Chapter 3
Plant Photosynthetic Pigments: Methods and Tricks for Correct Quantification and Identification

Beatriz Fernández-Marín, José Ignacio García-Plazaola, Antonio Hernández, and Raquel Esteban

1 Introduction to Photosynthetic Pigments

1.1 Leaf Composition of Photosynthetic Pigments

Chloroplast of green photosynthetic tissues in the Viridiplantae (monophyletic group that includes green algae and terrestrial plants) is characterised by a relatively conserved composition of pigments (Esteban et al. 2015). Leaves of virtually all plant species invariably contain chlorophyll (Chl) *a* and Chl *b*, and six carotenoids. Five of them are xanthophylls (carotenoids that contain oxygen): neoxanthin (Neo), lutein (Lut), violaxanthin (Vio), antheraxanthin (Ant) and zeaxanthin (Zea). The remaining carotenoid is a carotene (no oxygen in the molecule): β-carotene (β-Car). Additionally, certain taxa contain a second carotene: α-Car, which partially substitutes β-Car in some species under low light environment (Young and Britton 1989; Esteban and García-Plazaola 2016). Some species phylogenetically unrelated also include lutein epoxide (Lx), a xanthophyll likewise related to shade acclimation (Matsubara et al. 2005; Esteban et al. 2009b). Regarding pigment concentration, in agreement with the relatively conserved composition of pigments across green photosynthetic organisms, and because the maximum Chl concentration per leaf is limited by specific and physiological constrains (Niinemets 2007), photosynthetic pigments are restricted within specific ranges of concentrations. Highly reliable ranges of pigment content for non-stressed plants that were obtained from two databases comprising more than 800 species can be found in (Esteban et al. 2015; Fernández-Marín et al. 2017) (summarized as reference in Table 3.1).

B. Fernández-Marín (✉) · J. I. García-Plazaola · A. Hernández · R. Esteban
Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Bilbao, Spain
e-mail: beatriz.fernandezm@ehu.es

A. M. Sánchez-Moreiras, M. J. Reigosa (eds.), *Advances in Plant Ecophysiology Techniques*, https://doi.org/10.1007/978-3-319-93233-0_3

Table 3.1 Reliable ranges for chlorophyll contents and photosynthetic pigment ratios of non-stressed plants and their expected trends under high light or stress conditions

	Chl *a* + *b* (μmol m^{-2})	Chl *a* + *b* (μmol g^{-1} DW)	Chl *a*/*b* (mol mol^{-1})	Neo/Chl (mmol mol^{-1})
Non-stressed	145–800	0.2–15.8	2.2–4.2	24–65
Trend under high light or stress	Decrease	Decrease	Rise	Quite stable

	VAZ/Chl (mmol mol^{-1})	AZ/VAZ (mol mol^{-1})	Lut/Chl (mmol mol^{-1})	β-Car/Chl (mmol mol^{-1})
Non-stressed	22–177	0.05–0.35	68–283	27–157
Trend under high light or stress	Rise	Rise	Quite stable	Rise

Derived from (Esteban et al. 2015) and (Fernández-Marín et al. 2017)

Table 3.2 Spectral maxima of main photosynthetic pigments from green plants

	Chl *a*	Chl *b*	Neo	Vio	Lx	Ant	Lut	Zea	α-Car	β-Car
MaxAbs	435	469	437	441	441	446	446	451	446	451
λ(nm)	666	656	466	471	471	476	476	481	471	480

Wavelengths correspond to extracts in acetone obtained by the Photo-Diode Array (PDA) detector of the HPLC (García-Plazaola and Becerril 1999)

1.2 Location and Functions in the Chloroplast

Individual photosynthetic pigments have specific locations and functions within the photosynthetic apparatus. Chlorophyll *a* is located in the reaction centres (RCs) and the antennae (light harvesting complexes, LHCs) of both photosystems (PSI and PSII). Chlorophyll *b*, by contrast, is only bound to LHCs (Croce 2012). Both Chls show slightly different absorption spectra (Table 3.2, see Sect. 2.2) and function as the main light collectors in PSI and PSII. Carotenoids, on the other hand, play multiple roles in photosynthesis: first as light harvesters by broadening the spectrum of light collected by Chls (thanks to their absorbance of blue and blue-green light and to their capacity to transfer the absorbed energy to Chl); and second, as photoprotectants due to their ability to quench singlet oxygen and triplet Chl under excess light conditions. Additionally, they take part in the assembly of photosystems, thereby, altering the structure and function of the photosynthetic apparatus. Moreover, a relatively small fraction of the xanthophyll molecules, is not directly bound to any protein complex (i.e. is free in the lipidic membranes), where directly participate in thylakoid membrane stabilization (Havaux 1998; Dall'Osto et al. 2007b; Polívka and Frank 2010). Such is the remarkable case of some molecules of Zea (Havaux et al. 2004; Gruszecki and Strzalka 2005; Dall'Osto et al. 2010). β-Carotene is mainly found in the core complexes of PSII and PSI and also in LHCI, where it has an important role as quencher of singlet oxygen and triplet Chls (Dall'Osto et al. 2007b; Cazzaniga et al. 2012, 2016), while xanthophylls are mostly bound to antenna complexes (Moradzadeh et al. 2017).

Lutein is the most abundant xanthophyll in the photosynthetic apparatus and is located in LHCs. It can directly quench triplet Chls (Dall'Osto et al. 2006) but additionally plays a crucial role in the stability of LHC trimers (Lokstein et al. 2002) and has a photoprotective role (Dall'Osto et al. 2007a).

Neoxanthin is thought to play mainly a structural role in the assemblage of antenna protein complexes. It is located in the periphery of LHCII where it scavenges singlet oxygen (Dall'Osto et al. 2007a), but additionally, it has been evidenced very recently that Neo competes with Vio in the binding to LHCII, influencing the inter-conversions of Vio to Ant and Zea in the so-called VAZ-cycle (Wang et al. 2017). The conversion of Vio towards Ant and Zea is related to conformational changes in the antennae and with enhanced dissipation of energy as heat, that overall has an important photoprotective role in the photosynthetic apparatus (Demmig-Adams 1998; Johnson et al. 2011).

In parallel to the VAZ-cycle, an inter-conversion from Lx to Lut can also take place in some species (particularly common among some families, as Lauraceae) mainly acclimated to low light environments (i.e. forest understorey) in the so called LxL-cycle (Esteban et al. 2009c; 2010; Esteban and García-Plazaola 2014).

1.3 Dynamics of Photosynthetic Pigments in Response to the Environment

One of the most remarkable features of plant photosynthetic pigments is that their composition and proportion are highly dynamic (in particular, in response to changes in light intensity, and in general, in response to any stress factor), reflecting changes in photosynthetic and photoprotection processes. Environmental stresses (e.g. low temperature, drought, desiccation, salt stress, nutrition deficit, pollutants etc.) depress photosynthesis and consequently, lead to an excess of energy absorbed by Chls that cannot be converted into photochemistry. On a daily scale, cycles of synthesis/degradation of antenna components (including Chls) (Fukushima et al. 2009) and inter-conversion of xanthophylls within the VAZ-cycle (Demmig-Adams et al. 1996) or within the LxL-cycle (Esteban et al. 2009b) occur. The first (present in all the species from the Viridiplantae studied until now) consists on the conversion (de-epoxidation) of Vio into Zea via Ant under stress (i.e. excess irradiance at midday). Under non-stress (i.e. at night), the opposite reaction takes place, giving rise to VAZ-cycle that usually operates following a diurnal rhythm (day/night). This cycle modulates the efficiency of light energy conversion protecting the photosynthetic apparatus from photodamage but also being able to reduce plant productivity up to 20% under fluctuating light conditions (Kromdijk et al. 2016).

A second xanthophyll cycle, the LxL-cycle operates when a sunfleck suddenly increases the irradiation incident on seedling leaves. A fraction of the Lx molecules are de-epoxidised to Lut, which could enhance dissipation and photoprotection in addition to Zea. In contrast to the VAZ-cycle, the re-epoxida-

tion of Lut back to Lx under low light conditions is much slower and it has probably a pre-emptive role in case of repetitive sunflecks (Esteban et al. 2010). The operation of the VAZ-cycle under stressors other than light has been evidenced upon desiccation/rehydration cycles and also under anoxia or heat stress (Fernández-Marín et al. 2009, 2011a, b). Weather the same response is developed by LxL cycle or not, remains still unexplored. Apart from light stress, endogenous circadian rhythms are also among regulatory factors of chlorophyll and carotenoid contents in leaves on a daily basis (García-Plazaola et al. 2017).

Variations in photosynthetic pigments, estimated either as absolute content (per leaf area or per leaf mass) or as ratios (relative amounts of some pigments in comparison to others), can be extremely informative if few considerations are taken into account before data interpretation. First: location of the pigments in the LHCs and/or the RCs; second, if they are usually bound to protein complexes or free in the thylakoid membranes, and third, what their functions are. Thus, differential pigment contents (and proportions) can be found after analysis, regarding leaf acclimation (i.e.: sun *vs* shade exposition) or stress level. Probably due to their large amounts and to its important structural role in the photosynthetic apparatus Neo content is among the most stable under stress (Esteban et al. 2015; Fernández-Marín et al. 2017). By contrast, total Chl content, Chl *a*/*b* ratio, VAZ/Chl and to higher extent AZ/VAZ (de-epoxidated state of VAZ-cycle (Ant+Zea)/(Vio+Ant+Zea)), are among the most variable parameters either in response to high irradiance or to stress. Thus, when comparing sun with shade-exposed leaves, or stressed with non-stressed plants, Chl content is generally lower, while Chl *a*/*b*, VAZ/Chl and AZ/VAZ ratios are higher. Low temperature and drought are among the abiotic stresses inducing more evident changes in the VAZ-cycle. Thus, winter acclimation and also desiccation of photosynthetic tissues can lead to high levels or AZ/VAZ even if stress occurs in the dark (Esteban et al. 2009a; Fernández-Marín et al. 2009, 2011b, 2013; Míguez et al. 2017). Finally, different pigment composition can also reflect inter-specific or inter-ecotypes differences on leaf structure, anatomy and morphology (Camarero et al. 2012; Esteban et al. 2015; Fernández-Marín et al. 2017).

Different methodological approaches allow for the identification and quantification of photosynthetic pigments. Two main types of methodologies can be distinguished: (i) analytical procedures that imply destructive sampling, and (ii) optical methods that allow non-destructive assessment of photosynthetic pigments (Fig. 3.1).

While analytical procedures require the extraction of leaf pigments in an organic solvent, several optical methods can be applied over intact plants at different scales from leaf to landscape. Near-Infrared Reflectance Spectroscopy (NIRS) represents a methodology in between, since it requires destruction of samples, but non-extraction is needed once calibration for a certain sample-type is done. In this chapter, both invasive and non-invasive approaches will be covered with special focus on the analytical (U)HPLC procedure, for which a detailed and reproducible method is specifically provided.

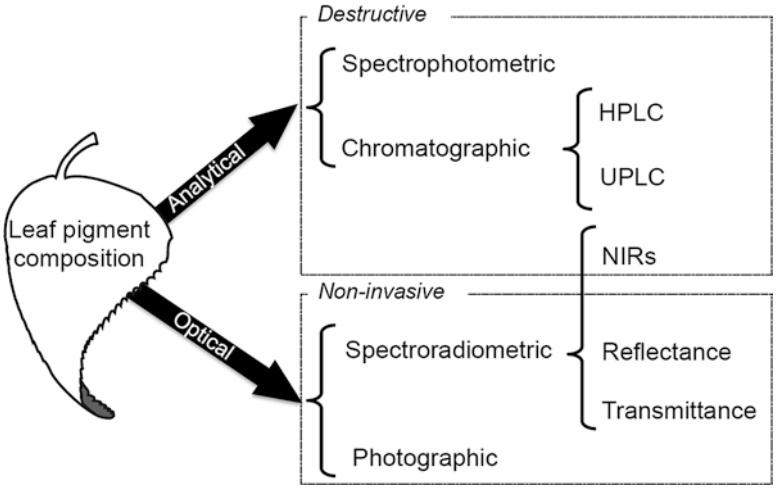

Fig. 3.1 Summary of main procedures currently available for the quantification of photosynthetic pigments: analytical, as spectrophotometric and chromatographic, and optical, as spectroradiometric and photographic

2 Destructive Methods to Quantify Photosynthetic Pigments

2.1 Sample Collection, Storage and Grinding

2.1.1 Sample Collection

When researchers are planning an experiment, in which the determination of chlorophylls and carotenoids is foreseen, special care should be taken at different steps of the procedure. Proper sample collection, (mainly in field studies) is particularly critical since leaf biochemical composition may vary enormously, as a result of individual differences and changing environmental conditions (as explained in the Sect. 1). This step is, therefore, extremely important due to the intrinsic capacity of pigments to quickly respond to the environmental fluctuations (i.e. within seconds (Peguero-Pina et al. 2013)) and the highly variable conditions in field studies. Primarily determinant for changes in the photosynthetic pigments are light, temperature and drought (Esteban et al. 2015; Fernández-Marín et al. 2017). Under this prerequisite, sample collection should be, whenever possible, performed under comparable conditions in order to exclude undesired variations (Tausz et al. 2003) (Fig. 3.2). This means that sampling must preferably be conducted at the same time of the day, sun orientation, relative position of leaf within the crown, etc. As an example, under non-stressful circumstances, north oriented leaves could be sampled at predawn from the lower part of the crown (i.e. low AZ/VAZ will be expected, Fig. 3.2). All these things considered, immediate freezing of leaf sample in liquid nitrogen is strongly recommended to prevent biochemical modifications, whenever available. If liquid nitrogen is not available, rapid desiccation of small leaf samples

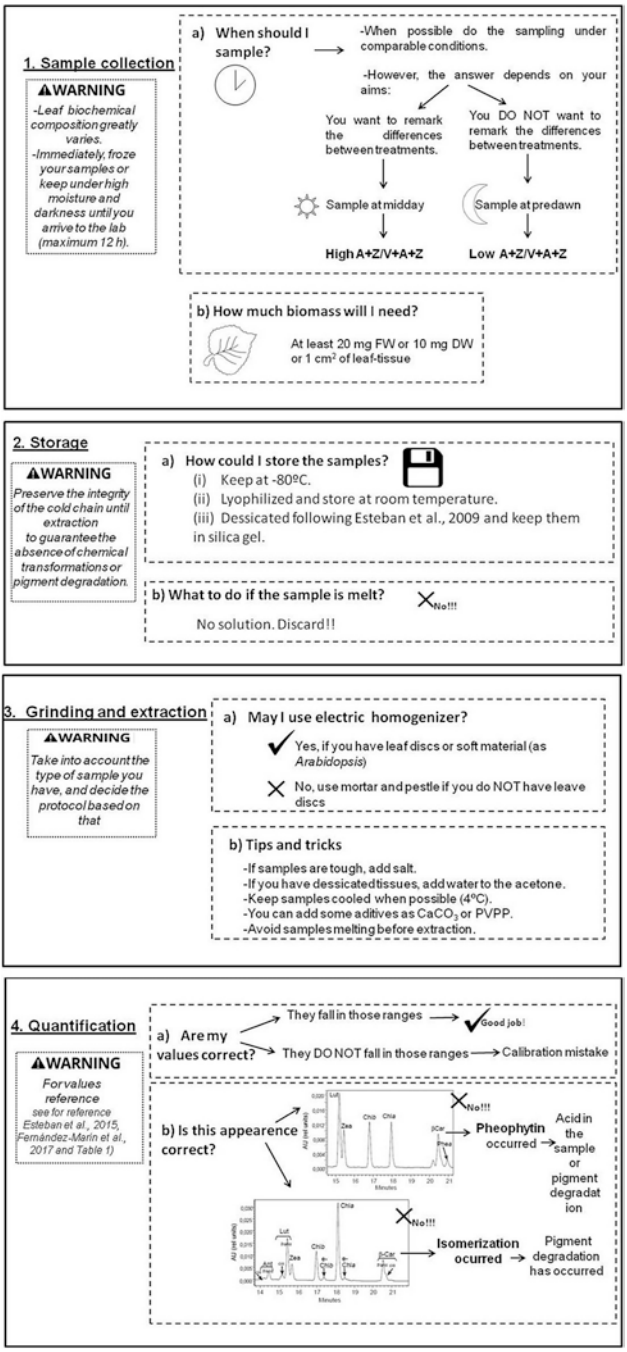

Fig. 3.2 Summary of the procedure for photosynthetic pigment analysis by (U)HPLC, including tricks and tips for a successful assessment

by using silica-gel is a secondly commendable approach, although non-reliable de-epoxidation index will be obtained during the quantification (Esteban et al. 2009a) (Fig. 3.2).

Alternatively, whenever the individual content of Ant, Vio, Zea or the AZ/VAZ are irrelevant for the aim of the experiment, scinded leaves can be collected in the field, keep under saturated atmosphere (to avoid dehydration) overnight and collect samples for pigments in the lab (this procedure is sometimes referred to as "artificial predawn"). When using liquid nitrogen all safety protocols need to be strictly followed.

2.1.2 Storage of Samples

After freezing, plant material should be stored at −80 °C until analyses (Young et al. 1996). Special care has to be taken to prevent sample melting during transfer from Dewar flask to the freezer since keeping the cold chain until the extraction is absolutely crucial to prevent any chemical transformations and/or pigment degradation. If a sample accidentally melts, even for a few seconds, irreversible chemical modifications may occur, altering its chemical composition. This sample should be then discarded for the pigment analyses (Esteban et al. 2009a) (Fig. 3.2). As an alternative to storage at −80 °C, samples can be lyophilized, which implies freezing of the samples and the subsequent sublimation of water under vacuum below the triple point (the temperature and pressure at which the three phases gas, liquid and solid of a substance coexist in thermodynamic equilibrium) (Cherian and Corona 2006). This procedure stabilises and improves the storability of the samples that can be preserved with silica gel at room temperature for several days, and at −20 °C for months. Even so, storage in the dark is recommended to avoid photo-deterioration of the pigments. Finally, when liquid nitrogen is not available (as in many remote areas), samples can be alternatively desiccated (explained in Sect. 2.1.1) and stored in silica according to the procedure of (Esteban et al. 2009a) (Fig. 3.2).

2.1.3 Commendable Amount of Sample and Methods for Grinding

Physical properties of the photosynthetic tissue of interest (e.g. leaf type: broad-leaf, needle, etc.; or the phylogenetic group of the species, etc.) represent another major critical point during sampling. Depending on this, the optimal amount of tissue collected for each sample, the grinding procedure (by mortar and pestle or with electronic devices such as homogenizer, mill, or dismembrator) and/or the final pigment expression (per fresh or dry weight, per area or per chlorophyll) may vary (Fig. 3.2). For a typical broad leaf and 1 mL volume of final extraction, approximately 20 mg fresh weight, 10 mg dry weight, or 1 cm^2 of leaf-tissue, are recommended per replicate.

Conservation protocols explained above generate two types of samples: desiccated (and consequently, dehydrated) or frozen (and hydrated), which differ in

chemical terms by the presence of water. This water is very important in the extraction procedure to facilitate the extraction of most polar pigments such as some xanthophylls (e.g. Neo, or Vio). For frozen samples, which contain water, pure organic solvent is used, but lyophilized or desiccated samples should be extracted with water-diluted organic solvents 95–98%.

2.2 Spectrophotometric Assay of Photosynthetic Pigments

Photosynthetic pigments are among the few plant metabolites that absorb light in the visible range. Furthermore, low polarity of these pigments allows relatively high specificity in the extraction and prevents interference with other coloured metabolites such as anthocyanins or betacyanins, which are hydrophilic. Consequently, its quantitative determination by spectroscopic techniques should be possible in crude pigment extracts. Given the fact that Chl *a* and *b* absorb red light at distinct wavelengths and that the absorbance of carotenoids is negligible in this range (Table 3.2), quantification of both Chls in green leaf extracts containing a mixture of photosynthetic pigments should be easy. In the 1940s, the first spectrophotometric equations for the simultaneous determination of Chl *a* and Chl *b* became available, and in fact those described by (Arnon 1949) are still in use by many scientists. However, several decades later it was evidenced that Arnon equations were inaccurate and were replaced by newer and more precise ones (Porra 2002). Nowadays, a wide range of equations optimized for the spectroscopic properties of chlorophylls and carotenoids extracted in different organic solvents (pure acetone, 80% acetone, chloroform, diethyl ether, dimethyl sulphoxide, methanol, 90% methanol, dimethyl formamide, ethanol and 95% ethanol) are available (see Table 3.3). Furthermore, some of them have been optimized for its use with spectrophotometers of high and low resolution (0.1–0.5 nm or 1–4 nm, respectively (Wellburn 1994).

In a basic protocol, pigment quantification starts with the grinding of plant material and extraction of photosynthetic pigments with an organic solvent (see Sect. 2.1.3). When other non-photosynthetic pigments are present, this step is usually enough to eliminate them from the extract, preventing any potential interference. The protocol simply finishes with the determination of absorbance at several (usually 3) wavelengths that represent the average maximum absorbance of carotenoids (470 nm), Chl *b* (642–653 nm) and Chl *a* (660–666 nm).

This method allows the estimation of the bulk of carotenoids, but the exact composition of each individual carotenoid cannot be estimated in the pigment mixture. The quantification of each carotenoid in principle would require separation analysis by HPLC (see Sect. 2.3). However, some alternative protocols, based on multi-wavelength measurement, have been developed to quantify spectrophotometrically each carotenoid on pigment mixtures (Küpper et al. 2007). Nevertheless, the usefulness of this method is limited by the fact that it does not allow a reliable estimation of carotenoids with identical absorption spectra, such as Zea and β-Car, of great interest for physiological or nutritional studies.

Table 3.3 Equations for the simultaneous determination of Chl *a* and Chl *b* in different organic solvents

Solvent	ABS λ_1	ABS λ_2	Factor A1	Factor A2	Factor B1	Factor B2	Refs
Acetone	661.6	644.8	11.24	2.04	20.13	4.19	*3*
	662	645	11.75	2.35	18.61	3.96	*4*
Acetone 90%	664	647	11.93	1.93	20.36	5.5	*2*
Acetone 80%	663	645	12.7	2.69	22.9	4.68	*1*
	663	646	12.21	2.81	20.13	5.03	*4*
	663.2	646.8	12.25	2.79	21.5	5.1	*7*
	663.6	646.6	12.25	2.55	20.31	4.91	*5*
Chloroform	656.6	647.6	11.47	2	21.85	4.53	*7*
	666	648	10.91	1.2	16.38	4.57	*7*
Diethylether	660.6	642.2	10.05	0.97	16.36	2.43	*3*
	662	644	10.05	0.766	16.37	3.14	*4*
	662	644	10.1	1.01	16.4	2.57	*6*
	662	644	10.05	0.89	16.37	2.68	*8*
Diethylether Water free	660	641.8	9.93	0.75	16.23	2.42	*3*
Diethylether Water sat.	661.6	643.2	10.36	1.28	17.49	2.72	*3*
DMFA	663.8	646.8	12	3.11	20.78	4.88	*5*
	664	647	11.65	2.69	20.81	4.53	*7*
DMSO	665	649	12.19	3.45	21.99	5.32	*7*
	665.1	649.1	12.47	3.62	25.06	6.5	*7*
Ethanol	665	649	13.95	6.88	24.96	7.32	*4*
Ethanol 95%	664.2	648.6	13.36	5.19	27.43	8.12	*3*
Methanol	665.2	652.4	16.72	9.16	34.09	15.28	*7*
	665.2	652	16.29	8.54	30.66	13.58	*5*
	666	653	15.65	7.34	27.05	11.21	*4*

References cited in the table: 1. Arnon (1949), 2. Jeffrey and Humphrey (1975), 3. Lichtenthaler and Buschmann (2001), 4. Lichtenthaler and Wellburn (1983), 5. Porra et al. (1989), 6. Smith and Benitez (1955), 7. Wellburn (1994), 8. Ziegler and Egle (1965). Table shows wavelengths and factors for the two following general equations:

Equation 1: **Chl *a* (μg/mL) =** (Factor A1 × ABS λ_1)–(Factor A2 × ABS λ_2)

Equation 2: **Chl *b* (μg/mL) =** (Factor B1 × ABS λ_2)–(Factor B2 × ABS λ_1)

Overall, the spectrophotometric assay of pigment composition is a simple, fast and reliable when is properly carried out. However, pigment studies are particularly prone to the occurrence of errors (as revised in (Fernández-Marín et al. 2015) caused by the careless use of analytical protocols. In the following lines, some typical problems, frequently encountered during the pigment determinations, are described, together with some alternatives:

- **Low extraction yield**: The extraction yield is not optimal. Differences among solvents, extraction procedures and species properties may account for unrealistic pigment composition in the extract. Optimize the grinding (the finer the

powder obtained, the easier the extraction will be) and check always the best extraction medium for each type of plant material.

- **Water in the tissue**: When extracting pigments from fresh or frozen leaves, a certain amount of water present in the tissue is unavoidably added to the extraction medium. This can have effects on the extraction yield of some pigments or in the spectroscopic properties of pigments (and consequently the accuracy of equations). If this issue needs to be solved the best option is to use only freeze-dried plant material that does not contain water. In any case, be aware of the polarity of the solvent (Fig. 3.2).
- **Turbidity**: A plant extract is turbid, and must be centrifuged to remove particles. Once the extract is completely clear, absorbance at 750 nm should be close to zero since photosynthetic pigments do not absorb at this specific wavelength. Nevertheless if, after the clearing process, some turbidity remains, consider that the lower the wavelength is, the higher the scattering of light will be. This effect causes a higher apparent absorbance and leads to an overestimation of Chl *b* respect to Chl *a* and of carotenoids respect to Chls. In some equations, this effect is compensated by subtracting A_{750}. However, the most straightforward way to correct this undesirable effect is to perform a new step of centrifugation and/or filtration.
- **Absorbance out of range**. To obtain realistic measurements, the absorbance range should be 0.3–0.85 (Lichtenthaler and Buschmann 2001). If not, the accuracy or the linearity of the measurement decreases. At this point the solution is to concentrate (by evaporation) or dilute the sample. If some previous measurements can be done, is always better to adjust the extraction volume and the amount of plant tissue to the desired range of absorbance values. Fluxing cold extracts with a gas N_2 gas stream accelerates the evaporation of solvent (concentration of the sample) with minimum risk for sample deterioration.
- **Presence of anthocyanins and other pigments**. The presence of anthocyanins (and other red pigments) is common in leaves from stressed plants. When these pigments cannot be completely excluded from the extract, their quantification equations can be modified by a term that considers and subtracts the residual absorbance of anthocyanins (i.e. A_{537}) (Sims and Gamon 2002).
- **Solvent and equation used do not match each other**. A problem commonly found in the literature is that the solvent used for the extractions is not exactly the same as the one used to derive the equations. Since the spectroscopic properties of pigments, that settle the base for the development of equations, vary (sometimes dramatically) with the nature of solvent, the correspondence between equation and solvent should always be checked directly in the original source, where the method is described. Be critical and avoid using and copying of the method that has been always used in your lab.

2.3 (U)HPLC Method to Assess Photosynthetic Pigment Composition

2.3.1 Generalities About (U)HPLC Methodology for Pigment Quantification

Liquid chromatography is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. High performance liquid chromatography (HPLC) is one of the best options to characterise the exact pigment composition in a plant tissue, and it is the standard method to quantify individual carotenoids. An alternative to HPLC is UHPLC that refers to ultra high performance liquid chromatography. This is an evolution of the former HPLC systems that offers several advantages, such as higher peak capacities, smaller peak widths, enhanced sensitivity and higher chromatographic resolution (Maurer et al. 2014). The shorter runtimes also considerably save mobile phase solvents. Nevertheless, UHPLC also has disadvantages in comparison with traditional HPLC: i.e. is more sensitive to complex matrix in the sample, and to buffers of the extraction medium.

An (U)HPLC is a computer-guided instrument that includes at least: mobile phase reservoirs and pumps, injector system, column (usually inserted in a thermoregulable module) and detector. A few reservoirs hold the solvents (mobile phase) that are fluxed by a high-pressure pump at a specified flow rate. An injector (sample manager or autosampler) is able to introduce the sample into the flowing mobile phase stream. This carries the sample into the HPLC column that contains the chromatographic packing material (called the stationary phase because it is held in place), where the separation of different metabolites from the sample takes place. The compounds will elute from the HPLC column at different times and will be detected by the detector.

Liquid chromatography for measuring photosynthetic pigments commonly uses a photodiode array (PDA) detector with UV–visible absorption detection to measure absorbance of individual pigments, once they have been separated in a reversed-phase column. Reversed phase chromatography uses a hydrophobic stationary phase (polymeric C18 or C30 particles into the column), with a strong affinity for hydrophobic compounds, as carotenoids and chlorophylls; and a polar mobile phase (commonly an aqueous-organic mixture). A gradient system using two different solvents (first the polar one, second the hydrophobic) optimizes the elution of the pigments. As a result, most hydrophobic molecules as β-carotene in the polar mobile phase tend to adsorb to the hydrophobic stationary phase and are retained for longer time during their pass through the column. Consequently, more polar molecules (such as xanthophylls) have higher affinity for the mobile phase and will pass faster through the column. As a result more polar molecules will elute quicker (at shorter retention times) than apolar pigments. Separation of pigments typically last around 30 min in HPLC and about 5 min in UHPLC (although this times are tremendously variable depending on the method, column length, and solvent flow used).

2.3.2 Example of a Reliable Method for Pigment Quantification by HPLC

Several (U)HPLC methods are available in the bibliography to determine carotenoids and Chls. Most of them use reversed phase HPLC systems with a visible light absorption detector and allow reproducible results when conditions (solvents, flow, column characteristics, etc.) are reproduced (Gilmore and Yamamoto 1991; Maurer et al. 2014; Junker and Ensminger 2016b). Here we show a HPLC/UHPLC method derived from (García-Plazaola and Becerril 2001). This method includes a UV-fluorescence detector for the determination of tocopherols in addition to pigments in the same injection. However, because of the scope of this chapter, we will refer only to the pigment analysis. In the next lines, we detail the procedure, some preventive measures and tricks to achieve good results in pigment quantification using HPLC.

- **Extraction**

  1. Main factor that can undesirably alter pigments during extraction are: light, high temperature and acids. So, keeping the extract cold, protected from direct light and from exposure to acids is recommended along the extraction and injection procedure. Extract fresh or frozen leaf sample in ice-bathed 100% acetone (its polarity index provides a good compromise for extracting both the most and the least hydrophobic pigments). In case of freeze-dried samples is commendable to proceed with a double extraction starting with 95% acetone and re-suspending the pellet in a second extraction with pure acetone, what leads to a final extract of 97.5% acetone on average. This will allow good yield-extraction of most polar (in the first extraction) and most apolar (in the second extraction) pigments. In order to prevent pigment degradation due to internal organic acids of the samples, the addition of $CaCO_3$ (0.5 g L^{-1}) in the extraction medium is commendable when HPLC is used (but must be avoided in UHPLC systems, since precipitation of the salt can obstruct the tubing). A suitable relationship between amount of plant material and extraction-medium should be used (typically 10–50 mg leaf fresh weight or 0.5–1 leaf cm^2 per mL). Soft fresh or frozen samples (i.e. small pieces of spinach or Arabidopsis leaves) can be directly homogenized with the extraction-medium by using an electric tissue homogeniser, or immediately before the extraction by using mortar and pestle. In this case, chill the mortar with liquid N_2 before adding samples and homogenise to powder just before adding the acetone. Collect the mixture in a 2 mL eppendorf tube. For tough samples you can either add a bit of sand into the mortar, or alternatively, freeze-dry the samples and powder them with a ball-mill or dismembrator before the extraction. In this case, after adding the acetone, vortex the sample vigorously for 10 s. Regardless the chosen extraction method, it is important to keep the samples refrigerated (≤4 °C) and to protect them from direct light (Fig. 3.2).
  2. Extract must be thereafter centrifuged at 4 °C and 16,000 g for 20 min to obtain the supernatant. The pellet can be re-extracted if it contains visible

chlorophylls (Fig. 3.2). If so, repeat the extraction step until the green colour of the pellet had gone and pool it together with the first supernatant.

3. Syringe-filtered the supernatant through a 0.22 μm PTFE filter. The first drops that pass the filter should be discarded to avoid contamination. Fill the HPLC vial and close it immediately with a cap. Extracts can be stored in the freezer (−20 °C) for few days but it is strongly recommended to immediately inject the samples in the HPLC. Long storage of extracts may provoke pigment isomerisation (Esteban et al. 2009a, b, c). The estimated time for the preparation of 24 samples (from extraction to insertion in the HPLC) is of ~2 h.
4. After each sample, wash the mortar, homogenizer, syringes, etc. generously with ethanol absolute or pure acetone.

- **HPLC conditions**

1. The mobile phase in (García-Plazaola and Becerril 2001) consists of two solvents. The solvent A, acetonitrile:methanol:water (84:9:7) v/v/v with TRIS-HCl buffer 10 mM pH 8 and the solvent B, methanol:ethyl acetate (68:32) v/v. Solvents must be HPLC-grade and it is recommended to filter them before using. Vacuum filtering also removes dissolved gases that otherwise could bubble along the chromatographic system. Most modern instruments ultrasonically eliminate gases from solvent when functioning.
2. Photosynthetic pigments are eluted using a linear gradient from 100% A to 100% B for the first 12 min, followed by an isocratic elution of 100% B for the next 6 min. This is followed by 1 min linear gradient from 100% B to 100% A. Finally, an isocratic elution with 100% A is established for a further 6 min, to allow the column to re-equilibrate with solvent A prior to the next injection (see Table 3.4 also for equivalences in case of UHPLC systems).
3. The solvent flow rate is 1.2 mL min^{-1} (0.5 mL/min for UHPLC).
4. Injected sample volume is 15 μL (1 μL for UHPLC).
5. HPLC chromatography is carried out in a Tracer Spherisorb ODS-1 reversed phase column (i.e.: Tracer Spherisorb or Waters® Spheridorb® ODS1). Column is 250 mm long with 4.6 mm diameter and 5 μm of particle size. The use of a guard column preceding the main column is strongly recommended to pro-

Table 3.4 Detailed HPLC gradient for the analysis of photosynthetic pigments after (García-Plazaola and Becerril 2001)

Step	Minutes	Flow (mL/min)	% A	% B
1	0	0	100	0
2	10 (2.4)	1.2 (0.5)	0	100
3	16 (3.6)	1.2 (0.5)	0	100
4	17 (3.8)	1.2 (0.5)	100	0
5	25 (5)	1.2 (0.5)	100	0

The corresponding modifications for its transfer to an UHPLC system are depicted in brackets within the table. Solvent A: acetonitrile 84%, Methanol 9%, H_2O-Tris (10 mM pH 8) 7%. LC-grade water instead of TRIS is recommended in solvent A for UHPLC systems. Solvent B: Methanol 68%, Ethyl acetate 32%

long its life-span and to enhance chromatographic separation (elution) of the pigments. An appropriate guard column would be Nova-Pak C-18 (50 × 3.9 mm; 4 μm). UHPLC column would be Waters® Acquity® UPLC HSS C18 SB (100 × 2.1 mm, 1.8 μm), with a frit filter (0.2 μm, 2.1 mm). Select the temperature of the column into the oven at 30 °C what usually guaranties repeatable separation conditions along the year in non-acclimated laboratories. High temperature accelerates the elution of the pigments but worsens the separation of some of them (i.e. Lut and Zea can be particularly tricky).

6. Each sample is scanned by the PDA in the range 250–700 nm, and peaks are detected and integrated at 445 nm for the quantification of carotenoids and Chls.

- **Pigment identification**

Once the chromatogram is obtained check that all pigments are present and sufficiently resolved (no overlapping peaks). A test of the system can be done by extracting pigments from a green leaf sample (such as spinach or Arabidopsis) and carefully assessing the chromatogram. All green leaves have at least 6 pigments and they should appear in the same order than in the method followed. With the method by García-Plazaola and Becerril (2001) the order of the pigments (with increasing retention times) is Neo, Vio, Lut, Chl *b*, Chl *a*, and β-Car (Fig. 3.3). Retention times and relative order of pigments may vary depending on the method (solvents, extraction medium, etc.). When leaves are illuminated or exposed to a severe stress prior to the sampling, two pigments add to the mentioned list: Ant, eluting between Vio and Lut, and Zea eluting close after its isomer Lut (Fig. 3.3). Peak-pigment identity is confirmed by comparing the visible absorption spectra of each peak (recorded with the PDA) to the literature. Although maximum wavelength of absorption may slightly vary depending on the solvents, most carotenoids and chlorophylls show a characteristic absorption spectrum that allows an almost unequivocal identification (Britton et al. 2004).

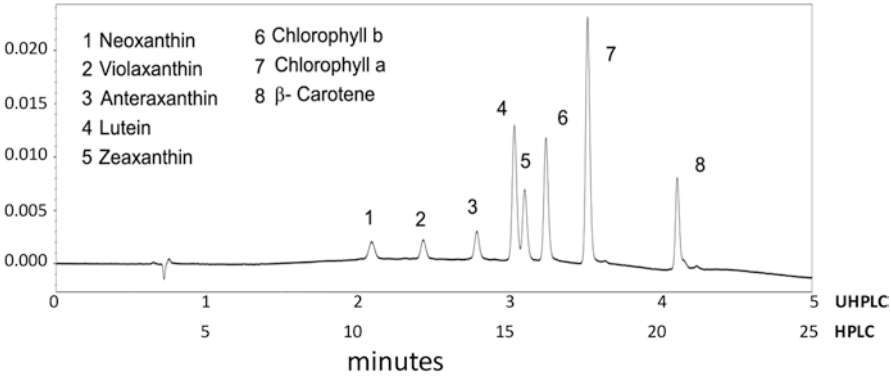

Fig. 3.3 Example of a pigment chromatogram integrated at 445 nm following the method by (García-Plazaola and Becerril 2001)

When a new method is being set up, a useful trick to check the resolution of Zea is to force a high content in the test-sample by placing a leaf under intense illumination for 15 min before sampling. When comparing the chromatograms from a dark-incubated and an illuminated leaf, Vio should decrease and concomitantly Zea should increase. If Zea does not appear or does not increase, it is likely that Zea and Lut (both xanthophylls are isomers) are eluting together. In fact, given the importance of Zea on plant responses, a bad separation of Lut and Zea is the most frequent problem that compromises the usefulness of a chromatographic protocol for ecophysiological studies.

- **Pigment quantification**

Although plant pigments have maximum absorbance at different wavelengths, usually integration of area of different peaks is done at the same wavelength for all compounds (typically 445 nm) where all carotenoids and chlorophylls sensitively absorb. The (U)HPLC software usually integrates all peaks in the chromatogram and gives a table with the retention time of each peak and their area. However, it is almost indispensable to make a manual correction to assure that all peaks areas correspond with the correct pigments. Some pigments like Neo and β-Car can show multiple peaks (*cis* and *trans* isomers) with the same absorption spectra, but this should not represent a problem since peak-areas can be summed. If the noise in the chromatogram is high, is better to increase plant sample amount to solvent ratio during the extraction than increasing the injection volume. Peak area is proportional to pigment amount but the exact relationship between peak area and pigment must be known to make the conversion using equations. For this purpose pigment standards must be used for calibration. The conversion of each peak area to mol or g pigment can be done using a spreadsheet (excel) or using the (U)HPLC software. Although it is possible to program the (U)HPLC software to make automatically all the process (integration, identification, and units conversion) all phases should be thoroughly supervised by the scientist. Final amount of pigment can be expressed in mol or weight units, both per leaf weight or area. Most frequently employed units are: μmol pigment m^{-2}, nmol cm^{-2}, μmol pigment g^{-1} dry weight. Comparison of obtained concentrations with data from the literature are crucial to avoid mistakes in the quantification or in the use of units (Esteban et al. 2015; Fernández-Marín et al. 2015).

- **Signs of pigment degradation**

The occurrence of double peaks indicating presence of isomers/epimers in our sample, as well as the occurrence of unexpected metabolites such as pheophytine can represent signs of sample degradation (Fig. 3.2). Exposition of samples to light, heat or acid (i.e. also intrinsic acids of sample), and also long-storage of samples under inadequate conditions usually explains the formation of isomers and epimers from carotenoids and the degradation of Chls to pheophytine. In Fig. 3.2 several tips and tricks to avoid sample degradation are given at different steps during sampling, storage, processing and analysis.

2.4 Near-Infrared Reflectance Spectroscopy (NIRS) for Pigment Quantification

Near-infrared reflectance spectroscopy (NIRS) analyses the diffuse reflectance of samples and it is based on the absorption of light in the range from 780 to 2500 nm. Each sample produces a unique spectral signature due to specific absorption of bonds such as O–H, C=O, C–N or N–H, characteristic of organic matter. Samples must be grinded and freeze-dried before NIRS analysis, since water has a high absorption in the near-infrared that could invalidate the measurements. Calibration (i.e. comparison of NIRS spectra with HPLC obtained contents) is needed for each individual metabolite (i.e. pigment) and sample type (i.e.: species, leaf developmental stage, etc.). Several recent examples of successful quantification of photosynthetic pigments by NIRS can be found in the literature (Pintó-Marijuan et al. 2013; Fernàndez-Martínez et al. 2017).

3 Non-invasive Analysis of Photosynthetic Pigments

Pigment concentration is undoubtedly most accurately measured by extraction in a solvent followed by its analytical determination. Alternatively, however, it is possible to estimate pigments content using non-destructive and *in situ* optical techniques. When light reaches a leaf most, but not all, of the visible wavelengths (400–700 nm) are absorbed by both Chls and carotenoids. However, these pigments do not capture so efficiently the green light, and as a result (unless there are anthocyanins or betacyanins in the cuticle), leaves display this colour. Although all plants look equally green (to human eye), it is possible to analyse non-absorbed light to derive plant pigment contents and/or relationships between pigments. Both, light reflected by the leaf surface, or light transmitted (light that cross/go through the leaf) can be used to estimate pigments content (Fig. 3.4).

3.1 Measurement of Pigments by Light Transmittance Through the Leaf

Several portable (handheld) models measuring light transmittance are available, such as *CCM-200 (Opti-Science), Spad (Minolta), CL-01 (Hansatech) and Dualex (Dx Force-A)*. All of them are able to estimate Chl content per area by estimating leaf light absorption of red radiation (around 650 nm) and near infrared (NIR) radiation (approximately 850–940 nm). NIR is not absorbed by photoreceptors, so it is used as a reference to correct the detour effect (light scattering) to give a meter output (Shrestha et al. 2012). Furthermore, *Dualex* device combines absorbance and fluorescence measurements in the UV-A band, in the Red and in the NIR to estimate

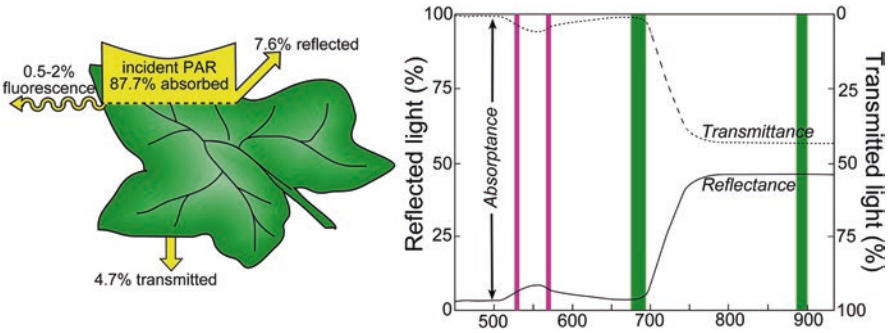

Fig. 3.4 Optical behaviour of incident visible light on a idealised leaf (left) and its spectrum of transmittance and reflectance (right). Pink (wavelengths used for PRI) and green (wavelengths used for NDVI) vertical bars highlight wavelength ranges typically used by non-invasive procedures for quantification of photosynthetic pigments

parameter others than Chl contents as nitrogen balance index, and the polyphenol content (leaf flavonols and anthocyanins indexes) (Cerovic et al. 2012). An important limitation of these devices is that measurements of the chlorophyll content are given in relative units and not in absolute units, and even more, the meter outputs are not totally linear with chlorophyll content (Parry et al. 2014). This means that when the exact chlorophyll content has to be calculated, a calibration with actual chlorophyll contents and relative units has to be done. In addition, the response is species-dependent, which means that a calibration for each plant species is needed. As the chlorophyll content in leaves is closely related with nitrogen content in plant, specifically in crops, the main field for these devices is agronomy where some of them are sold as nitrogen tester for crops. However, they have also been used for research work because they have several advantages: they produce fast, reproducible and non-destructive measurements (Parry et al. 2014), and the devices are also affordable and handheld.

3.2 Measure of Pigments by Leaf Light Reflectance

Vegetation reflectance can be measured using spectroradiometers that detect plant reflected light in the visible and NIR spectrum at different spatial and temporal scales, by *in situ* or in remote measurements. At the canopy scale many factors condition light reflectance such as leaf angle, leaf area, illumination, soil optical properties, and consequently a plethora of indexes and correction coefficients developed under different conditions have been widely used to remotely (airborne and satellite) assess changes in vegetation properties (Lu et al. 2015). However, most handy measurements can be done using *in situ* reflectance handheld instruments. Two main indexes, using reflectance at several wavelengths, are used to assess both, chlorophyll contents and VAZ-cycle pigments, the Normalised Difference Vegetation Index (NDVI), and the Photochemical Reflectance Index (PRI), respectively.

The PRI index reflects light use efficiency. Gamon and collaborators (2001) showed that energy dissipation can be monitored by changes in PRI as this values correlated with xanthophyll cycle pigment epoxidation state. This index uses relative reflectance at 531 and 570 nm to assess the VAZ pigment conversion (Gamon et al. 2001). As explained above, these three xanthophylls interconvert each other depending on balance between light use efficiency and light dissipation. The PRI is formulated as follows: [PRI = (R531 − R570)/(R531 + R570)], where R531 and R570 represent the reflectance at 531 and 570 nm respectively. Leaf surface properties (highly reflective cuticle, wax, hair or trichomes presence) can change the pattern of light reflectance, so R570 is used as the reference (Gamon et al. 2001). When light interception exceeds light utilization in photosynthesis Vio converts to Zea to help energy dissipation. Zea, but not Ant, absorb at 531 nm, so R531 and PRI accordingly decrease to zeaxanthin increases. For this reason, this index currently indicates vegetation health (high values) or plant stress (low values).

The NDVI is a vegetation index that increases depending on Chl density per area. Considering that Chls absorb red wavelength (600–700 nm approximately) but not in the NIR, reflectance in the red (R) is inversely related to green biomass. Since the reflectance is also influenced by structural properties in the leaf, this index includes the NIR correction in the calculation (NIR-R/NIR + R). Typically red is measured at 660–670 nm and NIR at 730–740 nm. Several handheld instruments using fixed wavelengths reflectance are available to measure *in situ* NDVI and/or PRI indexes: *PlantPen PRI 200 & NDVI 300 (Photon Systems Instruments), GreenSeeker Handheld (Trimble), RapidSCAN CS-45 Crop (Holland Scientific) and Spectral Reflectance Sensor (Decagon).* Handheld spectroradiometer as *SpectraPen SP 100 and SpectraPen LM 500 (PSI)* and more versatile full-range portable Spectroradiometer as *Apogee Instruments Spectroradiometer, SVC HR-640i (Spectra Vista Corporation) or ASD FieldSpec® spectrometer (Analytical Spectral Devices*) are able to detect reflectance at any wavelength, which allows to calculate custom-made reflectance indexes. Another equipments as *Geenseaker-Crop Sensing System (Trimble) and Crop Circle ACS-430 (Holland Scientific*) are designed for agronomical uses, as they are thought to be used with farming equipment.

It must be advised that PRI, NDVI and, in general, any reflectance index provide relative measurements, but not absolute values of plant pigment contents. Before any buying decision, we strongly recommend visiting the web sites of the manufacturers and comparing the specifications and options of the different instruments to check whether they measure the parameters of interest. Even more important, is to check the literature and applications of these non-invasive measurements.

3.3 Photographic Analysis

Besides spectral analysis of light, ordinary digital photographs can also be used to analyse plant pigment composition by using image programs that discompose photographs into the three RGB (red, green, and blue) channels. A few indexes have

been developed from the colour channels information to assess greenness at canopy level and individual leaves (see (Junker and Ensminger 2016a, b) for further details).

References

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenolxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoids: handbook. Springer, Basel

Camarero JJ, Olano JM, Arroyo Alfaro SJ, Fernández-Marín B, Becerril JM, García-Plazaola JI (2012) Photoprotection mechanisms in *Quercus ilex* under contrasting climatic conditions. Flora 207:557–564

Cazzaniga S, Li Z, Niyogi KK, Bassi R, Dall'Osto L (2012) The Arabidopsis szl1 mutant reveals a critical role of β-carotene in photosystem I photoprotection. Plant Physiol 159:1745–1758. https://doi.org/10.1104/PP.112.201137

Cazzaniga S, Bressan M, Carbonera D, Agostini A, Dall'Osto L (2016) Differential roles of carotenes and xanthophylls in photosystem I photoprotection. Biochemistry 55:3636–3649. https://doi.org/10.1021/acs.biochem.6b00425

Cerovic ZG, Masdoumier G, Ben GN, Latouche G (2012) A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiol Plant 146:251–260. https://doi.org/10.1111/j.1399-3054.2012.01639.x

Cherian M, Corona E (2006) Lyophilisation of biologicals. In: Briefings T (ed) Bioprocessing and biopartnering. Touch Briefings, London, pp 20–21

Croce R (2012) Chlorophyll-binding proteins of higher plants and cyanobacteria. In: Eaton-Rye J, Tripathy B, Sharkey TD (eds) Photosynthesis: plastid biology, energy conversion and carbon assimilation, Advances in Photosynthesis and Respiration. Springer, Dordrecht, pp 127–149

Dall'Osto L, Lico C, Alric J, Giuliano G, Havaux M, Bassi R (2006) Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection in vivo under strong light. BMC Plant Biol 6:32. https://doi.org/10.1186/1471-2229-6-32

Dall'Osto L, Cazzaniga S, North H, Marion-Poll A, Bassi R (2007a) The Arabidopsis aba4-1 mutant reveals a specific function for neoxanthin in protection against photooxidative stress. Plant Cell 19:1048–1064. https://doi.org/10.1105/tpc.106.049114

Dall'Osto L, Fiore A, Cazzaniga S, Giuliano G, Bassi R (2007b) Different roles of α- and β-branch xanthophylls in photosystem assembly and photoprotection. J Biol Chem 282:35056–35068. https://doi.org/10.1074/jbc.M704729200

Dall'Osto L, Cazzaniga S, Havaux M, Bassi R (2010) Enhanced photoprotection by protein-bound vs free xanthophyll pools: a comparative analysis of chlorophyll b and xanthophyll biosynthesis mutants. Mol Plant 3:576–593. https://doi.org/10.1093/mp/ssp117

Demmig-Adams B (1998) Survey of thermal energy dissipation and pigment composition in sun and shade leaves. Plant Cell Physiol 39:474–482

Demmig-Adams B, Gilmore AM, Adams WW (1996) In vivo functions of carotenoids in higher plants. FASEB 10:403–412

Esteban R, García-Plazaola JI (2014) Involvement of a second xanthophyll cycle in non-photochemical quenching of chlorophyll fluorescence: the lutein epoxide story. In: Demmig-Adams B, Garab G, Adams WW III, Govindjee (eds) Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. Springer, Dordrecht, pp 277–295

Esteban R, García-Plazaola JI (2016) Nonubiquitous carotenoids in higher plants: presence, role in photosynthesis, and guidelines for identification. In: Pessarakli M (ed) Handbook of photosynthesis, 3rd edn. CRC Press, Boca Raton, pp 589–600

Esteban R, Balaguer L, Manrique E, Rubio de Casas R, Ochoa R, Fleck I, Pintó-Marijuan M, Casals I, Morales D, Jiménez MS, Lorenzo R, Artetxe U, Becerril JM, García-Plazaola JI

(2009a) Alternative methods for sampling and preservation of photosynthetic pigments and tocopherols in plant material from remote locations. Photosynth Res 101:77–88. https://doi.org/10.1007/s11120-009-9468-5

Esteban R, Becerril JM, García-Plazaola JI (2009b) Lutein epoxide cycle, more than just a forest tale. Plant Signal Behav 4:342–344. https://doi.org/10.4161/psb.4.4.8197

Esteban R, Olano JM, Castresana J, Fernández-Marín B, Hernández A, Becerril JM, García-Plazaola JI (2009c) Distribution and evolutionary trends of photoprotective isoprenoids (xanthophylls and tocopherols) within the plant kingdom. Physiol Plant 135:379–389. https://doi.org/10.1111/j.1399-3054.2008.01196.x

Esteban R, Matsubara S, Jiménez MS, Morales D, Brito P, Lorenzo R, Fernández-Marín B, Becerril JM, García-Plazaola JI (2010) Operation and regulation of the lutein epoxide cycle in seedlings of *Ocotea foetens*. Funct Plant Biol 37:859–869. https://doi.org/10.1071/FP10014

Esteban R, Barrutia O, Artetxe U, Fernández-Marín B, Hernández A, García-Plazaola JI (2015) Internal and external factors affecting photosynthetic pigment composition in plants: a meta-analytical approach. New Phytol 206:268–280. https://doi.org/10.1111/nph.13186

Fernández-Marín B, Balaguer L, Esteban R, Becerril JM, García-Plazaola JI (2009) Dark induction of the photoprotective xanthophyll cycle in response to dehydration. J Plant Physiol 166:1734–1744. https://doi.org/10.1016/j.jplph.2009.04.019

Fernández-Marín B, Míguez F, Becerril J, García-Plazaola J (2011a) Activation of violaxanthin cycle in darkness is a common response to different abiotic stresses: a case study in *Pelvetia canaliculata*. BMC Plant Biol 11:181. https://doi.org/10.1186/1471-2229-11-181

Fernández-Marín B, Míguez F, Becerril JM, García-Plazaola JI (2011b) Dehydration-mediated activation of the xanthophyll cycle in darkness: is it related to desiccation tolerance? Planta 234:579–588. https://doi.org/10.1007/s00425-011-1420-1

Fernández-Marín B, Kranner I, Sebastián MS, Artetxe U, Laza JM, Vilas JL, Pritchard HW, Nadajaran J, Míguez F, Becerril JM, García-Plazaola JI (2013) Evidence for the absence of enzymatic reactions in the glassy state. A case study of xanthophyll cycle pigments in the desiccation-tolerant moss *Syntrichia ruralis*. J Exp Bot 64:3033–3043. https://doi.org/10.1093/jxb/ert145

Fernández-Marín B, Artetxe U, Barrutia O, Esteban R, Hernández A, García-Plazaola JI (2015) Opening Pandora's box: cause and impact of errors on plant pigment studies. Front Plant Sci 6:148. https://doi.org/10.3389/fpls.2015.00148

Fernández-Marín B, Hernández A, García-Plazaola JI, Esteban R, Míguez F, Artetxe U, Gómez-Sagasti MT (2017) Photoprotective strategies of Mediterranean plants in relation to morphological traits and natural environmental pressure: a meta-analytical approach. Font Plant Sci 19:1051. https://doi.org/10.3389/fpls.2017.01051

Fernàndez-Martínez J, Joffre R, Zacchini M, Fernández-Marín B, García-Plazaola JI, Fleck I (2017) Near-infrared reflectance spectroscopy allows rapid and simultaneous evaluation of chloroplast pigments and antioxidants, carbon isotope discrimination and nitrogen content in *Populus* spp. leaves. For Ecol Manag 399:227–234. https://doi.org/10.1016/j.foreco.2017.05.041

Fukushima A, Kusano M, Nakamichi N, Kobayashi M, Hayashi N, Sakakibara H, Mizuno T, Saito K (2009) Impact of clock-associated Arabidopsis pseudo-response regulators in metabolic coordination. Proc Natl Acad Sci 106:8791–8791. https://doi.org/10.1073/pnas.0904226106

Gamon JA, Field CB, Fredeen AL, Thayer S (2001) Assessing photosynthetic downregulation in sunflower stands with an optically-based model. Photosynth Res 67:113–125. https://doi.org/10.1023/A:1010677605091

García-Plazaola JI, Becerril JM (1999) A rapid high-performance liquid chromatography method to measure lipophilic antioxidants in stressed plants: simultaneous determination of carotenoids and tocopherols. Phytochem Anal 10:307–313

García-Plazaola JI, Becerril JM (2001) Seasonal changes in photosynthetic pigments and antioxidants in beech (*Fagus sylvatica*) in a Mediterranean climate: implications for tree decline diagnosis. Aust J Plant Physiol 28:225–232. https://doi.org/10.1071/PP00119

García-Plazaola JI, Fernández-Marín B, Ferrio JP, Alday JG, Hoch G, Landais D, Milcu A, Tissue DT, Voltas J, Gessler A, Roy J, Resco de Dios V (2017) Endogenous circadian rhythms in pigment composition induce changes in photochemical efficiency in plant canopies. Plant Cell Environ 40:1153–1162. https://doi.org/10.1111/pce.12909

Gilmore AM, Yamamoto HY (1991) Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. J Chromatogr A 543:137–145. https://doi.org/10.1016/S0021-9673(01)95762-0

Gruszecki WI, Strzalka K (2005) Carotenoids as modulators of lipid membrane physical properties. Biochim Biophys Acta Mol basis Dis 1740:108–115. https://doi.org/10.1016/j.bbadis.2004.11.015

Havaux M (1998) Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci 3:147–151. https://doi.org/10.1016/S1360-1385(98)01200-X

Havaux M, Dall'Osto L, Cuiné S, Giuliano G, Bassi R (2004) The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana*. J Biol Chem 279:13878–13888. https://doi.org/10.1074/jbc.M311154200

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochem und Physiol der Pflanz 167:191–194. https://doi.org/10.1016/S0015-3796(17)30778-3

Johnson MP, Goral TK, Duffy CD, Brain AP, Mullineaux CW, Ruban AV (2011) Photoprotective energy dissipation involves the reorganization of photosystem II light-harvesting complexes in the grana membranes of spinach chloroplasts. Plant Cell 23:1468–1479. https://doi.org/10.1105/tpc.110.081646

Junker LV, Ensminger I (2016a) Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing *Acer saccharum* leaves. Tree Physiol 36:694–711. https://doi.org/10.1093/treephys/tpv148

Junker LV, Ensminger I (2016b) Fast detection of leaf pigments and isoprenoids for ecophysiological studies, plant phenotyping and validating remote-sensing of vegetation. Physiol Plant 158:369–381. https://doi.org/10.1111/ppl.12512

Kromdijk J, Glowacka K, Leonelli L, Gabilly ST, Iwai M, Niyogi KK, Long SP (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. Science 354(6314):857–861

Küpper H, Seibert S, Parameswaran A (2007) Fast, sensitive, and inexpensive alternative to analytical pigment HPLC: quantification of chlorophylls and carotenoids in crude extracts by fitting with Gauss peak spectra. Anal Chem 79:7611–7627. https://doi.org/10.1021/ac070236m

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. In: Current protocols in food analytical chemistry. Wiley, Hoboken

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Lokstein H, Tian L, Polle JEW, DellaPenna D (2002) Xanthophyll biosynthetic mutants of *Arabidopsis thaliana*: altered nonphotochemical quenching of chlorophyll fluorescence is due to changes in photosystem II antenna size and stability. Biochim Biophys Acta Bioenerg 1553:309–319. https://doi.org/10.1016/S0005-2728(02)00184-6

Lu S, Lu X, Zhao W, Liu Y, Wang Z, Omasa K (2015) Comparing vegetation indices for remote chlorophyll measurement of white poplar and Chinese elm leaves with different adaxial and abaxial surfaces. J Exp Bot 66:5625–5637. https://doi.org/10.1093/jxb/erv270

Matsubara S, Naumann M, Martin R, Nichol C, Rascher U, Morosinotto T, Bassi R, Osmond B (2005) Slowly reversible de-epoxidation of lutein-epoxide in deep shade leaves of a tropical tree legume may "lock-in" lutein-based photoprotection during acclimation to strong light. J Exp Bot 56:461–468. https://doi.org/10.1093/jxb/eri012

Maurer MM, Mein JR, Chaudhuri SK, Constant HL (2014) An improved UHPLC-UV method for separation and quantification of carotenoids in vegetable crops. Food Chem 165:475–482. https://doi.org/10.1016/j.foodchem.2014.05.038

Míguez F, Fernández-Marín B, Becerril JM, García-Plazaola JI (2017) Diversity of winter photoinhibitory responses: a case study in co-occurring lichens, mosses, herbs and woody plants from subalpine environments. Physiol Plant 160:282–296

Moradzadeh M, Sadeghnia HR, Tabarraei A, Sahebkar A (2017) Anti-tumor effects of crocetin and related molecular targets. J Cell Physiol 233:2170–2182. https://doi.org/10.1002/jcp.25953

Niinemets Ü (2007) Photosynthesis and resource distribution through plant canopies. In: Eaton-Rye J, Tripathy B, Sharkey T (eds) Photosynthesis: plastid biology, energy conversion and carbon assimilation, Advances in Photosynthesis and Respiration. Blackwell Publishing Ltd, Dordrecht, pp 1052–1071

Parry C, Blonquist JM, Bugbee B (2014) In situ measurement of leaf chlorophyll concentration: analysis of the optical/absolute relationship. Plant Cell Environ 37:2508–2520. https://doi.org/10.1111/pce.12324

Peguero-Pina JJ, Gil-Pelegrín E, Morales F (2013) Three pools of zeaxanthin in *Quercus coccifera* leaves during light transitions with different roles in rapidly reversible photoprotective energy dissipation and photoprotection. J Exp Bot 64:1649–1661. https://doi.org/10.1093/jxb/ert024

Pintó-Marijuan M, Joffre R, Casals I, De Agazio M, Zacchini M, García-Plazaola JI, Esteban R, Aranda X, Guàrdia M, Fleck I (2013) Antioxidant and photoprotective responses to elevated CO2 and heat stress during holm oak regeneration by resprouting, evaluated with NIRS (near-infrared reflectance spectroscopy). Plant Biol 15:5–17. https://doi.org/10.1111/j.1438-8677.2011.00538.x

Polívka T, Frank HA (2010) Light harvesting by carotenoids. Acc Chem Res 43:1125–1134

Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73:149–156. https://doi.org/10.1023/A:1020470224740

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta Bioenerg 975:384–394. https://doi.org/10.1016/S0005-2728(89)80347-0

Shrestha S, Brueck H, Asch F (2012) Chlorophyll index, photochemical reflectance index and chlorophyll fluorescence measurements of rice leaves supplied with different N levels. J Photochem Photobiol B Biol 113:7–13. https://doi.org/10.1016/j.jphotobiol.2012.04.008

Sims DA, Gamon JA (2002) Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sens Environ 81:337–354. https://doi.org/10.1016/S0034-4257(02)00010-X

Smith JHC, Benitez A (1955) Chlorophylls: analysis in plant materials. In: Modern methods of plant analysis/Moderne Methoden der Pflanzenanalyselant analysis. Springer, Berlin/Heidelberg, pp 142–196

Tausz M, Wonisch A, Grill D, Morales D, Jiménez MS (2003) Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation. J Exp Bot 54:1505–1510. https://doi.org/10.1093/jxb/erg175

Wang K, Tu W, Liu C, Rao Y, Gao Z, Yang C (2017) 9-cis-Neoxanthin in light harvesting complexes of photosystem II regulates the binding of violaxanthin and xanthophyll cycle. Plant Physiol 174:86–96. https://doi.org/10.1104/pp.17.00029

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144:307–313. https://doi.org/10.1016/S0176-1617(11)81192-2

Young AJ, Britton G (1989) The distribution of α-carotene in the photosynthetic pigment-protein complexes of higher plants. Plant Sci 64:179–183. https://doi.org/10.1016/0168-9452(89)90022-8

Young A, Phillip D, Savill J (1996) Methods for carotenoid analysis. In: Pessaraki M (ed) Handbook of photosynthesis. Marcel Dekker Inc, New York, pp 597–622

Ziegler R, Egle K (1965) Zur quantitativen Analyse der Chloroplastenpigmente. I. Kritische. berprüfung der spektralphotometrischen Chlorophyll-Bestimmung. Beitr Biol Pflanz 41:11–37