrum of the same extracts 3 days after extraction (red and blue dotted lines); DMSO extract preserved in the dark at room temperature (13-15°C) and aqueous-acetone extract preserved at -20°C. Tannin acid VIS-spectrum in DMSO (continuous green line). (B) Absorption readings at 750 nm of DMSO extracts (4 h extraction at 65°C) in leaves of different forest and shrub species (*Laurus nobilis*, *Buddleja davidii*, *Arbutus unedo*, *Magnolia grandiflora*, *Quercus agrifolia* and *Quercus ilex*). Nine leaves extracted per species.

characteristics of sun-exposed leaves (Lichtenthaler, 1987). Chls concentration during the vegetative period varied in the range 18-119 $\mu\text{g}/\text{cm}^2$ (Table S3 [suppl]). High SPAD-pigment calibration correlations ($\mu\text{g}/\text{cm}^2$) were obtained for all pigments: $R^2=0.90$, $\text{RMSE}=0.16$, $p<0.0001$ for Chl_a ; $R^2=0.91$, $\text{RMSE}=0.16$, $p<0.0001$ for Chl_b and $R^2=0.90$, $\text{RMSE}=0.02$, $p<0.0001$ for carotenoids ($N=167$). The calibration equations obtained for the sum of Chl_{a+b} and carotenoids in $\mu\text{g}/\text{cm}^2$ were: $\text{Chl}_{a+b}=\exp(1.8244+0.0408*\text{Spad})$ and $C_{x+c}=(0.2907-0.0039*\text{Spad})^{-1}$. Filtered tissue powder after extraction was completely decoloured (Fig. S1 [suppl]). The weaker oxidizing properties of aqueous-acetone, the low temperature and the shorter extraction time prevented the oxidation of co-extracted polyphenolic compounds when this extraction method was used.

DMSO extracts of several perennial forest and shrub species were additionally analysed to confirm our results (Fig. 2B). All species had absorbance at 750 nm under 0.010 with the exception of *Q. ilex* that exhibited the maximum values (0.050). Furthermore, the ratio $\text{Chl}_a/\text{Chl}_b$ was under 2 only in the *Q. ilex* samples. The differences in foliar phenolic concentration between species (Hermes & Hedges, 2004) could explain the differences in the rapid development of colored interferences under the same oxidizing conditions. Analysis of total polyphenols using the Folin-Ciocalteu procedure in forest and shrub species extracted by DMSO were performed (mg gallic acid (GA) per gram dry leaf tissue). The *Q. ilex* leaves had the highest total polyphenol contents of all samples analyzed: 18.8 mg/g GA while all other leaf species were in the range 2.4 mg/g GA (*Laurus nobilis*) to 7.2 mg/g GA (*Arbutus unedo*). The development of colored interferences in photometric pigment determinations by DMSO could be also possible in leaves of other forest species with high polyphenol contents.

As conclusions, the use of the DMSO solvent should be avoided for photometric pigment analysis in *Quercus ilex* leaves. Cold extraction of finely homogenized foliar material with aqueous-acetone, free of interferences is recommended as we have proved that it provides more reliable pigment concentrations.

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