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Alternative Methods for Sampling and Preservation of Photosynthetic Pigments and Tocopherols in Plant Material from Remote Locations

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Abstract Current methods for the study of pigments involve freezing in liquid nitrogen and storage at –80 °C or lyophilization until HPLC analysis. These requirements greatly restrict ecophysiological research in remote areas where such resources are hardly available. We aimed to overcome such limitations by developing several techniques not requiring freezing or lyophilization. Two species with contrasting foliar characteristics (*Olea europaea* and *Taraxacum officinale*) were chosen. Seven preservation methods were designed, optimized and tested in a field trial. These protocols were compared with a control immediately frozen after collection. Pigments and tocopherols were analyzed by HPLC. Main artifacts were chlorophyll epimerization or phaeophytinization, carotenoid isomerization, altered de-epoxidation index and tocopherol degradation. Among all methods, sample desiccation in silica-gel provides robust samples (pigment composition was unaffected by storage time or temperature) and almost unaltered pigment profiles, except for a shift in epoxidation state.Although liquid nitrogen freezing and subsequent lyophilization or freezer storage were preferred, when these facilities are either not available or not suitable for long-distance transport, desiccation with silica-gel, passive extraction in acetone, and/or storage of fresh samples in water vapour saturated atmospheres, enable a complete pigment characterisation. Silica-gel is advisable for long-term sample conservation.

Key words: HPLC · liquid nitrogen · lyophilization · silica gel · xanthophylls

Introduction

After the development of resolutive chromatographic techniques, several hundreds of papers dealing with the functions of photosynthetic pigments (carotenoids and

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chlorophylls) in plants have been published during the last 25 years. These studies have revealed the ubiquitous presence of two chlorophylls (*a* and *b*) and six carotenoids (β-carotene, lutein, neoxanthin, antheraxanthin, zeaxanthin and violaxanthin) in photosynthetic tissues of plants and green algae (Young et al. 1997a). Besides, chloroplasts of some higher plants also contain significant amounts of non-ubiquitous carotenoids: lactucaxanthin, lutein-5,6-epoxide, α-carotene or rhodoxanthin. A large number of these studies have been focused on wild plant species, aiming to establish ecophysiological and adaptative role of chloroplastic pigments. As a result, a wide range of habitats and floras have been surveyed, including tropical and subtropical environments (Logan et al. 1996; Thiele et al. 1998; Barth et al. 2001; Tausz et al. 2001), deserts (Barker et al. 1998; 2002), high mountains (Streb et al. 1998; Zarter et al. 2006), deciduous forests (Niinemets et al. 1998), Mediterranean vegetation (Kyparissis et al. 1995) or boreal ecosystems (Slot et al. 2005).

An inherent limitation to biochemical field studies is that leaf samples must be preserved and safely transported from the field to the analysis laboratory. Typically, the analysis of photosynthetic pigments implies the immediate freezing of leaf samples in the field by the use of liquid nitrogen (Abadía and Abadía 1993; Young et al. 1997b). After deep-freezing and prior to analyses, samples are either stored at –80 °C in a freezer and/or in dry ice, or lyophilized and stored at room temperature (Tausz et al. 2003). The former method guarantees the absence of chemical transformations or pigment degradation, but it is extremely sensitive to cold chain failures. Another limitation of this method lays on the fact that on aircrafts, dry ice and liquid nitrogen are highly restricted as hold luggage, and not permitted as hand luggage for security reasons. The second method, lyophilization, implies the freezing of the samples and the sublimation under vacuum below the triple point. If applied correctly, this technique

provides rather robust samples that can be stored at room temperature and they are also easier to grind obtaining a homogeneous powder. In fact, the main goal of lyophilization is to enhance stability, decrease temperature sensitivity and improve shelf life of the resultant product (Cherian and Corona 2006).

The two methods explained above require some facilities nearby the field study site: liquid nitrogen, dry ice or a lyophilizer, which are hardly available in remote areas and difficult to transport, limiting enormously this kind of ecophysiological studies. Thus, with few exceptions (Thiele et al. 1998; Barth et al. 2001), almost all field studies on photosynthetic pigments have been performed in a limited number of countries where such technical facilities are easily available. In fact, when major biomes such as the tropical rainforest, have been investigated, researchers have frequently used plants growing in university campuses (Shiefthaler et al. 1999) or in artificial environments such as Biosphere 2 (Matsubara et al. 2005), and only rarely in the field (Matsubara et al. 2008).

Although several different analytical procedures have been described for carotenoid determinations in plant tissues (de las Rivas et al. 1989; Thayer and Björkman 1990; Gilmore and Yamamoto 1991; Abadía and Abadía 1993; García-Plazaola and Becerril, 1999; Young et al. 1997b), up to now there is no alternative method reported for sample preservation from collection to analysis. Therefore, aiming to overcome such limitation, the objective of the present study is to develop protocols for collection, transport, shipping and storage of leaf samples for pigment analyses in field sites where at least some of the standard method requirements are not met. We are aware that the best way to preserve plant material is by freezing it in liquid nitrogen immediately and store it at –80 °C, but also that this protocol limits greatly the geographical scope and, consequently, our knowledge. Several procedures for sample collection, transport and

storage have been described and tested in the present work: liquid nitrogen sampling and freezer storage (control; method A), *in situ* extraction with acetone (method B), passive extraction with acetone (method C), conservation with dimethyl sulfoxide (DMSO; 3% or 10%; methods D and E, respectively), liquid nitrogen sampling and lyophilization (method F), passive desiccation with silica gel (method G), and fresh samples storage in the dark in a water vapour saturated atmosphere (method H). Methods A and F have been employed by other authors (Tausz et al. 2003), while others have never been tested before. Finally, the main purpose of the present work is to guide the selection of the most reliable and suitable field method for different operational scenarios and research goals.

Methods

Plant material, sampling and experimental design

For all experiments two species with contrasting foliar morphology were used: a mesophytic herb *Taraxacum officinale* L. and a sclerophyllous xerophytic tree *Olea europaea* L. Except when indicated 3 mm ø discs were used. Eight different protocols were employed and evaluated for pigment and tocopherol conservation, transport and storage. One of them (method G) was also tested in a wider range of species and at different temperatures and times. Briefly:

-Method A (used as control): leaf discs were collected, immediately frozen in liquid nitrogen, and stored at –80 °C. For extraction, frozen leaf discs were ground in liquid nitrogen, homogenized and extracted with 2 ml ice-cold 100% acetone buffered with calcium carbonate.

-Method B (*in situ* acetone extraction): leaf discs were collected and immediately homogenized, extracted in the field site with the buffered acetone described for method A and filtered. Extracts were transported, and stored at room temperature for 48 h until HPLC analysis.

-Method C (passive acetone extraction): leaf discs were collected, immediately submerged in 2 ml of the buffered acetone described for method A, transported and stored at room temperature within 48 h before extraction. In the laboratory, leaf discs were homogenized and extracted with their own acetone as described in method A.

-Method D (DMSO 3%): leaf discs were collected, submerged in a 3% solution of dimethyl sulphoxide (DMSO) in water (v/v), transported and stored at room temperature within 48 h before extraction. After storage, discs were extracted with 2 ml of buffered acetone as in method A.

-Method E (DMSO 10%): As method D, but 10% DMSO in water (v/v) was used.

-Method F (Lyophilization): leaf samples were collected and immediately frozen in liquid nitrogen. Frozen material was lyophilized for at least five days, transported and stored protected from light and humidity until extraction. Samples for pigment determination were extracted as in method A.

-Method G (silica gel): leaf discs were collected and stored in paper envelopes filled with silica gel until extraction and transported to the analytical laboratory. Samples for pigment determination were extracted as in method A. In order to describe sample drying kinetics in this method, a different batch of leaf discs of both species were weighed and then stored in silica gel within paper envelopes. Then, two independent samples per species were weighed and removed at 2, 5, 10, 15, 30, 60, 120 and 250 min. The loss of weight in the box with silica was attributed to dehydration and compared with the loss of weight in an oven at 65 ºC for 24 h assuming this one as dry weight. Results were used for pigment concentration corrections. Fourth-order polynomials were fitted to relative percent moisture contents on a dry weight basis over time (adjusted $R^2 > 0{\cdot}95$). Drying rates were calculated as the first derivative of the fitted curves. This method was also validated for an additional set of ten species covering a wide taxonomic range: one liverwort (*Lunularia cruciata*), one fern (*Pteridium aquilinum*), one conifer (*Pinus radiata*), four schlerophyllous (*Buxus sempervirens, Quercus ilex*, *Eucalyptus globulus, Ocotea foetens*), one mesophytic (*Lonicera peryclimenum*) and two monocots (*Cortaderia setigera, Paspalum dilatatum*). The effects of temperature (from -20 °C to 40 °C) and storage time (1-4 weeks) were also tested for this method.

-Method H (*in vivo* storage): whole leaves were transported and stored in sealed plastic bags under a water vapour saturated atmosphere. After a dark period (12-16 h) at room

temperature (emulating pre-dawn conditions) leaves were sampled, frozen in liquid nitrogen and stored in liquid nitrogen as described in method A.

In order to test the reliability of each proposed method for pigment storage and conservation, a field trial comparing all these eight protocols simultaneously was performed in Tenerife (Canary Islands) in December 2007. This site was selected because of the technical facilities available at the University of La Laguna (lyophilizer and liquid nitrogen), the need of long-distance air transport from the field site to the chromatography laboratory, and because of the presence in the Canary Islands of the species used in a previous appraisal. With this trial we also aimed to highlight unexpected negative effects derived from storage conditions during air transport (i.e. related with particular environmental conditions within the aircraft). In this trial, conservation protocols were assayed within 48 hours after field collection, except in method F (five days) and in method H (16 h).

Analytical methods

All chromatographic analyses were performed at the Department of Plant Biology and Ecology of the University of the Basque Country in Bilbao (Spain), except for the optimization of method G, which was done at the Universidad Complutense of Madrid (Spain). Extracts obtained as in method A were centrifuged at 15000 *g*, and filtered through a 0.2 μm PTFE syringe filter (Waters). During all the extraction procedure, light was avoided and samples were maintained in a cool box. Extracts were injected (15 μL) in a reverse-phase Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001). The 717 plus autosampler was equipped with a thermostat which maintains temperature constant at 4 °C avoiding pigment degradation. Photosynthetic pigments were measured with a PDA detector (Waters model 996),

except tocopherols and phaeophytins that were quantified with a fluorescence detector (Waters model 474) set to λ_{exc}=295 nm and λ_{em}=340 nm for tocopherols and to λ_{exc}=413 nm and λ_{em}=669 nm for phaeophytins.

Several parameters, indicative of the feasibility of each protocol were compared: total chlorophyll content (including phaeophytins and epimeric derivatives), chlorophyll a/b ratio, formation of chlorophyll epimers (epimerization), formation of phaeophytin (phaeophytinization), xantophyll cycle pool (VAZ) to chlorophyll ratio, de-epoxidation index (A+Z/VAZ), total carotenoid to chlorophyll ratio, individual carotenoid (neoxanthin, lutein and β-carotene) to chlorophyll ratio, formation of carotenoid isomers (isomerization) and α-tocopherol to chlorophyll ratio. Ratios to chlorophyll are expressed on a total chlorophyll basis (including phaeophytins and epimers).

Statistical analysis

In this study, *in-situ* freezing and transport of leaf discs in liquid nitrogen served as the control method, *i.e.*, pairwise comparisons were made between this method and the alternative procedures tested for every pigment trait (content or percentage of alteration). All these comparisons were made by means of two-way analyses of variance (species x method). In the case of significant interaction between factors, departure from control in any of the study species was enough to reject the validity of the alternative method for the trait tested. All data were tested for normality and homogeneity of variances, log-transformed, if necessary, and when failed to meet ANOVA assumptions were analyzed using non-parametric Kruskal–Wallis ANOVA. The resulting *P* values were considered to be statistically significant if less than 0.05. Bonferroni correction for multiple pairwise comparisons was not applied to minimize the risk of a type II error, that is, the error of failing to reject the tested method when its

results differ significantly from those of the control. Calculated p-values, coefficients and regression lines are indicated on the figures whenever significant at $P > 0.01$. The statistical analyses were performed using Statistica (Statsoft, Inc., Tulsa, OK, USA) and SPSS 16.0 statistical package.

Results

Characterization of chemical alterations during storage and transport.

Deficient sample conservation generates several chemical alterations of pigment composition. These artifacts can be illustrated by Fig. 1, which shows HPLC patterns of *O. europaea* leaves stored using different extraction and conservation methods, in comparison with leaves frozen in liquid nitrogen (method A). Assays with *T. officinale* yielded similar results.

In the case of carotenoids, the most conspicuous effect was isomerization, characterized by a stoichiometric conversion of carotenoid isomers. This process affected all carotenoids, but was mostly noticeably for *cis*-N to *trans*-N and *trans*-L to *cis*-L transformations (methods B and C). Some treatments also led to a shift in de-epoxidation state of xanthophyll cycle. This can be observed in methods G and E (increase) and method H (decrease). Finally, a net degradation (not counterbalanced by isomer formation) was observed, especially in epoxidated xanthophylls (N, V and A) that were the most sensitive carotenoids to such alteration.

Degradation of chlorophyll was also remarkable in some treatments. In some cases, this effect led to a concomitant formation of chlorophyll epimers (methods B and C) or phaeophytins (methods D and E). In the DMSO treatments (methods D and E), phaeophytin formation did not compensate for the overall loss of chlorophyll, suggesting other degrading processes and a remarkable alteration of chlorophyll *a/b* ratio. In the case of α-tocopherol, we did not find any degradation product, but its content decreased significantly in DMSO treatments.

Method optimization

Preservation protocols compared in this study were previously optimized for our experimental conditions and for the two species with contrasting leaf characteristics. This was particularly relevant in the case of silica gel (method G). Drying kinetics of both species showed that drying rates decreased continuously from the beginning of storage in silica gel, which is indicative of a high drying intensity (Kemp et al. 2001). Drying rates exhibited an initial fast falling rate period followed by a slower one. Transition between these periods occurred at a leaf moisture content of roughly 10% of the initial value, which was reached at 50 and 85 min in *T. officinale* and *O. europaea*, respectively. Despite its speed, the drying process resulted in an artifactual increase in the de-epoxidation index. Different leaf disc sizes (ranging from 1 to 6 mm ø) were tested to avoid this artefact, but any significant effect was found neither in the de-epoxidation index, nor in the pigment profiles (data not shown). Thus, desiccation in silica provided reliable results for almost all analytical parameters. This fact, together with the novelty of the method, leads us to verify the suitability of this method under varied conditions. Therefore, the effects of temperature (in the range -20 to 40 °C) (Fig. 2) and storage time (up to one month) (Fig. 3) were also tested for this method. Pigment contents were not affected by storage temperature when compared with samples desiccated at room temperature (20 °C). When the effect of time was analysed, pigment composition also remained stable for the first three weeks of storage in silica (Fig. 3). During the fourth week, Chl a/b ratio and the contents of VAZ, N, β-C and L decreased significantly. The silica method was also tested through a wide taxonomic range, including ten species ranging from liverworts to monocots. As it is shown in figure 4, pigment concentrations from leaves stored in silica gel or frozen in liquid nitrogen were in most cases strongly correlated with slopes close to 1 in most cases. As observed in *T.*

officinale and *O. europaea*, the only exception to the adequate conservation of pigment profiles in silica gel, was the A+Z/VAZ ratio that suffered a species-dependent shift to higher values. Rates of chlorophyll epimerization (0.39 ± 0.04 % for silica and 0.38 ± 0.08 % for liquid nitrogen) and carotenoid isomerization (2.25 ± 0.26 % for silica and 1.99 ± 0.13 % for liquid nitrogen) were low and similar in both treatments. Main chemical artefact observed, when compared both methods was in the rate of phaeophytinisation (1.97 ± 1.29 % in silica vs. 0.53 ± 0.08 % in liquid nitrogen). This effect was due to the high formation of phaeophytin in needles of *Pinus radiata* desiccated in silica gel (13.51%).

Passive and *in situ* extractions in pure acetone were also tried (methods B and C). The effect of the addition of antioxidants (ascorbate or tocopherol) on pigment conservation was tested, but no significant improvement was found (data not shown). Temperature and duration of storage were critical for sample conservation. Storage temperature affected sample conservation by increasing the formation of chlorophyll epimers, although carotenoid isomerization was basically temperature independent (Fig. 5).

In the case of conservation with DMSO, two different DMSO concentrations were tested 3% (method D) and 10% (Method E). Despite the apparent external preservation of structures, pigment profiles were strongly affected even at the lower DMSO concentration, with a large loss and degradation of carotenoids and chlorophylls. Both buffered DMSO solutions yielded essentially the same results.

Conservation of fresh leaves in a water - saturated atmosphere was performed following previously described protocols (Tausz et al. 2003). Samples were stored up to a maximum of 20 h in the dark, with no significant changes in pigment composition, except for an expected decrease in de-epoxidation index.

Comparative test

The field study performed in Tenerife (Canary Islands) allowed us to recreate an overseas experimental field study and to analyse some previously untested factors, such as the effects of aircraft conditions on sample stability. We did not find any restriction to the transport of samples, except for liquid nitrogen that was shipped through a freight carrier in a special container (Voyageur, Air Liquide) that follows the IATA regulations.

Pigment composition of control samples is shown in Table 1. For both species, samples were collected at full sunlight, as it is shown by the presence of significant amounts of A and Z. Content of chlorophylls, photoprotective pigments (L, VAZ and β-C) and tocopherol were significantly higher in *O. europaea* in consonance with its higher xerophily.

Results of this comparative test are summarized in Table 1. They clearly confirm that the use of DMSO affects almost all analyzed compounds. Extraction in pure acetone (passive or *in situ*) also affected significantly several components, but at least in this experiment, they had the advantage of maintaining the de-epoxidation index stable, due to a uniform degradation of the xanthophyll cycle components. Lyophilization also maintained tocopherol, carotenoids and A+Z/VAZ stable, but was not able to prevent the formation of chlorophyll derivatives. Desiccation in silica gel and storage in a water vapour saturated atmosphere, yielded acceptable results, except for the de-epoxidation index, in both methods, and phaeophytinization, in silica gel storage, that differed significantly from control values. As observed during the optimization process, desiccation tended to increase the de-epoxidation index, while in a water vapour saturated atmosphere, xanthophyll cycle recovered almost completely overnight. De-

epoxidation index and chlorophyll alteration were thus the most sensitive parameters studied (Fig. 6).

Discussion

Despite the general cautions taken in this study (e.g. avoidance of light, use of buffered solutions; Abadía and Abadía 1993; Young et al. 1997b), most of the alternative methods tested for conservation, transport and storage of pigment samples led to unreliable results due to artifacts. Among the latter ones, we identified formation of chlorophyll epimers, phaeophytins and carotenoid isomers. In some treatments (most noticeably in those using DMSO) formation of these products did not compensate for the overall loss of pigments, indicating the involvement of other degradation processes. These effects have been described by other authors in acetonic extractions (Hyvärinen and Hynninen 1999; van Leeuwe et al. 2006) in which pigments degradation is lower than in other solvents such as methanol, but chlorophyll transformation into allomers and epimers cannot be completely prevented.

We initially tested the possibility of using acetone as a preservative-stabilizer of leaf samples. Pure acetone is a good extraction solvent for a wide range of pigment polarities, and its use (pure and/or mixed with water) is generalized in plant studies (Dunn et al. 2004). Since extractions for HPLC studies can be done on fresh or frozen samples, and acetone extracts can be stored for long time at –20 °C without apparent changes (Abadía and Abadía 1993), we tried to verify how stable were samples extracted and/or stored in acetone. We found a strong formation of chlorophyll epimers and carotenoid isomers that was temperature-dependent irrespective of the mode of conservation (methods B and C). Thus, this method could be useful only when sample storage in a freezer is possible. Interestingly, all components of the xanthophyll cycle were altered in the same proportion irrespective of temperature, maintaining stable the de-epoxidation index. The DMSO solvent has been also used as an extraction medium alternative to acetone (Barnes et al 1992), and could be potentially used to stabilize

pigment composition in leaf samples. Among all methods tested, conservation in DMSO solutions (3% or 10%), generated the highest number of artefacts, with dramatic loss of pigments, and chemical transformations.

Overnight storage of leaf samples at room temperature in a water-saturated atmosphere has been widely used as a method to provide artificial predawn conditions in comparative studies (Tausz et al. 2003). In the present work, we have increased storage time to 18 h, and the only modification with respect to control leaves was an expected relaxation of xanthophyll cycle. This approach presents an excellent option for those experiments in which determination of de-epoxidation index is not necessary and is possible to freeze the samples in less than 24 hours, preferably within 16 hours. This storage time is roughly equivalent to the duration of a night period. Longer incubations should be reappraised for each species and experimental design.

A major outcome of this work is the development of a protocol based on sample desiccation with silica gel that provided unexpectedly good results. The only artefact that was regularly observed was the modification of the deepoxidation index. The high reliability of the results, together with the simple sample handling makes this method suitable for field screenings particularly common in ecological and evolutionary research. In these studies, the accurate determination of the de-epoxidation index is often of secondary interest, as it usually requires all samples to be collected within a restrictive time interval (i.e. predawn or midday). This new method does enable pigment studies in remote locations since samples can be desiccated at a wide range of temperatures and stored for at least three weeks. However it must be noted that this method should be reappraised for each experimental design, as was observed in needles of *Pinus radiata*, in which a high phaeophytin formation took place.

More than suggesting a unique alternative method to sample freezing, this work aims to generate an array of technical procedures that can be used depending on technical limitations, and scientific objectives. These proposals organized as a dichotomous key are summarized in Table 2. Following this scheme our suggestion is to use the standardized liquid nitrogen and storage at –80 °C whenever possible, but in case of transport restrictions (as in many air companies that reject dry ice or liquid nitrogen even in hold luggage), lyophilization is the recommended choice. In fact, the efficiency of extraction can be even significantly higher in freeze-dried samples (Tausz et al., 2003; van Leeuwe et al. 2006). When none of these two methods is feasible due to working restrictions, we recommend storage and transport of fresh leaves in plastic bags saturated with water vapour when the time between collection and analysis can be less than 20 h, and desiccation with silica gel when the time required for transportation is more than 20 h. When determination of the de-epoxidation state of xanthophyll cycle is needed, these methods should be combined with *in situ* extractions in acetone.

In conclusion, our findings show that the study of key functional traits, as leaf contents in chlorophylls, photoprotective pigments and tocopherols, is not limited by liquid-nitrogen availability or transport. Besides the optimal protocol (i.e. immediate freezing in liquid nitrogen followed by lyophilization or storage at –80 °C), easy sample transport and storage in saturated atmosphere or after being desiccated in silica gel can provide valid and reliable measurements of most traits. Eventually, these methods can be complemented with determinations of the de-epoxidation index after *in situ* acetonic extraction. We conclude that the array of methods and sampling strategies proposed in the present paper contribute to expand the range of plant populations, communities, species, ecosystems and regions eligible to be explored from an ecophysiological perspective.

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Table 1 Mean (± standard error, n = 5) leaf contents in chlorophylls and carotenoids, and percentages of pigment alteration (epimerization, phaeophytinization, and isomerization), across two contrasting species (*Olea europaea* and *Taraxacum officinale*) obtained with different methods (see text for further details). Total chlorophylls include both *a* and *b*, plus their epimers and phaeophytins. Carotenoid contents also include their isomers. Letter code indicates significant differences (ANOVA, $P < 0{\cdot}05$) between species in the control treatment (in bold). Pairwise ANOVA comparisons between methods revealed either non-significant (NS) or significant ($P < 0{\cdot}05$) differences from controls. These differences were considered significant if found in any of the two study species. In the case of significant departure of the alternative method from control, the percentage of overestimation (positive values) or underestimation (negative values) of the tested trait is shown. Asterisk (*) denotes that this percentage significantly differed between species (significant interaction between species and method; $P < 0{\cdot}05$).

Pigment	A *In situ* liquid-nitrogen freezing (control)		B Acetone *in situ* extraction	C Acetone passive extraction	D DMSO 3%	E DMSO 10%	F Lyophilization	G Silica gel	H *In vivo* storage
	O. europaea	*T. officinale*							
Total Chlorophylls (μmol m^{-2})	**395•46 ± 51•21** [a]	**221•82 ± 13•90** [b]	NS	NS	NS	NS	NS	NS	NS
Chlorophyll *a/b* (mol mol^{-1})	**2•75 ± 0•07** [a]	**2•58 ± 0•04** [a]	-2	+6 *	-20 *	-33 *	NS	NS	NS
Chlorophyll *a* epimerization (%)	**0•28 ± 0•05** [a]	**0•42 ± 0•04** [a]	+662 *	+1524 *	NS	+54	+299 *	NS	NS

Phaeophytinization (%)	**0•43 ± 0•01** [a]	**0•45 ± 0•06** [a]	+52 *	+310 *	+6022 *	+8054 *	+38	+28	NS
VAZ (mmol mol^{-1} chl)	**178•98 ± 28•18** [a]	**105•14 ± 5•80** [b]	-34	-26	-24	-39	NS	NS	NS
A+Z/VAZ (mol mol^{-1})	**0•67 ± 0•03** [a]	**0•37 ± 0•04** [b]	NS	NS	-1 *	+20 *	NS	+15	-78 *
β-Carotene (mmol mol^{-1} chl)	**131•62 ± 6•70** [a]	**113•80 ± 1•10** [b]	-36 *	-11 *	-17	-27	NS	NS	NS
Lutein (mmol mol^{-1} chl)	**148•40 ± 6•65** [a]	**122•67 ± 1•45** [b]	-17	NS	NS	NS	NS	NS	NS
Neoxanthin (mmol mol^{-1} chl)	**45·35 ± 1·89** [a]	**40•34 ± 0•87** [b]	-11	-14	-4 *	NS	NS	NS	NS
Carotenoid Isomerization (%)	**3·05 ± 0·55** [a]	**2•52 ± 0•14** [a]	+729 *	+619	+149	+169	NS	NS	NS
α-Tocopherol (mmol mol^{-1} chl)	**2090•57 ± 290•59** [a]	**62•13 ± 12•42** [b]	-32	-32	-65 *	-63 *	NS	NS	NS

Table 2 Dichotomic key for guiding the choice of the most suitable protocol for a given technical limitation or research goal.

1a. Operating conditions and facilities enable *in situ* immediately liquid nitrogen freezing……..……..2
1b. Samples can not be frozen *in situ*……..……3

2a. Samples can be transported in liquid nitrogen or dry ice to the analysis laboratory……....**Method A**
2b. Samples can not be frozen-transported but a lyophilizer is available in a support laboratory…….**Method F**

3a. Less than 20 h between sample collection and analysis……... 4
3b. More than 20 h between sample collection and analysis……..…… 5

4a. A+Z/VAZ is to be measured …….**Methods B+H**
4b. A+Z/VAZ is not measured ……..……**Method H**

5a. A+Z/VAZ is to be measured……..**Methods B+G**
5b. A+Z/VAZ is not measured……..…….**Method G**

Fig. 1 Examples of HPLC carotenoid and tocopherol profiles at 445 nm (left side) or fluorescence signal (right side) in extracts from leaves of *O. europaea* obtained by different procedures: *In situ* freezing with liquid nitrogen, (method A), acetone passive extraction (method C), DMSO 10% (method E), lyophilization (method F), silica gel (method G), in vivo storage (method H). Methods B and D did not differ qualitatively from C and E, respectively. Absorbance at 445 nm. *c*-N, *cis*-neoxanthin; *t*-N *trans*-neoxanthin; *c*-V *cis*-violaxanthin; *t*-V *trans*-violaxanthin; *c*-A, *cis*-anteraxanthin; *t*-A, *trans*-anteraxanthin; *c*-L, *cis*-lutein; *t*-L, *trans*-lutein; *t*-Z *trans*-zeaxanthin; Ch *b*, chlorophyll *b*; e-chl *b*, chlorophyll *b* epimer; Ch *a*, chlorophyll *a*; e-chl *a*, chlorophyll *a* epimer; *c*-β-C, *cis*-β-carotene; *t*-β-C, *trans*-β-carotene; Ph *a*, phaeophytin *a*; Ph *b*, phaeophytin *b*; e-Ph *a*, phaeophytin *a* epimer; α-T, α-tocopherol.

Fig. 2 Effect of storage temperature on photosynthetic pigments and α-tocopherol of leaf samples kept in silica gel from collection to analysis. Statistical significance was determined using a two-way ANOVA, with species (*O. europaea* and *T. officinale*) and temperature (-20, 4, 20, 40 ºC) as fixed factors. Two-way interaction was never significant. Data are presented as across-species means (± SE, n = 10). Different letters denote significant ($P < 0{\cdot}05$) differences between temperature treatments (Tukey's HSD test after significant ANOVA results).

Fig. 3 Effect of storage time on photosynthetic pigments and α-tocopherol of leaf samples kept in silica gel from collection to analysis. Statistical significance was determined using a two-way ANOVA, with species (*O. europaea* and *T. officinale*) and time (from 7 to 28 days) as fixed factors. Two-way interaction was never significant. Data are presented as across-species means (± SE, n = 10). Different letters denote

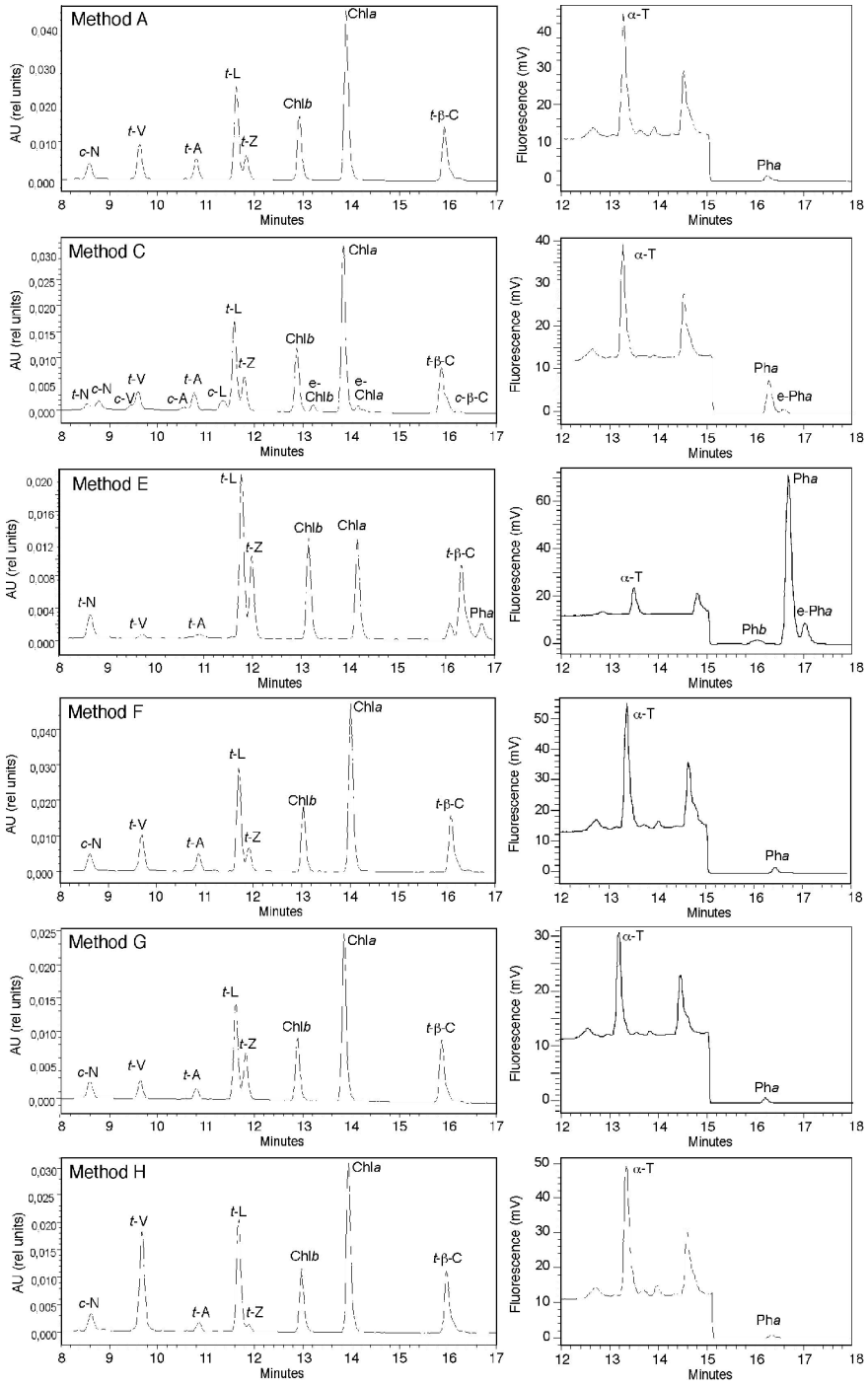
significant ($P < 0{\cdot}05$) differences between storage periods (Tukey's HSD test after significant ANOVA results).

Fig. 4 Relationships between pigment composition in samples from ten different plant species frozen in liquid nitrogen or desiccated in silica gel. Dotted line represents the theoretical optimal regression with slope 1. Data are presented as the mean for each species (±SE, n=5). All correlations were significant at $P < 0.01$ except for A+Z/VAZ ($P = 0.051$).

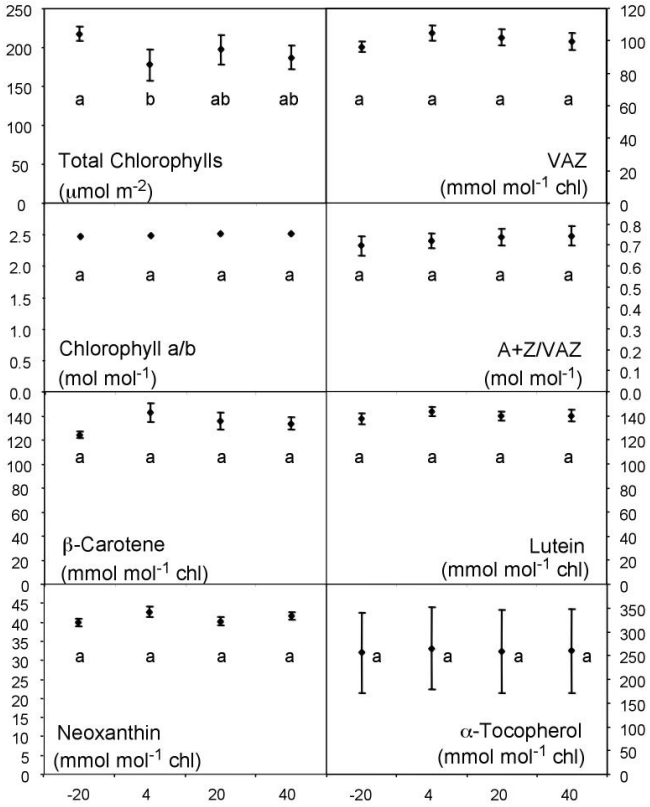
Fig. 5 Temperature dependence of chlorophyll epimers and carotenoid isomers formation in relation to control. Rates of *cis*-lutein and chlorophyll *a* epimer formation in passive acetonic extracts of *T. officinale* and *O. europaea* leaves are shown. Similar trends were found for neoxanthin, violaxanthin and chlorophyll *b*.

Fig. 6 Relationships between A+Z/VAZ in control samples, and samples extracted *in situ* in acetone, stored fresh in a water vapour saturated atmosphere, desiccated in silica or lyophilized. Determination error increases with distance from diagonal. Filled symbols correspond to *O. europaea* and empty symbols to *T. officinale*.

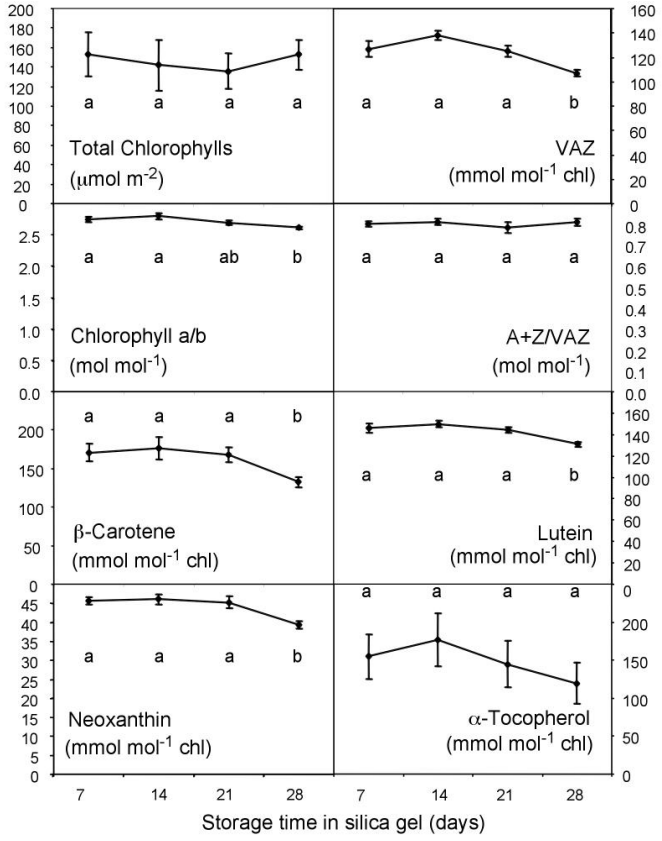
1 Figure 1


Method A
AU (rel units)
Minutes
c-N
t-V
t-A
t-L
t-Z
Chlb
Chla
t-β-C
α-T
Pha
Fluorescence (mV)
Method C
t-N
c-N
c-V
c-A
c-L
e-Chlb
e-Chla
c-β-C
e-Pha
Method E
Phb
Method F
Method G
Method H

2

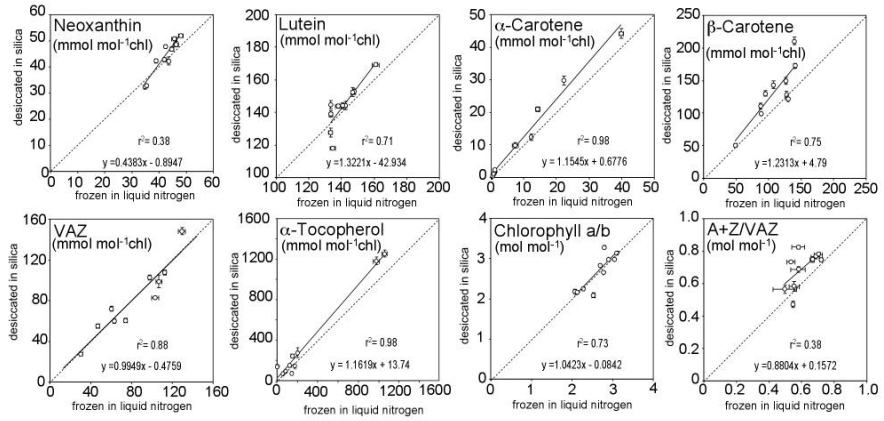

Total Chlorophylls
(μmol m-2)
a b ab ab
VAZ
(mmol mol-1 chl)
a a a a
Chlorophyll a/b
(mol mol-1)
a a a a
A+Z/VAZ
(mol mol-1)
a a a a
β-Carotene
(mmol mol-1 chl)
a a a a
Lutein
(mmol mol-1 chl)
a a a a
Neoxanthin
(mmol mol-1 chl)
a a a a
α-Tocopherol
(mmol mol-1 chl)
a a a a
-20 4 20 40 -20 4 20 40
Storage temperature in silica gel (°C)

1


Total Chlorophylls
(μmol m-2)
VAZ
(mmol mol-1 chl)
Chlorophyll a/b
(mol mol-1)
A+Z/VAZ
(mol mol-1)
β-Carotene
(mmol mol-1 chl)
Lutein
(mmol mol-1 chl)
Neoxanthin
(mmol mol-1 chl)
α-Tocopherol
(mmol mol-1 chl)
Storage time in silica gel (days)

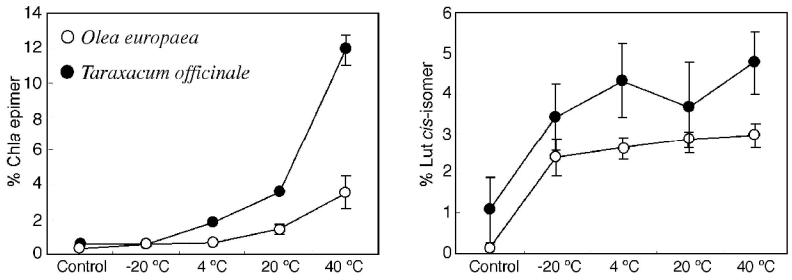
1

1 Figure 4.


Neoxanthin
(mmol mol-1chl)
r2= 0.38
y =0.4383x - 0.8947
Lutein
(mmol mol-1chl)
r2= 0.71
y =1.3221x - 42.934
α-Carotene
(mmol mol-1chl)
r2= 0.98
y = 1.1545x + 0.6776
β-Carotene
(mmol mol-1chl)
r2= 0.75
y =1.2313x + 4.79
VAZ
(mmol mol-1chl)
r2= 0.88
y =0.9949x - 0.4759
α-Tocopherol
(mmol mol-1chl)
r2= 0.98
y = 1.1619x + 13.74
Chlorophyll a/b
(mol mol-1)
r2= 0.73
y =1.0423x - 0.0842
A+Z/VAZ
(mol mol-1)
r2= 0.38
y =0.8804x + 0.1572
desiccated in silica
frozen in liquid nitrogen

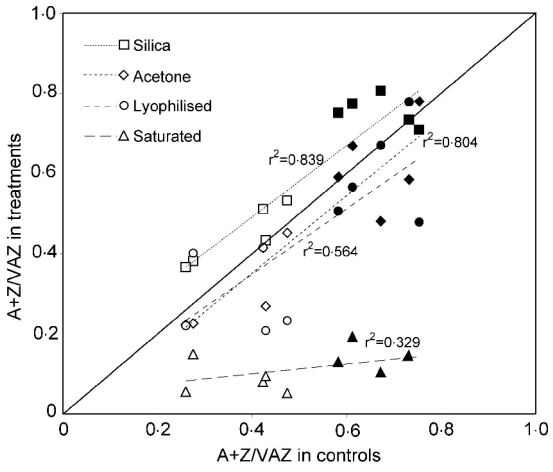
2

1 Figure 5.


Olea europaea
Taraxacum officinale
% Chla epimer
% Lut cis-isomer
Control
-20 °C
4 °C
20 °C
40 °C

2

1 Figure 6.


1·0
0·8
0·6
0·4
0·2
0
0
0·2
0·4
0·6
0·8
1·0
A+Z/VAZ in treatments
A+Z/VAZ in controls
Silica
Acetone
Lyophilised
Saturated
r²=0·839
r²=0·804
r²=0·564
r²=0·329

2

3