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METHODS

Application of Reflectance Spectroscopy for Analysis of Higher Plant Pigments

M. N. Merzlyak*, A. A. Gitelson, O. B. Chivkunova*, A. E. Solovchenko*, and S. I. Pogosyan***

**Department of Physiology of Microorganisms, Faculty of Biology, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia;*
e-mail: mnm@6.cellimm.bio.msu.ru, m_merzlyak@mail.ru
***Center for Advanced Land Management Information Technologies, School of Natural Resource Sciences, University of Nebraska, Lincoln, USA*

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Abstract—Nondestructive techniques developed by the authors for assessment of chlorophylls, carotenoids, and anthocyanins in higher plant leaves and fruits are presented. The spectral features of leaf reflectance in the visible and near infrared regions are briefly considered. For pigment analysis only reflectance values at several specific wavelengths are required. The chlorophyll (Chl) content over a wide range of its changes can be assessed during leaf ontogeny using reflectance near 700 nm and, in the absence of anthocyanins, at 550 nm. The approaches used for elimination of Chl interference in the analysis of carotenoids (reflectance at 520 nm) and anthocyanins (at 550 nm) are described. The suitability of reflectance spectroscopy for estimates of carotenoid/chlorophyll ratios during leaf senescence and fruit ripening is demonstrated. The algorithms developed for pigment analysis are presented, and the conditions of their applicability are considered. Further perspectives for the application of reflectance spectroscopy including remote sensing for estimation of plant pigment content and physiological states are discussed.

Key words: chlorophyll - carotenoids - anthocyanins - reflectance spectroscopy - leaves - fruits

INTRODUCTION

Chlorophylls (Chl) and carotenoids (Car) are essential pigments of higher plant assimilatory tissues responsible for variations of color from dark-green to yellow. Other pigments involved in leaf and fruit coloration are flavonoids (yellow) and anthocyanins (red). The absolute amounts of the pigments as well as their ratios are important physiological characteristics of the leaf, whole plants, and plant communities. The content of Chl, the dominant pigment of green leaves, determines to a great extent the amount of PAR absorbed by the leaf, the photosynthetic rate, and plant productivity [1–3]. Carotenoids are involved in light harvesting and other physiologically important functions, preventing, via several mechanisms, the damages to plants caused by excessive fluxes of visible radiation [4–7]. The induction of synthesis and accumulation of certain phenolic compounds (flavonoids) is an important mechanism of plant protection from damages by UV-A and UV-B radiation [8, 9]. Red-colored flavonoids, anthocyanins (Anth) are able to exert protective effect against damage induced by radiation in the visible part of solar spectrum [10–12]. The pigment content undergoes directional and specific changes in the course of plant growth and development, during adaptation to unfavorable environmental conditions, and under various stresses and damages [3–5, 7, 9–15].

As a rule, plant pigment analysis in physiological and biochemical studies is performed with spectrophotometry of tissue extracts. The application of this methodology involves tissue destruction; it is time-consuming and is coupled with artifacts due to pigment instability, incomplete extraction, the presence of light-absorbing impurities, etc. [16, 17]. These circumstances make the nondestructive estimation of pigment content with reflectance spectroscopy of intact tissues an attractive alternative to wet chemical methods. Indeed, both qualitative and quantitative changes in pigment content of plant tissues should be inevitably apparent in tissue optical properties. For example, reflectance spectra of leaves and fruits undergo remarkable changes under deficiency of mineral nutrition, pollution, different stress conditions, during adaptation to variable solar irradiation, and in the course of senescence [2, 6, 7, 11, 14, 15, 18–24]. The application of nondestructive optical methods for pigment assessment is advantageous since they allow rapid measurements on a large number of samples, which thereafter remain intact and could be used for further analysis. Recently, commercially available reflectometers suitable for field measurements, providing reliable spectral data from both very small surface and whole plants are designed [14, 23, 25]. Furthermore, reflectance spec-

Abbreviations: Anth—anthocyanins; Car—carotenoids; Chl—chlorophyll(s); NIR—near infrared (750–800 nm); PAR—photosynthetically active radiation.

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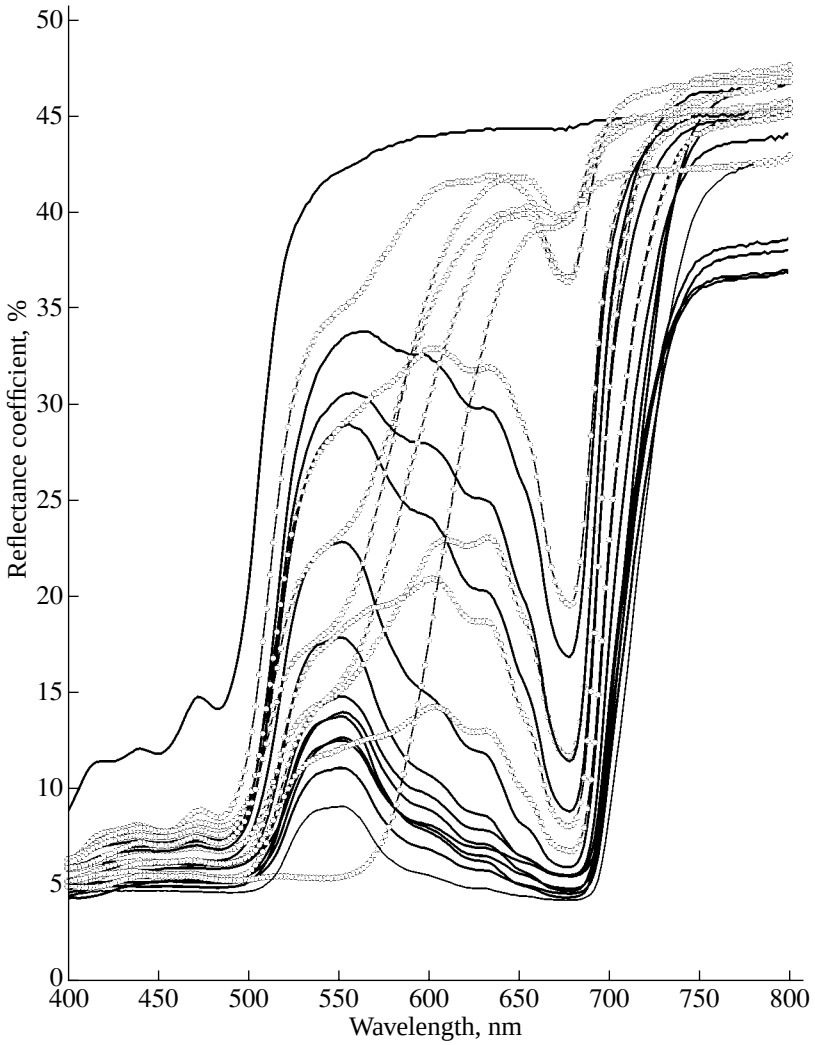

Reflectance spectra of maple (*Acer platanoides* L.) leaves collected in August–September 1999.
The chlorophyll content in leaves ranged from 0.1 to 60 nmol/cm^2 (see red–orange region in upper and lower spectra, respectively). Spectra of anthocyanin-free (<1 nmol Anth/cm^2) leaves are shown by solid lines; lines with symbols correspond to leaves with Anth content ranging from 2.1 to 40.8 nmol/cm^2 (upper and lower spectra, respectively). The spectra were recorded on a black background.

troscopy is of wide use in global remote monitoring of agro- and phytocenoses. In recent years these approaches have also been implemented in "precision agriculture" technologies [2, 3, 14, 26–29].

The basic theory of diffuse reflectance developed by Kubelka and Munk for a homogenous layer of "infinite thickness" yields a simple relationship between the intensity of reflected light and absorption and scattering coefficients of the medium (see [30]). A plant leaf consisting of several structures with different refraction indices (cuticle, epidermis, and mesophyll) and containing high amounts of pigments represents a complex optical system [31–33]. Although detailed investigations of leaf optical properties have appeared in the literature [32, 33], the most fruitful approaches for quantitative pigment analysis *in situ* were developed by considering the leaf as a "black box". Significant amount of research was dedicated to the development of techniques for nondestructive analysis of plant pigments, and these issues attracted much attention during the last decade [2, 14, 18, 20, 24, 26, 27, 34–43]. This paper is a brief review of techniques for quantitative estimation of Chl, Car, and Anth in leaves and fruits with reflectance spectroscopy developed in our laboratory.

GENERAL FEATURES OF PLANT REFLECTANCE SPECTRA

In order to develop nondestructive technique for plant pigment estimation, one needs thorough understanding of their *in vivo* spectroscopy, localization within tissues, and patterns of their changes during physiological processes in plants. Senescing leaves, with their dramatic changes in Chl, Car, and Anth con-

tent, are suitable for demonstration of the influence of pigments on leaf reflectance spectra (those of maple, as an example, are shown in figure [6, 21, 22]). Leaves with low Chl and Anth content exhibit high reflectance coefficients (35–48%) at wavelengths above 600 nm. Measurements performed on leaves with extremely weak pigmentation showed that the leaf tissues possessed high reflectance without discernible spectral features in the NIR and almost entire visible (up to the blue range) parts of the spectrum. These investigations also revealed that apparent absorption of light by leaves (amounting to 10–15%) in the range of 750–800 nm, described repeatedly in the literature, is likely to result from incomplete collection of transmitted light with an integrating sphere [44]. The pigments occurring at very low amounts, which are difficult to quantify analytically (e.g., Chl content of 0.3–0.4 nmol/cm^2), gave rise to distinct troughs in the red region of reflectance spectra of fruits and leaves (figure, see also [6, 21, 22, 44]). With an increase in pigment content up to 10–12 nmol/cm^2, leaf spectra acquired pronounced features of Chl (pale-green leaves), Car (yellow leaves), and Anth (red leaves) absorption. The reflectance spectra of green (Chl content ca. 30 nmol/cm^2) and especially of dark-green leaves were poorly resolved.

The leaf reflectance in the main bands of Chl *a* absorption (near 440–450 and 670–680 nm) became saturated at Chl content of 10–15 nmol/cm^2. However, reflectance in these spectral regions did not drop below 4–5% even at very high concentrations of the pigment (figure, see also [6, 15, 21, 22, 44]). This could be explained by reflection of light by superficial leaf structures (cuticle and epidermis) containing very low amounts of pigments (see epidermis spectra in [9]). Distinct bands attributable to Car absorption could be distinguished only in reflectance spectra of senescing (yellow) leaves at terminal stages of Chl degradation (see uppermost curve in figure and spectra in [6, 15, 21, 44]). The absorption of Anth usually manifests itself as a shoulder or a band near 540–550 nm usually superimposed on a considerable background of Chl and Car absorption [11, 22, 24]. Leaves containing no Chl accumulated Anth in high amounts (over 40 nmol/cm^2), which determined very low reflectance of red leaves in the green part of the spectrum (figure, [22]). Fruits (e.g., apples and lemons) demonstrated spectral reflectance features similar to those of leaves [12, 15, 21, 24].

Even this brief consideration illustrates the complications associated with nondestructive analysis of plant pigments using reflectance spectra. In order to overcome these constraints, we adopted the following strategy of our investigations.

(1) The detection of reflectance spectral bands governed predominantly by absorption of pigment of interest and sensitive to this pigment content.

(2) The development of models (algorithms) relating reflectance at certain wavelengths with pigment content in the entire range of its variation.

(3) Finding a way for elimination of Chl interference in the spectral bands employed for Car and Anth assay.

In these studies, leaves and fruits of several plant species were analyzed at all stages of their development and at a wide range of changes in their pigment content. Along with leaves possessing common pattern of ontogenetic changes in coloration (from green to yellow) we used some species predisposed to accumulate high amounts of Anth. This was the case in juvenile or senescing plants and sometimes in leaves and fruits exposed to strong sunlight.

The following criteria were used for validation of the approaches developed: (i) the algorithms should be sensitive only to a certain pigment and insensitive to other pigments or morphological–anatomical features of plants and (ii) they should be applicable to independently obtained data sets. For testing the second criterion, we used leaves collected in different years.

CHLOROPHYLL

In healthy Anth-free leaves, Chl is the only pigment absorbing in the green to far-red spectral range [6, 15, 44]. In earlier investigations reflectance minimum at 670–680 nm was employed for Chl analysis. Although the algorithms developed for these wavelengths showed a good sensitivity and linearity at low Chl content range, they became rapidly saturated with an increase in the pigment content over 10–15 nmol/cm^2 [26, 36, 45].

Our analysis showed that spectral regions where reflectance coefficients are sensitive to wide-range variations of Chl content (from 0 to 50–60 nmol/cm^2) are situated aside from the red maximum of Chl absorption: in the green (broad band near 550–600 nm) and red (narrow band at 700–705 nm) parts of the spectrum. In particular, these regions were revealed in the spectrum of standard deviation of reflectance calculated for leaves with broad variation of Chl content [20, 36]. Then, it was found that reflectances in these bands were hyperbolically related to Chl content [20, 38, 41]. It should be noted that Chl absorbtion coefficients are very low in these bands. This apparently universal feature of plant reflectance spectra (the linear dependence of inverse reflectance in certain spectral regions on pigment content) was used as a basis in the development of algorithms for estimation of Chl and other pigment.

One of the requirements for reliable algorithms of pigment analysis is their low sensitivity to morphological–anatomical traits of plant tissues. For leaves differing in pigment content, the lowest variation of reflectance at wavelengths longer than 500 nm was found in the NIR region [20, 36]. Since leaf pigments possess no measurable absorption in the NIR, the tissue reflectance in this region is apparently determined by “internal” optical properties related to leaf thickness, water content, and light scattering. The scattering within plant tis-

sues arises at interfacial boundaries separating phases with different refraction indices [2, 31, 33, 44, 45].

Taking into account the above circumstances, the algorithms for estimation of Chl content were suggested in the form of simple ratios of reflectance coefficients at certain wavelengths: R_{NIR}/R_{550} and R_{NIR}/R_{700}. Note that R_{NIR} is insensitive and R_{700} and R_{550} are highly sensitive to Chl content. Both ratios were highly sensitive to Chl content in a wide range of its changes in leaves and fruits of diverse plant species and depended linearly on the pigment content [20, 24, 36, 37, 41, 43]. Furthermore, Chl assessment could be performed in broader spectral ranges: the algorithmic expressions $[R(\lambda)^{-1} - (R_{NIR})^{-1}]R_{NIR}$ were shown to provide highly precise and linear estimates of leaf Chl content in the wavelength ranges of 530–580 and 695–735 nm [43].

It is noteworthy that the R_{NIR}/R_{550} and R_{NIR}/R_{700} ratios possessed similar sensitivity to Chl content, which was due to high correlation between reflectance coefficients at 550 and 700 nm characteristic of healthy anthocyanin-free leaves. Furthermore, there is ground to believe that this correlation represents the universal feature of leaf reflectance spectra in the ranges predominantly or exclusively governed by Chl absorption [20, 21, 38, 41, 43]. Thus, accumulation of Anth (figure, see also [22, 24]) leads to a significant decrease in R_{550} relative to R_{700}. This greatly complicates the application of the R_{NIR}/R_{550} index for Chl assessment in red leaves. At the same time, our studies showed that the R_{NIR}/R_{700} index could be used for Chl analysis even at high Anth content [22]. Further details of devising the indices and their characteristics are considered in [38, 43].

Several other approaches allowing efficient analysis of Chl in leaves were developed using reflectance in the red region of the spectrum. Thus, the pigment content could be retrieved from the amplitude and position of the peak in the first derivative of a reflectance spectrum between 685 and 706 nm, a so-called "red edge" [2, 46]. It is important that the position of this peak was linearly related to leaf reflectance coefficient at 700 nm [45]. The functions

$$\int_{705}^{750} [R(\lambda)/R_{555} - 1]d\lambda \text{ and } \int_{705}^{750} [R(\lambda)/R_{705} - 1]d\lambda]$$

also displayed good correlation with the leaf chlorophyll content [23, 46].

ANTHOCYANINS

The lack of close correlation between reflectance coefficients at 550 and 700 nm in red leaves became a basis for our approach to nondestructive assessment of Anth. It should be stressed that the decrease in correlation between $R_{550}$and R_{700} takes place even at very low Anth content (ca. 1–2 nmol/cm^2).

Thus, sensitive assessment of these "stress pigments" turns feasible. Spectral analysis revealed that Anth (represented mainly by cyanidin derivatives) localized in vacuoles within leaf and fruit cells possessed an absorption maximum near 540–550 nm [11, 22, 24]. Since chlorophyll absorption is significant in this region (figure), the contribution of Chl to reflectance should be taken into account while assaying Anth content. We proposed to remove the Chl contribution in the green spectral region by using reflectance at 700 nm [22, 24]. Thus, we devised an ARI index (Anthocyanin Reflectance Index) for Anth assessment. This was defined as $R_{NIR}(1/R_{550} - 1/R_{700})$, where the first term in parentheses corresponds to the combined absorption by Anth and Chl and the second one is due to Chl absorption only. In leaves of four plant species, ARI proved to be a highly sensitive linear indicator of Anth content in the concentration range up to 40 nmol/cm^2 [22]. The effectiveness of ARI was confirmed by its successful application to leaves of other plant species and apple fruits [24].

CAROTENOIDS

The analysis of carotenoids absorbing in the blue region of the spectrum is greatly complicated by an overlapping absorption of Chl present in high amounts in plant tissues [6, 15, 24]. Moreover, the ability of Car to participate in light absorption by green leaves was even questioned [5]. Additional obstacles to the Car analysis in plants are due to the occurrence of several xanthophylls whose pools undergo unproportional changes during leaf ontogeny and upon adaptation of leaves to variable light conditions [4–7].

Nevertheless, the results of our analysis showed the possibility of quantitative assessment of Car using reflectance at 510–520 nm; some algorithms have been developed for this purpose [6, 24, 47]. In its basic form, CRI (Carotenoid Reflectance Index) was suggested as $R_{NIR}(1/R_{510} - 1/R_{550})$, where the first term in parentheses corresponds to light absorption by Car and Chl, and the second one is related to Chl absorption [6, 24]. Along with R_{550}, reflectance coefficient at 700 nm could also be employed as a Chl-sensitive term. The applications of this algorithm to leaves of several plant species [6] and apple fruits [24] have confirmed its suitability for assessment of carotenoids in the whole range of their changes in the course of leaf ontogeny and fruit ripening. It should be mentioned, however, that CRI is not applicable to Anth-containing plant tissues.

CHLOROPHYLL TO CAROTENOID RATIO

The ratio of Car to Chl is an important characteristic of plant photosynthetic apparatus. The most dramatic changes in the content of these pigments occur at terminal stages of leaf and fruit development in many plant species. At these stages plant tissues retain certain amounts of Car or Car synthesis is stimulated on the background of Chl degradation. In chloroplasts of senescing plant tissues, the chloroplast plastoglobules

rather than thylakoids become the predominant sites of Car localization [4, 6, 7, 21, 48]. The analysis of green leaves with different pigment content revealed a strong correlation between reflectance coefficients in the red maximum of Chl absorption (near 678 nm) and in spectral band near 500 nm governed by combined absorption of Chl and Car [15, 21]. In senescing coleus (*Coleus blumei* Benth.) leaves, characterized by a remarkably synchronous disappearance of both Chl and Car resulting in whitish leaf coloration, a high correlation of reflectances at these wavelengths was retained until the late stages of Chl breakdown. By contrast, during chlorophyll degradation in yellowing leaves of deciduous trees (maple and chestnut) and in ripening fruits (e.g., apples and lemons) R_{678} increased significantly higher than R_{500}. As a result, a close correlation between reflectance coefficients at these wavelengths characteristic of tissues with high chlorophyll content was disrupted [15, 21]. In such fruits and leaves, Car content was higher than that of Chl [15, 24].

For detection of relative changes in Chl and Car content, Plant Senescence Reflectance Index (PSRI) was suggested that incorporates reflectance coefficients at 500 and 678 nm along with NIR reflectance: $(R_{678} - R_{500})/R_{NIR}$ [15]. This index exhibited high correlation with the molar Car/Chl ratio in senescent maple leaves [15] and ripening apple fruits [24].

In leaves and fruits containing high amounts of Chl, R_{500} was somewhat higher than R_{678}, which resulted in negative PSRI values. As mentioned earlier, the reflectance at 678 nm changed faster in the course of leaf senescence than that at 500 nm, which made PSRI positive. Therefore, the stage when PSRI turns to zero (i.e., $R_{678} = R_{500}$) was chosen as a criterion for the onset of senescence in plants exhibiting Car retention. Our experiments showed that the induction of Car synthesis occurs at different stages of Chl degradation and the rates of relative changes in Chl and Car content vary between plant species [15]. PSRI was successfully used for characterization of the rates of apple and lemon ripening [15, 24] and for studying ethylene effects on the kinetics of ripening of lemon fruits [15].

TISSUE BROWNING

Reflectance spectra of plants undergo significant changes during tissue browning and necrotization, which is due to accumulation of colored products of polyphenol oxidation. Browning of leaves occurs during senescence [21] and as a result of air pollution [14], whereas fruit browning represents a symptom of superficial scald [49] and sunburn [12]. Analyses of apple fruit reflectance spectra during development of superficial scald and artificial browning induced by *n*-hexane treatment revealed the most significant changes of reflectance in the green (near 550 nm) and NIR spectral regions, whereas the red band of Chl absorption was much less affected [49]. For quantification of tissue browning the Browning Reflectance Index (BRI) was suggested: $(1/R_{550} - 1/R_{700})/R_{NIR}$. This algorithm could be used for estimation of superficial scald development in Anth-free fruit on the background of Chl and Car absorption [49].

CONCLUSIONS AND PROSPECTS

The results obtained during the last decade considerably extended possible applications of reflectance spectroscopy for the estimation of pigment content and for assessment of physiological state of plants. These achievements are really impressive, because some time ago reflectance spectroscopy was considered inadequate to provide useful information about plant organisms due to their low reflectance and poorly resolved spectra that seemed similar in different species [25]. We consider these similarities as evidence of common organization of the photosynthetic apparatus and uniformity of its changes occurring during plant development and stress responses in higher plants.

The data presented in this review show that reflectance spectroscopy could be a useful and efficient tool for pigment analysis in plants. Remarkably, the approaches for nondestructive assessment of Chl, Car, and Anth discussed here require knowledge of reflectance only at few certain wavelengths. However, the possibilities of application of this technique to leaves (fruits) of other plant species need further verification. Another problem which remains to be solved is the finding of approaches for selective assessment of Chl *a* and *b*. The developed algorithms provide sensitivity, linearity, and precision for analysis of Anth at a certain concentration range, but ARI becomes saturated at Anth content above 40–50 nmol/cm^2. Apparently, this problem could be solved with new approaches. Promising data were obtained that indicate the feasibility of nondestructive analysis of flavonoids absorbing in the near UV region of the spectrum. Accumulation of flavonoids in response to high-intensity solar radiation manifests itself in a pronounced decrease of fruit reflectance in the range of 350–420 nm [7, 11, 12]. All this facilitates the extensive application of reflectance spectroscopy for solving various issues of plant physiology at the level of individual leaves and fruits.

Our estimations showed that the developed algorithms could be employed in remote probing of plants for pigment analyses in broad spectral ranges [6, 22, 26, 28, 41]. Several features of leaf reflectance revealed in these studies provide a basis for the development of this technology [26, 28, 29].

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