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Non-invasive quantification of foliar pigments: Possibilities and limitations of reflectance- and absorbance-based approaches

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ABSTRACT

Established reflectance-based approaches for estimation of foliar pigment contents assume close relationship between leaf absorbance and reflectance. Complex organization and high pigment content of leaves may lead to violation of the essential assumptions under Kubleka-Munk theory relating reflectance and absorbance. We compared relationships of absorbance and reciprocal reflectance vs. pigment content in leaves collected across species, developmental stages and physiological states. As a result, limitations of reflectance-based spectroscopy for pigment quantification were revealed. We deduced in situ absorbance of foliar chlorophylls, carotenoids, and flavonoids (including red-colored anthocyanins) and introduced a concept of specific spectral response of the optical properties to each pigment group. Quantitative criteria of spectral range selection for the absorbance- and reflectance-based techniques yielding effect of each pigment on the background of other pigment absorption were suggested and validated. We argue that absorbance- and reflectance-based approaches to pigment estimation complement each other and can be used synergistically in advanced models for accurate estimating foliar pigments. The study provides a deeper insight into interception of light by photosynthetic and photoprotective pigments as function of physiological condition and developmental stage, which is important for plant biology as well as knowledge-driven selection of spectral bands for noninvasive pigment estimation models.

1. Introduction

Absorption of light by plant pigments allows tracking their content via effect of the absorption on spectra of optical properties, absorbance and reflectance. Accurate estimation of pigment content using absorbance (α) and reflectance (ρ) spectra requires their close linear relationships with specific pigment of interest. Importantly, these relationships should bear minimal effect of other pigments. Since absorption bands of the pigments often overlap, it is a challenging problem hence development of quantitative measures of α and ρ response to specific pigment content is a prerequisite for assessment of the potential for non-destructive technologies based either on α or ρ spectroscopy.

Kubelka-Munk theory [1,2] laid a basis for reflectance spectroscopy suggesting that the relationship between remission function, which in reality is reciprocal reflectance, ρ^{-1} [3], related to the ratio of absorption to scattering coefficients. However, this assumption was not tested for leaves containing widely variable pigment content and composition that makes uncertain limits of reflectance spectroscopy as well as requirements to spectral regions could be used for estimating contents of four types of foliar pigments—chlorophylls, Chl, carotenoids, Car, as well as phenolic compounds such as anthocyanins, AnC, and other flavonoids, Flv. Although strictly speaking AnC are flavonoids as well, in this paper we consider red-colored AnC separately from other Flv which display a weak coloration when present in high quantities. Thus, development and implementation of reflectance-based techniques requires answering three pivotal questions. First, is it possible to describe the leaf as a medium with a close, linear ρ^{-1} vs. α relationship throughout the visible and the NIR ranges of spectrum, as required by Kubelka–Munk theory? Second, what are spectral ranges where the abovementioned requirement is fulfilled? Third, what would be an objective criterion for discerning the ranges where it is not fulfilled? Answering these questions, requiring a thoughtful study of ρ^{-1} vs. α relationship in leaves with widely variable pigment (Chl, Car, AnC and Flv) contents, shall reveal the possibilities and limitations of the reflectance-based techniques. It may lay a background for informed

Abbreviations: AnC, Anthocyanins; [AnC], Anthocyanin content; Car, Carotenoid(s); [Car], Carotenoid content; Chl, Chlorophyll(s); [Chl], Chlorophyll content; Flv, Flavonoid(s); [Flv], Flavonoid content; ρ, Reflectance; α, Absorbance

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selection of spectral bands for devising new and improving existing models for reflectance-based estimation of the pigments as well as for developing absorbance-based techniques applicable in the cases where the reflectance-based approaches fail.

Main objective of the paper was to find quantitative measures of absorbance and reflectance responses to each pigment content allowing to recognize possibilities and limitations of reflectance- and absorbance-based approaches for estimating foliar pigment content. Solution of this problem we used as a springboard to devise noninvasive techniques for estimating foliar pigment content, namely chlorophyll, anthocyanin and flavonoids. Specifically, we focus on (a) finding spectral regions where assumption of close linear ρ^{-1} vs. α relationship holds, thus, reflectance-based spectroscopy can provide accurate estimation of foliar pigment content; and (b) estimating content of pigments that absorb in spectral regions where reflectance is insensitive to pigment content and absorbance-based techniques are the only way non-destructively assessing its content.

2. Methods

Two data sets were used in this study. Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.) characterized by a widely varying content of pigments (Table. 1), especially of AnC and Flv [4,5] served as model species for this study. Healthy and homogeneously colored leaves were randomly collected according to their coloration in a park at Moscow State University in 2016. AnC were abundant during spring and autumn in sunlit leaves. In these species, AnC were predominantly in the vacuoles of the cell layer of palisade parenchyma (see [6] for more details).

The procedure allowing simultaneous quantification of Chl, Car, Anc, and Flv in an extract from leaf zone used for transmittance measurements was employed essentially as described in Solovchenko et al. [7]. Leaf disks (total area of 3.8 cm^2) were ground in chloroform–methanol (2:1, vol/vol) in the presence of MgO. After completion of extraction, homogenates were filtered through a paper filter, and distilled water (1/5 of total extract volume) was added. Then extracts were centrifuged at 3000*g* for 10 min until phase separation.

Chl and Car concentrations were quantified spectrophotometrically in lower (chloroform) phase using coefficients reported by Wellburn [8]. The upper (water–methanol) phase was used for assay of Flv, which were quantified spectrophotometrically using molar absorption coefficient ε_{358} = 25.4 $mM^{-1}\,cm^{-1}$ determined for rutin in 80% aqueous methanol. After determination of Flv the water–methanol phase was acidified with HCl (final concentration of HCl = 0.1%) and used for quantification of anthocyanins by measuring absorbance at 530 nm; absorption coefficient of 30 $mM^{-1}\,cm^{-1}$ [9] was accepted. Flv (in equivalent amounts of rutin) as well as other pigment content were expressed relative to leaf surface area.

Table 1
Characteristics of the datasets used in this work.

Dataset (*n* of leaves)	ANGERS (308)	Virginia creeper (24)
Chlorophyll (μg cm^{-2})		
Average ± STD	34 ± 22	10.9 ± 8.4
Min/Median/Max	0.7/29/107	0.36/10.5/38
Carotenoids (μg cm^{-2})		
Average ± STD	8.8 ± 5.1	3 ± 2.1
Min/Median/Max	0/7.5/26	0.4/17/2.8
Anthocyanins (μg cm^{-2})		
Average ± STD	1.7 ± 2	13 ± 9.4
Min/Median/Max	0/1.2/17.2	0.06/17/28
Flavonoids (μg cm^{-2})		
Average ± STD	N/A	99 ± 48
Min/Median/Max		38/79/195

Leaf transmittance (T) and reflectance spectra were recorded with a 150–20 Hitachi spectrophotometer equipped with an integrating sphere against barium sulphate as a standard. The spectra were recorded at 2-nm sampling intervals in 350–800 nm range. Absorbance (α) was calculated via transmittance as $\alpha = -\lg T$.

Second data set used was the ANGERS measured in 2003 at INRA in Angers (France) includes 308 leaves of > 40 plant species [10]. Transmittance and reflectance spectra measured using ASD FieldSpec with the integrating sphere Li-Cor 1800–12 in the range 400 to 2450 nm. This data were used frequently and described in detail in ANGERS Leaf Optical Properties Database (https://ecosis.org/?result=2231d4f6-981e-4408-bf23-1b2b303f475e).

3. Results

3.1. Spectral Characteristics of Leaves Studied

The leaves of Virginia creeper with widely varying [AnC] and [Flv] and low-to-moderate [Chl] illustrate how variable leaf optical properties, the absorbance, transmittance and reflectance, are (Fig. 1). In the blue range (400–500 nm), absorbance was very high varying widely between 1 and 3 increasing towards shorter wavelength in all leaves studied being affected by [Chl], [Car], and [Flv]. Accordingly, transmittance of the leaves was below 0.1 and varied about 10-fold. In contrast, reflectance of the leaves converged to a narrow range around 0.1 with a small variability.

In the green range (500–600 nm), the absorbance and transmittance showed a high variability with two ranges of convergence—around 500 and 600 nm where change in pigment content only slightly affected both traits. Optical properties around 500 nm were governed by Chl, Car and AnC and beyond 530 nm—by Chl and especially AnC, which absorption in situ peaks around 550 nm. Reflectance in the green was variable but much less than absorbance and transmittance.

In the red range (600–690 nm), optical properties were affected by Chl absorption that peaks in situ around 670 nm; magnitude of absorbance peak increased gradually with increase in [Chl]. In the transmittance spectra, Chl absorption manifested itself as a trough which depth steadily grew with an increase in [Chl] and transformed into a deep minimum in the spectra of leaves with a high [Chl]. In the reflectance spectra, Chl absorption revealed itself as a trough (in the case of low [Chl]), although in the leaves with moderate-to-high [Chl], reflectance converged to a narrow range around 0.1. Thus, the behavior of reflectance in the red range (as in blue) differed from that of absorbance or transmittance.

The differences in the spectra depicting absorbance and reflectance were further studied for representative leaves from two datasets. In the Virginia creeper leaf with a moderate [Chl], when α was below 1.1, ρ^{-1} and α closely related in a linear manner (determination coefficient R^2 = 0.98) in spectral ranges between 690 and 750 nm and 515–600 nm (Fig. 2). However, at wavelength shorter than 515 nm and between 600 and 695 nm, the slope of the α vs. ρ^{-1} relationship decreased and a strong hysteresis appeared in the range 600–695 nm: for the same absorbance, reciprocal reflectance was significantly higher than in the blue range 400–515 nm. Moreover, in the shortwave blue range ρ^{-1} decreased with increase in absorbance. Thus, in Virginia creeper leaves with α ≥ 1.1, reciprocal reflectance cannot be considered as a proxy for absorbance.

The ANGERS data set contained leaves with a wide [Chl] and [Car] variation. In Fig. 3a, absorbance and reciprocal reflectance spectra are presented for leaf with much higher [Chl] than of the Virginia creeper leaf shown in Fig. 2. In the spectral ranges 520–560 nm and 695–750 nm α was below 1.2 and the ρ^{-1} vs. α relationship was linear with R^2 = 0.99 (Fig. 3b). However, in the spectral range 560–695 nm α exceeded 1.2 and the relationship was essentially non-linear with a strong hysteresis and negative slope between 650 and 680 nm. At shorter wavelengths (beyond 520 nm), the slope of the relationship

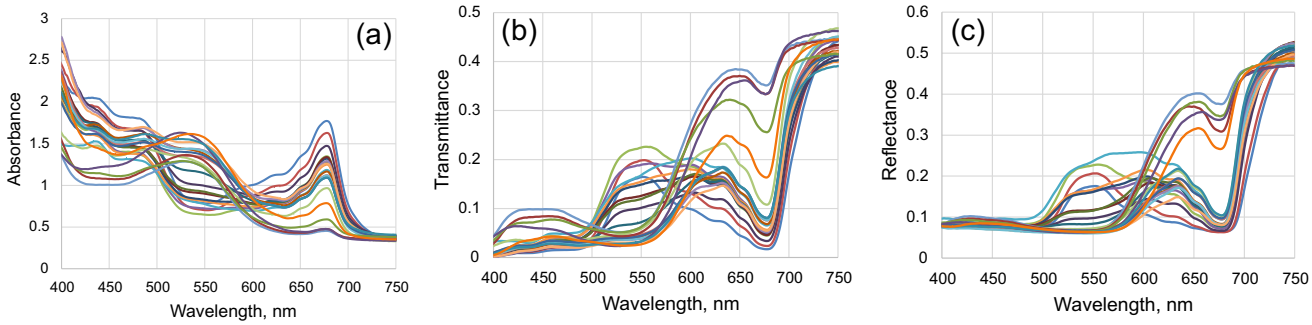

Fig. 1. Absorbance (a), transmittance (b) and (c) reflectance spectra of Virginia creeper leaves with widely varying pigment contents and composition.

decreased drastically and was close to zero at wavelengths $\lambda < 500$ nm; i.e., ρ^{-1} remained virtually invariant.

Thus, in both leaves presented in Figs. 2 and 3, the ρ^{-1} vs. α relationships were close and linear in the *green* (520–560 nm) and *red edge* (695–750 nm) ranges where $\alpha < 1.2$ and was essentially non-linear in the blue and red ranges This circumstance imposes a very strict limitation on the possibility of foliar pigment content retrieval via reflectance spectroscopy.

3.2. In Situ Specific Optical Properties of Foliar Pigments

The ANGERS is a dataset with probably widest [Chl] variation among existing data sets (Table 1). However, it does not represent leaves with moderate-to-high [AnC]: only in 12 of 308 leaves [AnC] exceeded 5 μg cm^{-2} with the maximal value of 17 μg cm^{-2}. Thus, we used this data set to study optical properties of leaves with high variability of [Chl] and [Car] contents against a slightly variable background of [AnC]. The Virginia creeper leaves had widely variable [AnC]; in addition, it is the only data set we know where [Flv] and optical properties are presented. This data set was used to study in situ optical properties of Flv and AnC.

To quantify the effect of each pigment content, [p], on absorbance and reciprocal reflectance, *α* vs. [p] and ρ^{-1} vs. [p] relationships were established at each wavelength (λ) and for each pigment. We calculated determination coefficient, R^2, for linear relationships *α* vs. [p] and ρ^{-1} vs. [p] as well as the slopes of these relationships at each wavelength. Determination coefficient is a quantitative measure of how well the best-fit function performs as a predictor of α or ρ^{-1}, specifically, how much of their variability can be explained by the variation in the corresponding pigment content. Slopes of *α* vs. [p] and ρ^{-1} vs. [p] relationships represent sensitivity of absorbance and reciprocal reflectance to the pigment content. However, none of the characteristics, either R^2 or the slope, is an accurate quantitative measure of each pigment effect on α and ρ^{-1}. The spectra of the slope per se do not impart the strength of the corresponding relationships and the R^2 spectra bear no information about the sensitivity of α or ρ^{-1} to each pigment content.

Quantitative measure of the effect of each pigment on absorbance combining these two parameters is α response, Rα, at each wavelength [11]:

$$R\alpha = (d\alpha/d[p])/NRMSE \quad (1)$$

where [*p*] is a pigment content, dα/d[*p*] and NRMSE are first derivative and normalized root mean square error of the *α* vs. [p] relationship, respectively.

In the same way, quantitative measure of the effect of each pigment on reciprocal reflectance, is ρ^{-1} response, $R\rho^{-1}$,

$$R\rho^{-1} = (d\rho^{-1}/d[p])/NRMSE \quad (2)$$

where [*p*] is a pigment content, $d\rho^{-1}/d[p]$ and NRMSE are first derivative and normalized root mean square error of ρ^{-1} vs. [p] relationship.

Both measures Rα and $R\rho^{-1}$ represent the spectral response of absorbance and reciprocal reflectance to content of specific pigment

The first question should be answered was how α and ρ^{-1} responded to [Chl] and how close the Rα vs. [Chl] and $R\rho^{-1}$ vs. [Chl] relationships are. We carried it out for the ANGERS data set with widest [Chl] variation. The main feature of R^2, Rα and $R\rho^{-1}$ spectra was disparate spectral behavior of absorbance and reciprocal reflectance (Fig. 4). In the blue range (400–500 nm), R^2 for the α vs. [Chl] relationship was above 0.7 but it was below 0.3 for the ρ^{-1} vs. [Chl] relationship (Fig. 4a). The same was the case in the range 600–680 nm. Only *in the green and red edge ranges* R^2 of both α and ρ^{-1} vs. [Chl] relationships were comparable reaching the R^2 around 0.9 in range 700–710 nm. Importantly, (i) in the ranges of highest Chl absorption—the blue (400–500 nm) and the red (around 670 nm), Rα was

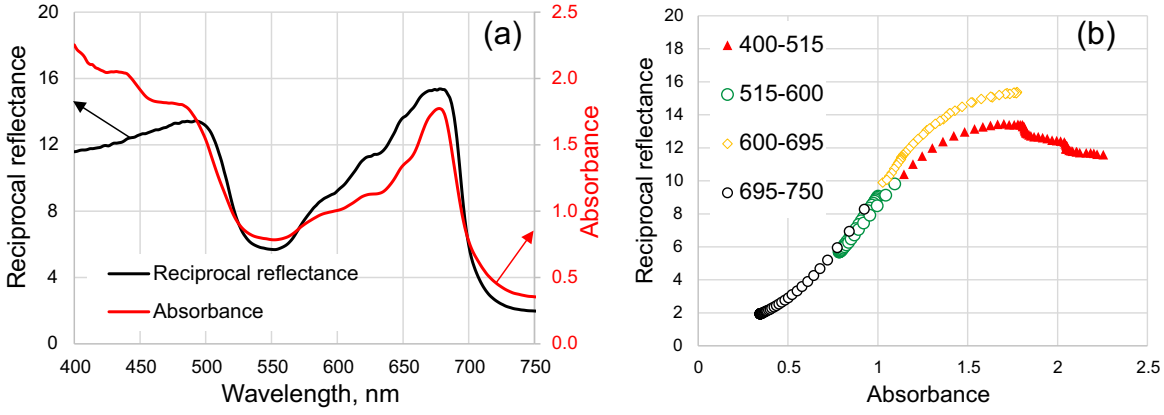

Fig. 2. (a) Absorbance and reciprocal reflectance spectra of Virginia creeper leaf with a moderate [Chl] = 22 μg cm^{-2} and [Car] = 5 μg cm^{-2}, a small [AnC] = 0.07 μg cm^{-2}, and a very high [Flv] = 165 μg cm^{-2}. (b) Reciprocal reflectance vs. absorbance of the same Virginia creeper leaf.

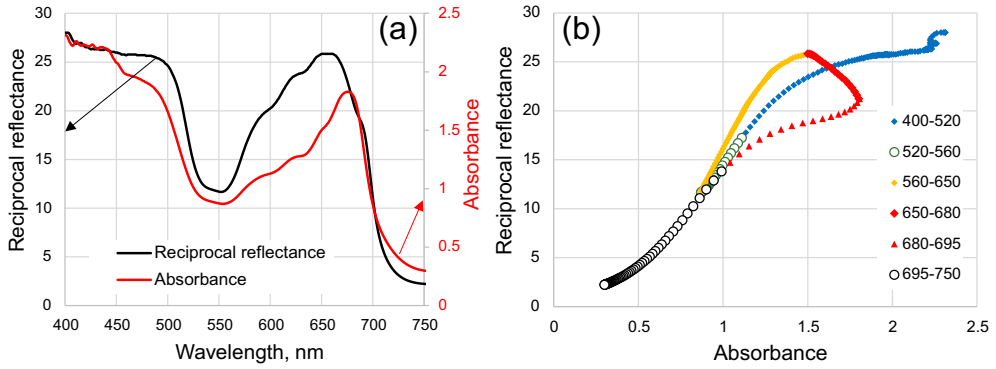

Fig. 3. (a) Absorbance and reciprocal reflectance spectra of a leaf from the ANGERS data set with a high [Chl] = 54 μg cm^{-2} and [Car] = 12 μg cm^{-2}, and a small [AnC] = 2.2 μg cm^{-2}; (b) Reciprocal reflectance vs. absorbance of the same leaf.

two-fold higher than Rρ^{-1} (Fig. 4b), and (ii) the green (520–570 nm) and the red edge (700–710 nm) were the only spectral ranges where Rρ^{-1} > Rα and Chl was the main factor governing ρ^{-1}.

Next step was to compare spectral responses of reciprocal reflectance to the contents of all three pigments identified in the ANGERS data set (Fig. 5). The Rρ^{-1} spectra for Chl and Car were almost identical. It is not surprising because in this data set [Car] correlated very closely with [Chl] (R^2 > 0.9). Thus, [Chl] and [Car] were not really independent variables. Actually, we did not find a data set where [Chl] and [Car] varied independently so we omitted Car estimation from this study.

The main spectral feature of the Rρ^{-1} spectra was constituted by small values of this trait in the blue (absorption bands of Chl and Car) and red (absorption band of Chl) ranges. Two distinguished peaks of Rρ^{-1} for Chl were around 600 and 700 nm. Importantly, both peaks were positioned in the ranges where absorption by Chl is much smaller than in the red absorption band where leaf reflectance is saturated at [Chl] smaller than 20 μg cm^{-2} [3,11].

Rρ^{-1} for [AnC] was high in the green range around 550 nm (the main AnC absorption region in situ, [5]). However, AnC Rρ^{-1} ≅ Chl Rρ^{-1} so reciprocal reflectance in this region was affected by Chl to the same degree as by AnC.

The responses Rα and Rρ^{-1} to [Chl], [AnC], and [Flv] were compared for Virginia creeper leaves with highly variable [AnC] and [Flv] and low-to-moderate [Chl]. As for the ANGERS data set containing leaves with much higher [Chl], Rα to [Chl] was higher than Rρ^{-1} to [Chl] in the spectral ranges of highest Chl absorption, blue and red (Fig. 6a). Chl Rρ^{-1} > Chl Rα around 640 and 700 nm that located far from red Chl absorption band. Notably, both Rα and Rρ^{-1} to Chl were negative in the green range (Fig. 6a) due to negative slopes of α vs. [Chl] and ρ^{-1} vs. [Chl] relationships. Major number of leaves in this

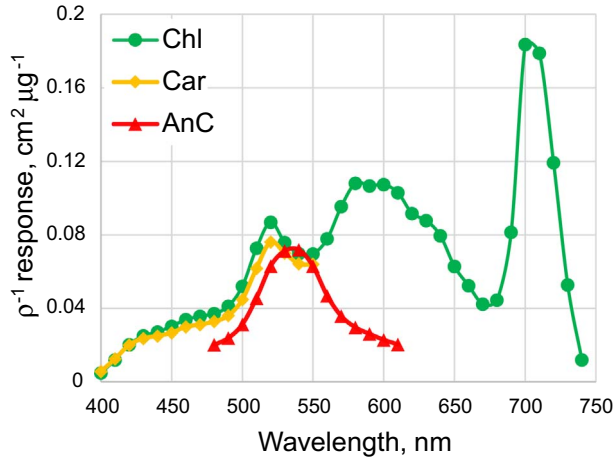

Fig. 5. Spectral response of reciprocal reflectance, Rρ^{-1}, to Chl, Car, and AnC contents for 308 leaves constituting the ANGERS data set. AnC content was identified in Feret et al. [13].

data set had high amount of [AnC] and small amount of [Chl]. In leaves with small [Chl], absorbance in green range was high governing by [AnC] and with increasing [Chl] it decreased due to decreasing [AnC]. For AnC, Rα > Rρ^{-1} was recorded in the range 520–560 nm, where absorption of AnC peaks in situ (Fig. 6b), due to saturation of reflectance at high [AnC] when α exceeded 1.2 (see 3.1).

For Flv, in the range 400–460 nm Rα was 5–7 fold higher than Rρ^{-1} (Fig. 6c). In this range Chl, Car and Flv absorb and reflectance saturated at a low [Chl], thus, ρ^{-1} became almost invariant with respect to pigment content (see Fig. 1c). It is illustrated well in Fig. 7A where

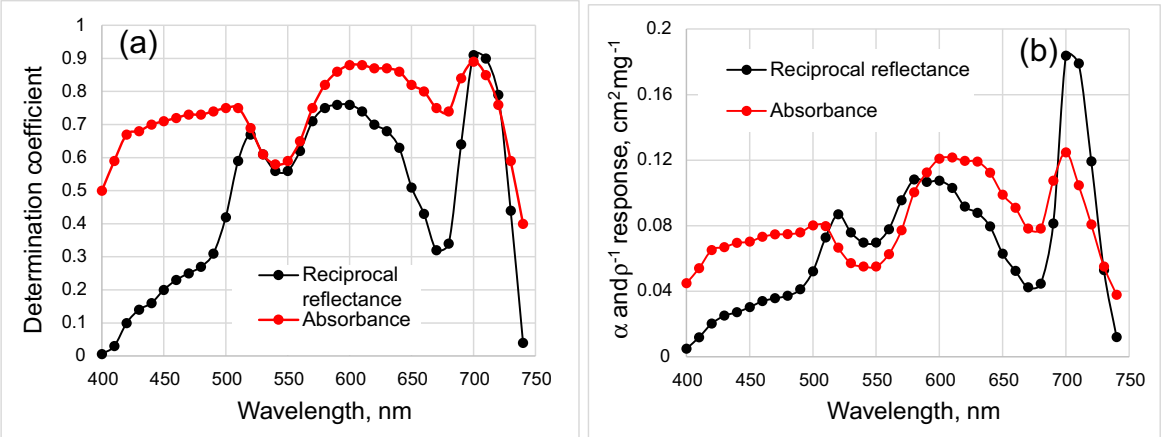

Fig. 4. Characteristics of α vs. [Chl] and ρ^{-1} vs. [Chl] relationships for the ANGERS data set: (a) spectra of determination coefficient, R^2, of the α vs. [Chl] and ρ^{-1} vs. [Chl] linear relationships, (b) spectra of α response, Rα, and reciprocal reflectance response, Rρ^{-1}, to Chl content.

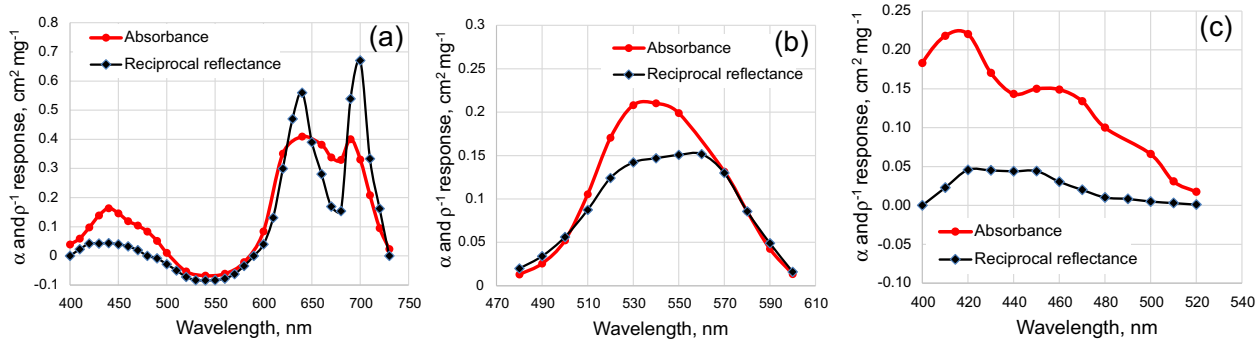

Fig. 6. Spectra of absorbance response, Rα, and reciprocal reflectance response, $R\rho^{-1}$, to pigment content of (a) chlorophyll [Chl], (b) anthocyanin [AnC] and (C) flavonoids [Flv] in leaves of Virginia creeper data set with highly variable [AnC] and [Flv] and low to moderate [Chl].

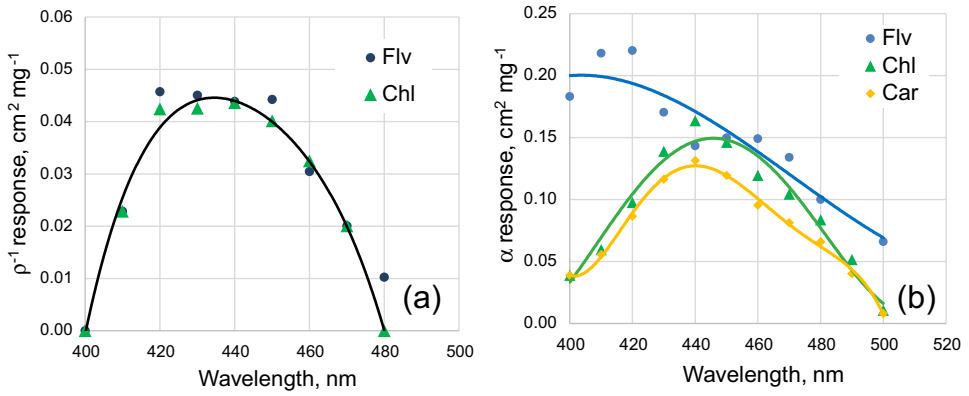

Fig. 7. The responses of reciprocal reflectance, $R\rho^{-1}$ (a) and absorbance, Rα (b) to pigment contents in the blue range of the spectrum for the Virginia creeper leaves. Note that $R\rho^{-1}$ to Flv and $R\rho^{-1}$ to Chl are identical (Fig. 7a) showing that there is no way to distinguish between these pigments using reflectance spectra. In contrast, absorbance response to Flv in range 400–430 nm was higher than that to Chl (Fig. 7b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

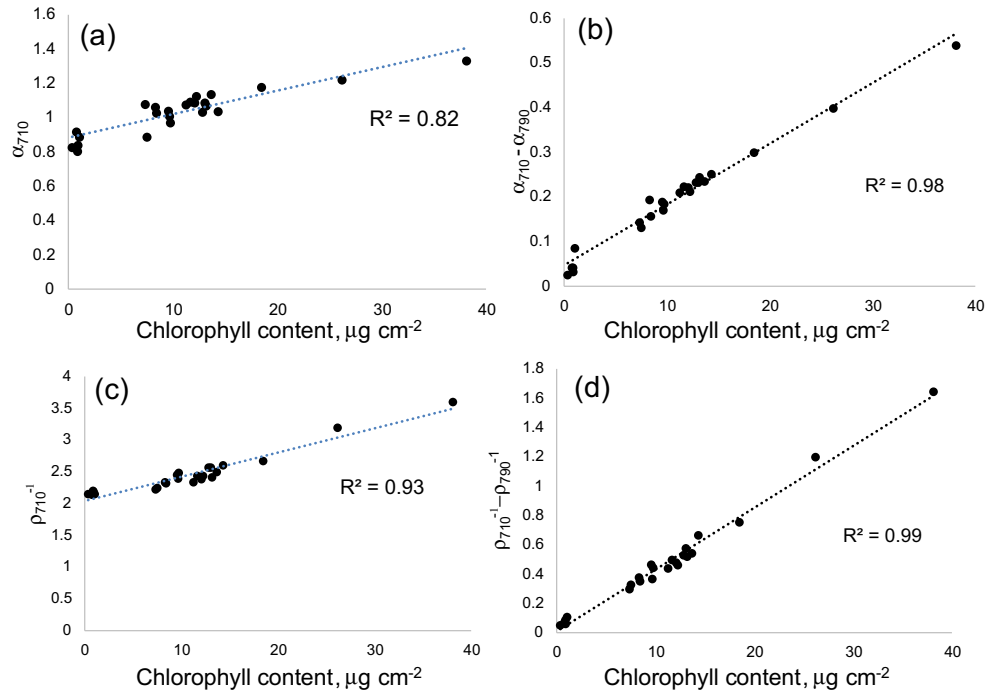

Fig. 8. Chlorophyll content in the Virginia creeper leaves plotted versus (a) absorbance at 710 nm, α_{710}, (b) difference of absorbance at 710 nm and in the NIR at 790 nm, $\alpha_{710} - \alpha_{790}$, (c) reciprocal reflectance at 710 nm, ρ_{710}^{-1}, and (d) difference $\rho_{710}^{-1} - \rho_{790}^{-1}$.

$R\rho^{-1}$ to Chl and $R\rho^{-1}$ to Flv were indistinguishable and very small. In contrast to $R\rho^{-1}$, responses of absorbance to [Chl] and [Flv] were much higher and, importantly, in spectral range 400–430 nm Rα to Flv was higher than Rα to Chl (Fig. 7b). This finding gives an important insight into identification of spectral range suitable to [Flv] retrieval from absorbance spectra.

3.3. Implications for Pigment Content Estimation

3.3.1. Chlorophylls

For individual leaves and two contrasting data sets was shown that Rα and $R\rho^{-1}$ to Chl are very different across the whole spectral range and greatly depend on pigment content and composition. Essentially, in the *red edge region* (around 700 nm) $R\rho^{-1} >$ Rα (Figs. 4b and 6a) and

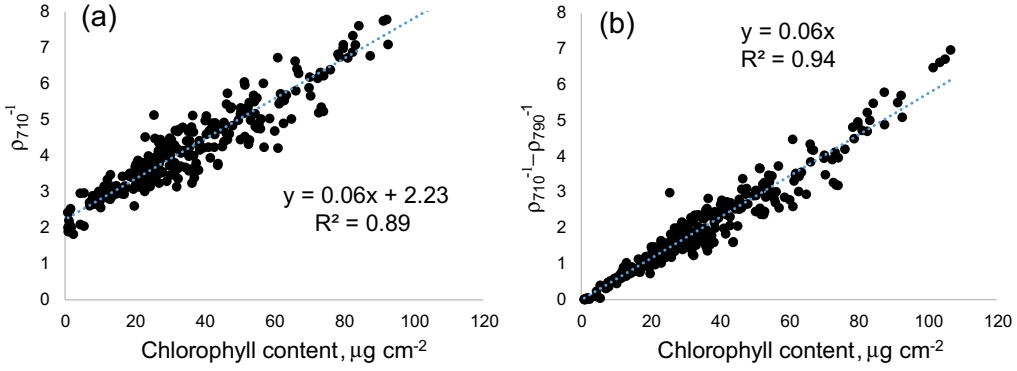

Fig. 9. Chl content in the leaves of the ANGERS data set plotted versus (a) reciprocal reflectance at 710 nm, ρ_{710}^{-1}, and (b) difference $\rho_{710}^{-1} - \rho_{790}^{-1}$.

spectral shape and magnitude of the responses were almost identical despite great variation in pigment content in the data sets studied. It means that ρ^{-1} in the red edge region may be used as a term in algorithms for accurate and, probably, generic measure of [Chl] (Fig. 8a and c). The only obstacle for achievement of a high accuracy of [Chl] estimation using ρ^{-1} is non-zero values of absorbance and reciprocal reflectance in the near infra-red (NIR) spectral range where Chl does not absorb (Fig. 1A). This is apparent absorbance caused by uncertainties of transmittance and reflectance measurement. These uncertainties may affect accuracy of [Chl] estimation especially for low [Chl] [3]. Thus, subtraction of α_{NIR} and ρ_{NIR}^{-1} for accurate [Chl] estimation is required. We suggest using the difference between absorbance and reciprocal reflectance in the red edge (α_{RE} and ρ_{RE}^{-1} around 710 nm) and in the NIR (α_{NIR} and ρ_{NIR}^{-1} beyond 760 nm) for accurate [Chl] estimation:

$$\text{Chl} \propto \alpha_{RE} - \alpha_{NIR} \quad (3)$$

$$\text{Chl} \propto \rho_{RE}^{-1} - \rho_{NIR}^{-1} \quad (4)$$

Subtraction of α_{NIR} and ρ_{NIR}^{-1} makes ($\alpha_{RE} - \alpha_{NIR}$) and ($\rho_{RE}^{-1} - \rho_{NIR}^{-1}$) almost proportional to [Chl] (i.e., relationships goes to origin) and it does bring significant increase in accuracy (Fig. 8b and d). Both models (3) and (4) yield very accurate [Chl] estimation using absorbance and reflectance with the determination coefficient above 0.98 and NRMSE < 2.4%.

Reciprocal reflectance ρ_{710}^{-1} alone was also a very accurate measure of [Chl] in the ANGERS data set with a wide [Chl] variability (Fig. 9a). To increase accuracy of estimating [Chl] in this data set, we applied model (4) with red edge band at 710 nm and NIR at 790 nm (Fig. 9b). The accuracy was high (R^2 = 0.94, NRMSE = 9.7%) confirming robustness of the approach.

3.3.2. Anthocyanins

In the Virginia creeper data set containing AnC-containing leaves (mainly red and dark red), large difference in Rα and $R\rho^{-1}$ in the range 520–550 nm was found (Fig. 6b). It shows that the highest accuracy of [AnC] estimation may be achieved using absorbance at 550 nm (Fig. 10a). Reciprocal reflectance around 570 nm where Rα = $R\rho^{-1}$ was also found an accurate proxy of [AnC] (Fig. 10c). However, in the range 550–570 nm (Fig. 5) Chl significantly affected α and ρ^{-1}. Thus, subtraction of this effect should be done using α and ρ^{-1} in the red edge range where they accurately represent [Chl]. The following models were capable of accurate estimation of [AnC] using absorbance and reflectance of leaves with widely variable pigment composition (Fig. 10):

$$[\text{AnC}] \propto \alpha_{550} - \alpha_{RE} \quad (5)$$

$$[\text{AnC}] \propto \rho^{-1}{}_{570} - \rho^{-1}{}_{RE} \quad (6)$$

3.3.3. Flavonoids

In the blue range 400–500 nm where optical properties are affected by all three pigments, Chl, Car and Flv, ρ^{-1} was either almost flat (Figs. 1c and 3b) or even decreased with α increase (Fig. 2b). The Rα and $R\rho^{-1}$ for [Chl] and [Flv] bring unique quantitative information on the response of α and ρ^{-1} which is specific for each pigment. As can be seen from Fig. 7a, $R\rho^{-1}$ to [Chl] and $R\rho^{-1}$ to [Flv] were equal showing that reflectance spectroscopy is unable to differentiate between these pigments. By contrast, in the range between 400 and 430 nm the Rα to [Flv] was higher than Rα to [Chl]. It means that for data used it is the only spectral band where [Flv] may be estimated using nondestructive absorbance spectroscopy (Fig. 7b). Another important finding is that Chl effect in the range 400–430 nm is still significant, and its subtraction would be necessary for accurate estimation of [Flv] (Fig. 7b).

Absorbance at 420 nm was quite an accurate proxy of [Flv] (Fig. 11a). Subtraction of α_{710} allowed decreasing Chl effect at 420 nm and the following model may be used for accurate non-destructive estimation of [Flv] in a wide range of their variation (Fig. 11b):

$$[\text{Flv}] \propto a_{420} - a_{710} \quad (7)$$

4. Discussion

Here, we compared the relationships between absorbance and reflectance vs. pigment content in leaves using large datasets collected across plant species, developmental stages and physiological states. The analysis made obvious certain limitations of reflectance-based quantification of the foliar pigments, especially in the blue and red manifesting itself as a failure of linear relationship between reciprocal reflectance and absorbance. These limitations can be understood in frame of Kubleka-Munk theory, which was developed for the case of a relatively weak absorber evenly distributed in a thick layer of a highly reflective substance [1]. Considering large extinction coefficients of Chl and other pigments [12], their high content in and structural complexity of the leaf and its photosynthetic apparatus [13], it becomes clear that in many cases the foliar pigments will violate these assumptions. Indeed, the leaves with absorbance exceeding 1 are rather "strong absorbers" in the Kubelka-Munk terminology, and distribution of pigments in the cells is far from uniform [6,14]. Furthermore, superficial structures of plants such as leaf [15] and fruit [16] cuticle give rise to backscattering. The contribution of the signal backscattered by weakly pigmented superficial structures of leaf (cuticle and epidermis) to the total leaf reflectance bears no information of the leaf pigment composition and decreases the "information payload" of total reflected signal. This contribution increases dramatically towards shorter wavelength of the visible part of the spectrum but bears only scarce information on the biochemical composition of the leaf.

These limitations obviously affect the spectral ranges suitable for [Chl], [Car], [AnC] and [Flv] estimation. As a result, reflectance-based approach can be implemented only in certain spectral ranges positioned outside the main absorption bands of the pigments, mainly in the long-wave part of the visible range, red edge and NIR (e.g., [3,5,11,17]). In view of these restrictions, it is important to have a quantitative criterion

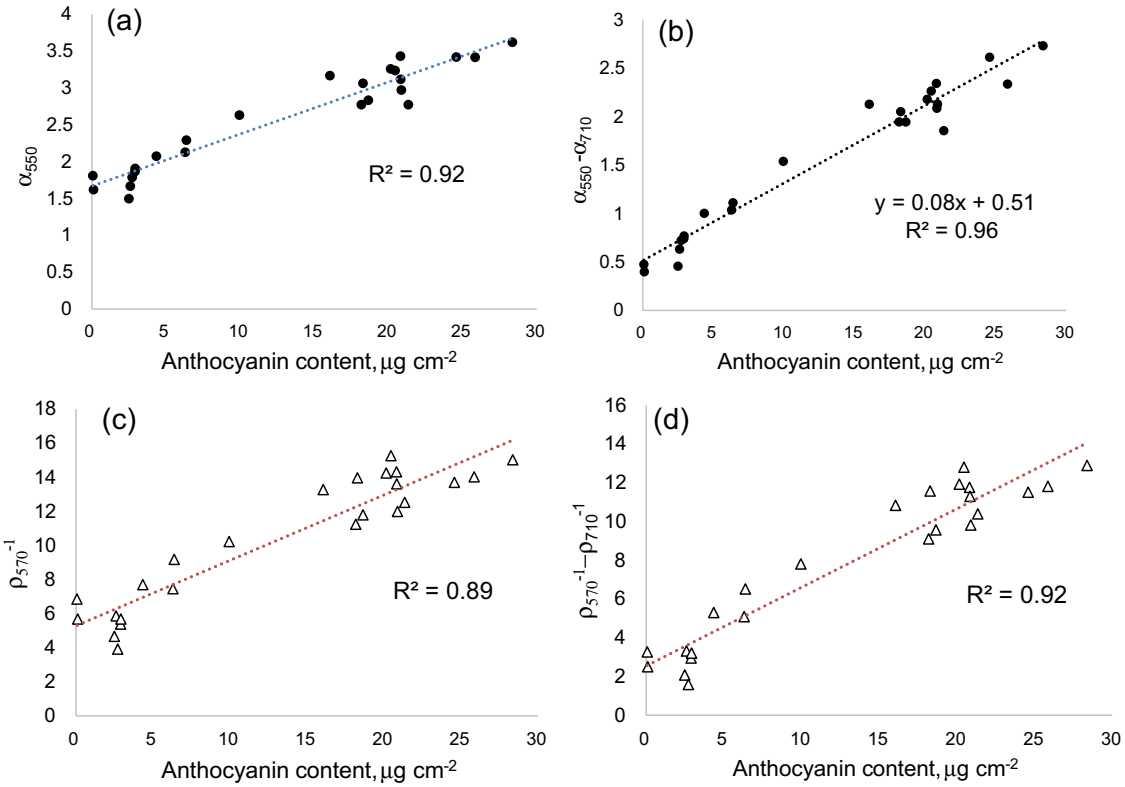

Fig. 10. Anthocyanin content in the Virginia creeper leaves plotted versus (a) absorbance at 550 nm, α_{550}, (b) difference of absorbance at 550 nm and 710 nm, $\alpha_{550} - \alpha_{710}$, (c) reciprocal reflectance at 570 nm, ρ_{570}^{-1}, and (d) difference $\rho_{570}^{-1} - \rho_{710}^{-1}$.

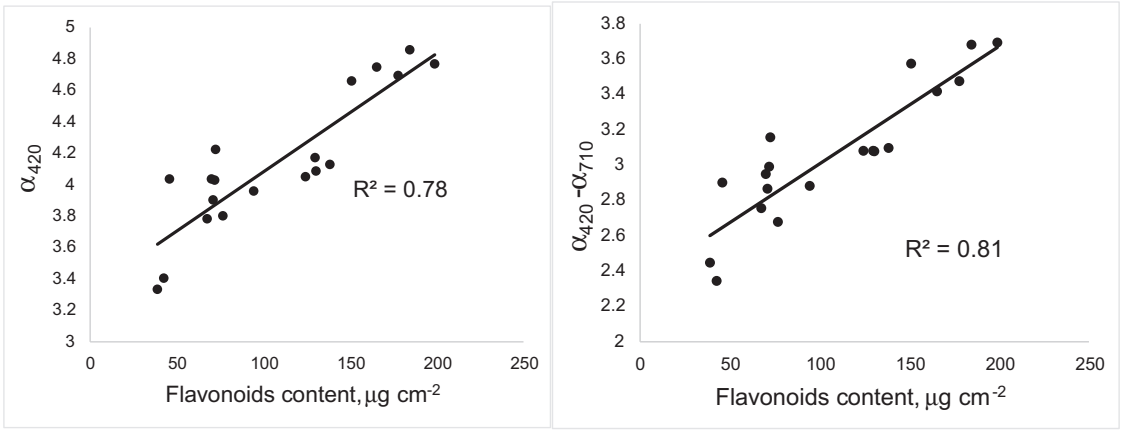

Fig. 11. Flavonoid content in Virginia creeper leaves plotted versus (a) absorbance at 420 nm, α_{420}, (b) difference of absorbance at 420 nm and 710 nm, $\alpha_{420} - \alpha_{710}$.

of the suitability of a certain spectral range for application of the reflectance-based techniques, which was not defined so far. In this work, we tried to close this gap by suggesting $R\alpha$ and $R\rho^{-1}$ as quantitative measures of the absorbance and reflectance response to content of each pigment.

It was shown for individual leaves and two contrasting data sets that $R\alpha$ and $R\rho^{-1}$ to Chl are very different across the spectral region and greatly depend on pigment content and composition. Thus, $R\alpha$ and $R\rho^{-1}$ complement pigment specific absorption coefficients (e.g., [13]) bringing quantitative effect of each pigment *with background of other pigments* on α and ρ^{-1}. Our findings using quantitative spectral responses to each pigment group is in accord with the results of previous studies identified optimal spectral bands for retrieval of foliar pigment content (e.g., [18–23]).

Clearly, it is important not only to distinguish the limits for application of the reflectance-based technics but also to find a more generic approach capable of overcoming these limitations. Towards this end, we deduced in situ absorbance of foliar Chl, Car, AnC and Flv and introduced a concept of specific absorbance response objectively showing the contribution of each pigment group to light absorption. Judging from the results of this study, foliar absorbance much less suffered from the limitations typical for the reflectance-based approaches. The absorbance-based algorithms demonstrated increased dynamic range and linear relationships with the leaf pigment content especially pronounced in the shortwave part of the visible spectrum.

Exploiting the absorption-based approach, one can improve dramatically non-invasive estimation of the pigments absorbing in the blue (flavonoids) and blue-green (carotenoids). This turned to be feasible even on the background of strong overlapping absorption of other pigments although estimation of smaller Flv quantities require further

testing. Analysis of light interaction with leaf in terms of absorbance improves our understanding of in situ light absorption properties of all key pigment groups paving way for further insights in plant physiology and photobiology. Basing on the comparative account of advantages and drawbacks of the reflectance- and absorbance-based pigment estimation, we argue that these approaches complement each other and can be used synergistically in advanced models for accurate estimation of foliar pigments. We believe that the response traits introduced in this work are very instructive for understanding the specific and combined effect of pigments on optical properties. Hence another outcome of this study is a deeper insight into the interception of light by photosynthetic and photoprotective pigments as function of physiological condition and developmental stage, which is of utter importance for plant physiology and photobiology. It is the foundation of knowledge-driven selection of spectral bands for creating new and improving existing models for noninvasive remote estimation of the pigments.

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