Semina: Ciências Agrárias
ISSN: 1676-546X
semina.agrarias@uel.br
Universidade Estadual de Londrina
Brasil

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Semina: Ciências Agrárias, vol. 37, núm. 3, mayo-junio, 2016, pp. 1277-1283
Universidade Estadual de Londrina
Londrina, Brasil

DOI: 10.5433/1679-0359.2016v37n3p1277

Estimating chlorophyll content from *Eucalyptus dunnii* leaves by reflectance values

Estimativa da concentração de pigmentos clorofilianos em folhas de *Eucalyptus dunnii* por valores de refletância

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Abstract

This study aimed to estimate photosynthetic pigments contents from leaves of *Eucalyptus dunni* Maiden based on values of reflectance spectra of red, green and blue colors obtained with a digital color analyzer. We collected fifty leaves from the lower third of the crown of twenty trees including young as well as mature leaves. From each leaf an area of 14 cm^2 of the leaf blade was cut in which we measured reflectance values on the red, green and blue spectra with a portable digital colorimeter, obtained relative index of chlorophyll with a SPAD – 502 and determined the content of the chlorophyll *a*, *b,* and *a* + *b* by classic method of solvent extraction. We submitted the data to multiple linear regression and nonlinear analysis at 5% of error probability. It was evaluated the occurrence of multicollinearity. The negative exponential model resulted in good fit when data from red spectrum was used for chlorophyll *a*, green spectrum for chlorophyll *b* and *a* + *b,* making possible correlation coefficients between the estimated values and the extracted above 0.85. Except for the chlorophyll *a* content, the accuracy in estimates of photosynthetic pigments were higher than estimated by the chlorophyll meter, even with linearity between methods. Therefore, it is possible to estimate photosynthetic pigments on *E. dunni* leaves through values of red and green wavelengths from a digital color analyser.
Key words: Chlorophylls. Colorimetry. Spectrum colors.

Resumo

O presente trabalho objetivou estimar a concentração de pigmentos clorofilianos em folhas de *Eucalyptus dunni* Maiden com base nos valores de refletância de cores no espectro eletromagnético no vermelho, verde e azul obtidos por um analisador de cores. Cinquenta folhas coletadas do terço inferior da copa de vinte árvores foram utilizadas abrangendo folhas jovens até folhas em processo de abscisão. De cada folha foi retirada uma área de 14 cm^2 de limbo foliar, e nesta mensurados os valores da refletância no espectro na faixa do vermelho, verde e azul com um colorímetro portátil, obtido o índice relativo de clorofila com um SPAD – 502 e determinada a concentração de clorofila *a*, *b* e *a* + *b* pelo método clássico de extração por solvente. Os resultados foram submetidos a análise de regressão linear múltipla e não linear a 5% de probabilidade de erro. Avaliou-se a ocorrência de multicolinearidade. Dos modelos ajustados foi realizada uma análise dos resíduos e correlacionados com a concentração extraída. Houve multicolinearidade entre os valores do espectro de cores, inviabilizando a estimativa das clorofilas por

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Recebido para publicação 01/04/15 Aprovado em 24/11/15

um modelo linear múltiplo. O modelo exponencial negativo possuiu maior aderência, quando utilizados os valores do espectro vermelho para a concentração de clorofila *a*, e do verde para a concentração de clorofila *b* e *a* + *b*, possibilitando coeficientes de correlação entre os valores estimados e os extraídos acima de 0,85. Com exceção da concentração de clorofila *a*. a precisão nas estimativas dos pigmentos clorofilianos foram superiores ao estimado pelo clorofilômetro, mesmo com linearidade entre métodos. Portanto, é possível estimar pigmentos clorofilianos em *E. dunni* por meio de valores de comprimentos de onda na faixa do vermelho e verde do espectro de cores.
Palavras-chave: Colorimetria. Clorofilas. Espectro de cores.

Chlorophylls are the main chloroplast pigments responsible for the capture of solar radiation, which plays a dominant control over the amount of solar radiation absorbed by plants. Therefore, foliar chlorophyll concentrations keep close relationship with photosynthetic rates and primary productivity.

The quantification of chlorophyll is of particular importance for plant studies to assess growth and development subjected to various environmental conditions.

The classical method for determination of chlorophyll from leaves is the extraction with solvents, followed by spectrophotometric reading (ARNON, 1949). Although easily performed, the method results in destructive collection of plant material samples, besides being burdensome in relation to the time and consumption of reagents.

With the arrival of remote sensing tools and portable analyzers that use of non-destructive optical principles associated with transmittance, absorbance or reflectance of radiation by leaves chlorophyll, it became possible to evaluate not only large spatial areas with high frequency of data acquisition and field measurements (BARRY et al., 2009; COSTE et al., 2010; RIGON et al., 2012).

Estimation of leaf chlorophyll content with SPAD 502 produced by Konica Minolta has been one of the most used protocol. The instrument measures the transmission of light in the red and infrared spectra, with a peak of transmittance near 650 nm and 940 nm, respectively, resulting in a dimensionless unit. However, the accuracy of the results can be influenced by the thickness and leaf relative water content, since the optical path traveled by a photon increases with the thickness, resulting in higher diffusivity and thereby overestimating chlorophyll content (MARENCO et al., 2009).

Color portable analyzer uses the spectral selective reflection method to determine the color of a sample, showing RGB (red, green, blue) values by means of color photo transmitters in a spectral scale of 400 to 700 nm which lies near the absorption of two primary wavelengths associated with the activity of chlorophylls (680 mm and 700 mm).

The analysis of the selective reflection of light allows to detect the reflectance peak in the region near 550 nm which is related to the total chlorophyll on the leaf inside visible range (SILVA et al., 2012) as well as detecting visible differences in the color of the leaf, due to a variable distribution of chloroplast pigments such as carotenes and the xanthophylls. Therefore, the selective reflectance of wavelength bands of red, green and blue by photosynthetic pigments enables its estimate. However, the accuracy, precision and usefulness of the color values of the leaf for color digital analyzers are little known.

This study aimed to estimate chlorophyll contents in leaves of *Eucalyptus dunni* based on the color reflectance values in the visible spectrum obtained by a color analyzer.

We collected leaves of *E.dunnii* from an eight years old monoculture belonging to State University of Western Paraná in Marechal Cândido Rondon County, PR in November 2013. The plantation is located with the coordinates of 24º 35 ‘42 “S and 53º 59’ 54” W and an altitude of 472 m.

The climate, according to Köppen's classification is a Mesothermal Humid Subtropical with hot summers (average temperature above 22.0 °C), tendency for concentration of rains, winters with little frequent frosts (average temperature less than 18.0 °C) and average annual rainfall of 1,500 mm. The soil of the experimental area was classified as rhodic hapludox (EMBRAPA, 2006).

The assay used approximately 500 leaves of 20 trees, collected from the lower third of the canopy. Of the total leaves described above, we used up to 50 leaves whose number was determined by a simple random sampling method, based on the variance of the concentration of chlorophyll *a* + *b* of 20 leaves. The leaf sample number was based on the acceptance of a 10% error limit with a 95% probability by the t Student "t test" for a population, which tends to finite, as suggested by Pellico Netto and Brena (1997) based on Equation 1.

$$NS = [(N * t^2) * S^2\mu] / [N (EL * \mu)] + (t^2 * S^2\mu) \quad (1)$$

Which are: NS: number of samples; N: total number of leaves; t: value of t Student (0.05; n-1); $S^2\mu$: sample variance; EL: decimal error limit; μ: arithmetic average of the sample.

Determination of chlorophyll content followed the methodology proposed by Arnon (1949) modified by Barbieri Junior et al. (2010) by removing stages of crushing and centrifugation. Leaf samples of 14.0 cm² (per sample) were placed in Falcon tubes of 15.0 cm³ previously coated with aluminum foil and filled with 10 mL of 80% acetone. The specimens were incubated at 25 °C for 48 h. At the end of the extraction period, 3 mL aliquots were transferred to 3.0 cm³ quartz cuvettes and the absorbance values read at 645 and 663 nm. The results for the concentration of chlorophyll *a*, chlorophyll *b*, and chlorophyll *a* + *b* were obtained according to the equations 2, 3 and 4 proposed by Arnon (1949) and the results expressed in μmol m², considering the volume of the extractor, the leaf area and the molar mass of chlorophylls *a* and *b*.

$$\text{Chlorophyll } a = 12.7\ A_{663} - 2.69\ A_{645} \quad (2)$$

$$\text{Chlorophyll } b = 22.9\ A_{645} - 4.68\ A_{663} \quad (3)$$

$$\text{Chlorophyll } a + b = \text{chlorophyll } a + \text{chlorophyll } b \quad (4)$$

To the color reflectance analysis in the spectra range of red, green and blue, we used the Digital Color Analyzer ACR-1023 (Instrutherm®) whose results were expressed as mean of three measurements per sample with area of 14.0 cm², closing the instrument on the leaf blade. In addition, mean values were obtained from the relative index of chlorophyll with a SPAD – 502 (Konica Minolta®) obtaining the average of five readings per sample.

Results were subjected to the test of Lilliefors to evaluate normality of residual distribution, followed by multiple linear regression analysis at 5% probability of error. It was noted the occurrence of multicollinearity between color spectra for the variance inflation factor (VIF) admitting VIF <10.0. If found multicollinearity we adopted the non-linear regression analysis. The selected model was the one who resulted in significance by the F test, with higher adjusted coefficient of determination and lower standard error of the estimate. In addition, to validate the adjusted models we conducted an analysis of the residues of the adjusted models and obtained simple linear correlation coefficients between observed with estimated values. Analyses were performed with Sigmaplot 12.0 (SIGMAPLOT, 2011). We used the values spectra of red, green and blue as independent variables.

All parameters quantified in leaves of *E. dunnii* showed normal distribution by Lilliefors test at 5% error probability. The coefficients of variation were 41.0%, 58.8%, 48.7% and 27.4% for chlorophyll content *a*, *b*, *a* + *b* and the relative index of chlorophyll respectively, indicating high variation

between samples. This behavior was expected, as were used for the analysis a variety of leaves from developing to mature leaves. For reflectance values, coefficients of variation were less than 10%.

The results of multiple linear regression analysis resulted in multicollinearity to the values of the lengths in red (VIF = 34.5) and blue (VIF = 25.7) spectra of the estimates of all chlorophyll pigments, which prevented the use of a multiple model for the estimates. For the values of the lengths in the green spectrum VIF value was 4.7. Multicollinearity results in model fit problems, causing negative impacts on the estimation of the parameters. The variance inflation factor (VIF) measures how much the variance of the beta coefficient is inflated by its collinearity with other independent variables (FREUND; WILSON, 1998).

Because it was not possible to use a multivariate model, only the reflectance values in the green spectrum did not accuse collinearity with the other color spectra, we decided to use a nonlinear regression analysis, which expressed significance to the negative exponential model with two parameters (Table 1).

Wavelength values in red spectrum allow a better estimate of the chlorophyll *a* content (Figure 1a) whose coefficient of correlation with the classical method was 0.86 (Figure 1b). However, in the estimation of content chlorophyll *b* and *a* + *b* values in the green spectrum presented greater adherence to the concentrations determined by the classical method (Figure 1c and 1e). Values obtained for reflectance in the blue spectrum did not allow good estimates of the concentration of chlorophylls, compared to other color spectra (Table 1). The blue spectrum reflects wavelengths between 400 and 500 nm, being less than the peak reflectance of chlorophylls (SILVA et al., 2012).

Table 1. Equations and regression parameters fitted between the spectra values of red (R), green (G) and blue (B) with the content of chlorophyll *a*, *b*, and *a* + *b* obtained from *E. dunnii* leaves.

	Spectrum	Equation	R^2	P	δ
Chrl *a*	R	$\hat{Y}= 248.7580^{*}\exp(-0.0121^{*}X)$	0.73	<0.0001	15.74
	G	$\hat{Y}= 201.8455^{*}\exp(-0.0085^{*}X)$	0.68	<0.0001	17.16
	B	$\hat{Y}= 1229.4442^{*}\exp(-0.0420^{*}X)$	0.55	<0.0001	20.31
Chrl *b*	R	$\hat{Y}= 557.0392^{*}\exp(-0.0197^{*}X)$	0.84	<0.0001	17.42
	G	$\hat{Y}= 461.9589^{*}\exp(-0.0149^{*}X)$	0.89	<0.0001	16.58
	B	$\hat{Y}= 6172.3378^{*}\exp(-0.0650^{*}X)$	0.64	<0.0001	26.54
Chrl *a* + *b*	R	$\hat{Y}= 734.3612^{*}\exp(-0.0157^{*}X)$	0.84	<0.0001	28.95
	G	$\hat{Y}= 616.5996^{*}\exp(-0.0117^{*}X)$	0.86	<0.0001	28.48
	B	$\hat{Y}= 5550.2076^{*}\exp(-0.0535^{*}X)$	0.64	<0.0001	43.31

Which are: R^2: coefficient of determination; P: significance probability by F-test; δ = standard error of estimate (μmol m^2).

Considering that, the concentration of chlorophyll pigments exponentially decreases with the increase in wavelength values in the green spectrum (Figure 1), it was indicated that the reflectance values in the red and green color in the visible spectrum are inversely related to the concentration of chlorophylls due to lower absorption of radiation by chlorophyll pigments. It is noteworthy that all adjusted equations met the conditions of residue analysis, presenting normal distribution of errors, model homoscedasticity and data independence.

The estimated chlorophyll pigments of leaves from various genotypes of *Zea mays* L. were studied by Amarante et al. (2010) using the relation h^{o}/(LxC),

which is the ratio of angle values by multiplying the brightness and chromaticity values. Those authors reported that the best estimates of chlorophyll *a*, *b* e *a* + *b* contents were achieved by a linear model and asymptotic respectively, obtained with a CR-400 MINOUTA colorimeter (Konica Minolta®). However, the genotypic diversity resulted in lower accuracy of the estimate of the concentration of chlorophyll *a*. Therefore, the concentration of chlorophyll is variable depending on the genotype resulting in increased estimation errors, as observed in this study (Figures 1a and 1b) as the correlation coefficient between the results of classic method and estimates was 0.86.

Additionally, the concentration of chlorophyll is associated with the reflectance of blue color readily observed in studies on paper chromatography, whose values in the color spectrum did not allow good estimates.

For the concentration of chlorophyll *a* + *b* wavelengths in the green spectrum resulted in better fits, less estimated error, since the leaf green appearance is related to its highest reflectance in this band, due to the chlorophyll content, being notorious for correlation coefficients of 0.94 and 0.93 respectively, between the classic method and the estimates (Figures 1d and 1f).

The values of the relative chlorophyll content obtained with the SPAD 502 chlorophyll-meter showed an exponential increase in the concentration of chlorophylls *a*, *b* and *a* + *b* in *E. dunnii* leaves with accuracy of 82%, 66% and 79%, respectively (Table 2), corroborating with the findings of Marenco et al. (2009) of six tropical tree species.

The use of reflectance values from red spectrum by color analyzer showed inferior accuracy in relation to value estimated with the SPAD chlorophyll for the concentration of chlorophyll *a* (Table 2), according to the lowest adjusted coefficient of determination and higher estimation error, as the reflectance of the color blue by the chlorophyll *a* did not enabled good estimates.

The greater precision of the color analyzer in relation to the SPAD to estimate the concentration of chlorophyll *b* (Table 2) results from the fact that in the visible green region (500 to 600 nm) the chlorophyll pigments absorb less energy, resulting in larger reflectance peaks. The results with chlorophylls *a* + *b* from the teste methods resulted in little difference detected in the precision, expressed by the coefficients of determination and errors associated with estimates of chlorophyll pigments.

It is noteworthy that the coefficients of correlation between the relative index of chlorophyll with those obtained in red and green spectra were –0.92 and –0.94 ($p < 0.0001$) enabled to estimate the concentration of chlorophyll pigments with acceptable accuracy and linearity to that achieved by a widely used portable chlorophyll meter. The color analyzer has national technology (50%) and the price is about three times less than the imported instrument.

Therefore, the concentration of chlorophylls *a*, *b* and *a* + *b* can be estimated by using the wavelength values in the range of red and green color spectra with a digital colorimeter.

Figure 1. Adjusted equations and analysis of residuals between red and green spectral values with the content chlorophyll *a* (a, b), *b* (c, d) and *a* + *b* (e, f) in *E. dunnii leaves*.

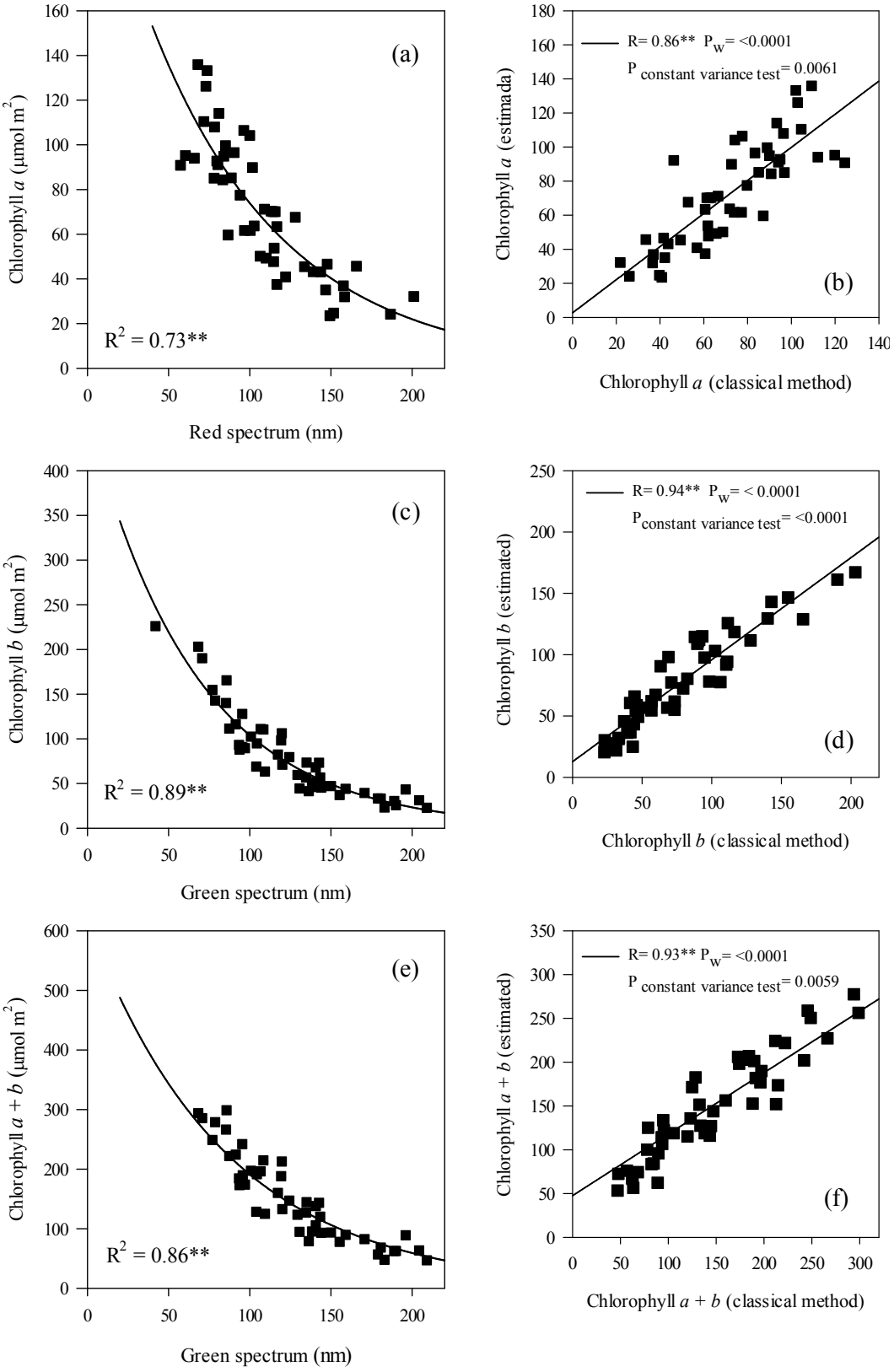

Table 2. Comparison of the accuracy of methods to estimate the content chlorophyll *a* and *b*, and *a* + *b* in equations adjusted with red spectrum values (R) or green (G) and relative index of chlorophyll (SPAD) obtained from *E. dunnii* leaves.

	Method	Equation	R^2	P	δ
Chrl *a*	R	$\hat{Y}= 248.7580^{*}\exp(-0.0121^{*}X)$	0.73	<0.0001	15.74
	SPAD	$\hat{Y}= 12.28^{*}\exp(0.0450^{*}X)$	0.82	<0.0001	12.75
Chrl *b*	G	$\hat{Y}= 461.9589^{*}\exp(-0.0149^{*}X)$	0.89	<0.0001	16.58
	SPAD	$\hat{Y}= 21.724^{*}\exp(0.0290^{*}X)$	0.66	<0.0001	19.68
Chrl *a* + *b*	G	$\hat{Y}= 616.5996^{*}\exp(-0.0117^{*}X)$	0.86	<0.0001	28.48
	SPAD	$\hat{Y}= 32.806^{*}\exp(0.0370^{*}X)$	0.79	<0.0001	32.46

Which are: R^2: coefficient of determination; P: significance probability by F-test; δ = standard error of estimate (μmol m^2).

Acknowledgements

We thank Fundação Araucária, CAPES and CNPq for granting scholarships to the authors.

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