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SHORT COMMUNICATION

The Chlorophyll Fluorescence Ratio F_{735}/F_{700} as an Accurate Measure of the Chlorophyll Content in Plants

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A remote sensing technique is presented to estimate the chlorophyll content in higher plants. The ratio between chlorophyll fluorescence at 735 nm and in the range 700–710 nm, F_{735}/F_{700} *was found to be linearly proportional to the chlorophyll content (with determination coefficient,* r^2*, more than 0.95), and, thus, this ratio can be used as a precise indicator of chlorophyll content in plant leaves. This new chlorophyll fluorescence ratio indicates chlorophyll levels with high precision- the error in chlorophyll prediction over a wide range of chlorophyll content (from 41 to 675 mg* m^{-2}*) was less than 40 mg* m^{-2}*. The technique was tested and validated in three plant species: beech (*Fagus sylvatica *L.), elm (*Ulmus minor *Miller), and wild vine (*Parthenocissus tricuspidata *L.).*

INTRODUCTION

The ratio of fluorescence (F) measured at 685 nm and 735 nm, F_{685}/F_{735}, was successfully used to determine the chlorophyll (Chl) content in leaves (e.g., Lichtenthaler 1987a; Lichtenthaler and Buschmann, 1987; Guyot and Major, 1988). The relationship F_{685}/F_{735} vs. Chl was expressed by a power function $F_{685}/F_{735}=m\text{Chl}^{-n}$. For different plant species, the coefficients m and n were different, but the common features of the relationships were i) high sensitivity of the ratio to low to medium Chl content (from near zero to about 100–150 mg m^{-2}—slightly green and yellowish-green leaves); and ii) the ratio F_{685}/F_{735} leveled off at moderate to high Chl content and its sensitivity to Chl>200 mg m^{-2} was fairly low.

Chlorophyll fluorescence depends to a great extent on pigment content and the absorption of leaves (e.g., Buschmann and Lichtenthaler, 1988; Guyot and Major, 1988; Dahn et al., 1992). In green leaves, about 90% of the emitted Chl fluorescence at 685 nm is reabsorbed by the Chl of the leaf (Gitelson et al., 1998). Recently, the effect of reabsorption of the Chl fluorescence was quantitatively estimated (Dahn et al., 1992; Gunther et al., 1994; Agati et al., 1993; 1995; Gitelson et al., 1998). It was found that the effect of reabsorption of the red Chl fluorescence at 685 nm is particularly large when leaves possess a moderate to high Chl content. In order to determine why the sensitivity of the ratio F_{685}/F_{735} to moderate to high Chl content is low and in which spectral bands fluorescence is more sensitive to Chl content, absorption and reflectance spectra of the leaves have to be studied together with emitted fluorescence.

It was recently found that reflectance in the range near 700 nm is consistently dependent on Chl content and is almost equally sensitive to Chl content throughout a wide range of its variation (Gitelson and Merzlyak, 1994; 1996; 1997). It would be expected that the use of fluorescence in the range near 700 nm, located farther

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away from the Chl absorption band near 680 nm, allows the extension the range of precise Chl estimation.

This article aims to:

- check the hypothesis that the F_{735}/F_{700} fluorescence ratio is sensitive to Chl content in a wide range of Chl variation and can be used as precise indicator of Chl content, and
- test the validity of the ratio F_{735}/F_{700} for different plant species—a beech tree (*Fagus sylvatica* L.), an elm tree (*Ulmus minor* Miller), and a wild vine shrub (*Parthenocissus tricuspidata* L.), and to determine the accuracy of Chl prediction by the ratio in a wide range of Chl levels from 40 mg to 675 mg m^{-2}.

MATERIALS AND METHODS

The leaves of a beech tree (*Fagus sylvatica* L.), an elm tree (*Ulmus minor* Miller), and a wild vine shrub (*Parthenocissus tricuspidata* L.) were sampled at the campus of the University of Karlsruhe in August and September 1996. Three sets of beech leaves (37 leaves altogether), two sets of wild vine leaves (18 leaves), and a set of elm leaves (9 leaves) were studied.

Leaf pigments, chlorophylls, and carotenoids were determined quantitatively at the same spot of the leaf where the reflectance, transmittance, and fluorescence spectra had been measured. The content of chlorophyll *a* and *b*, as well as that of total carotenoids, was spectrophotometrically determined (UV2101PC, Shimadzu, Kyoto, Japan) in an acetone (100%) extract solution using the reevaluated equations of Lichtenthaler (1987b).

The color of leaves varied from yellowish-green to green for wild vine, and from slightly green (2nd flush leaves) to dark-green for elm and beech. Among the leaves studied, the total Chl content varied in a very wide range (for elm leaves: 62.8–638 mg Chl m^{-2}; for beech: 85.5–675 mg Chl m^{-2}; for wild vine: 41.6–471 mg Chl m^{-2}). The thickness of the leaves varied from 0.085 mm (shade leaves of the beech) to 0.2 mm (sun leaves of the beech). The average leaf thickness was close to 0.15 mm.

Chl fluorescence emission spectra were taken from intact leaves using a spectrofluorometer (Luminescence spectrometer LS52, Perkin-Elmer, Germany). The excitation wavelength was set to 430 nm, 550 nm, and 630 nm. Reflectance (R) and transmittance (T) spectra were measured in the spectral range from 400 nm to 800 nm with data points every 2 nm, and a spectral resolution of higher than 0.8 nm. A spectrometer with integrating sphere (UV2101PC, Shimadzu, Kyoto, Japan) was used. In the spectrometer, diffraction grating is placed before sample compartment; thus, reflectance and transmittance were measured while leaves were exposed to light spectrum ranged not more than 1 nm from wavelength measured. The absorption of the leaves was calculated in percent as $A=100-T-R$. Reflectance, transmittance, and Chl fluorescence spectra data were saved on a PC and processed using a spreadsheet program (QUATTRO PRO).

For validation of the ratio F_{735}/F_{700} as a predictor of Chl content, the combined data set (Chl fluorescence spectra and total chlorophyll content collected for 64 beech, elm, and wild vine leaves) was separated into model-development and model-testing subsets. For the model-development subset, data collected for nine elm leaves were used. Validation was done using data collected for 55 beech and wild vine leaves. The predicted total Chl contents were calculated using measured (the model-testing subset) fluorescence ratio F_{735}/F_{700} in an equation with coefficients generated by regression within the model-development data-set. Predicted Chl content was compared to actually measured total Chl content, and the standard deviation of predicted values from actually measured Chl content was presented.

RESULTS AND DISCUSSION

Spectral properties of the leaves in the range of Chl fluorescence from 680 nm to 750 nm were studied. The following were common features of absorption and reflectance for all plant species investigated here (Fig. 1):

- Minimal absorption (near 10%) and maximal reflectance (45–50%) in the near-infrared (NIR) range of the spectrum; at wavelengths longer than 735 nm, the sensitivity of both reflectance and absorption to Chl content was minimal.
- Maximal absorption (80% to more than 90%) and minimal reflectance (5–8%) near 680 nm. While a slight increase in absorption (and decrease in reflectance) occurred with a Chl increase from 100 mg m^{-2} to 200 mg m^{-2}, both absorption and reflectance were insensitive to moderate to high (above 200 mg m^{-2}) Chl content.
- Absorption and reflectance in the range from 700 nm to 710 nm were found to be sensitive to Chl content across the 41 mg m^{-2} to 675 mg m^{-2} range. The absorption at 700 nm ranged from 40% to 85%, and the reflectance, from 33% to about 10%.

In the region of 680–750 nm, termed the "red edge," the leaf reflectance changes from very low in the chlorophyll red absorption band near 680 nm to very high in the near-infrared near 750 nm. It is caused by combined effect of strong Chl absorption and leaf internal scattering. When Chl increases, the leaf reflectance reached saturation level near chlorophyll absorption band. Figure 1B shows this phenomenon. At 680 nm, the saturation level is reached for Chl around 100 mg m^{-2}

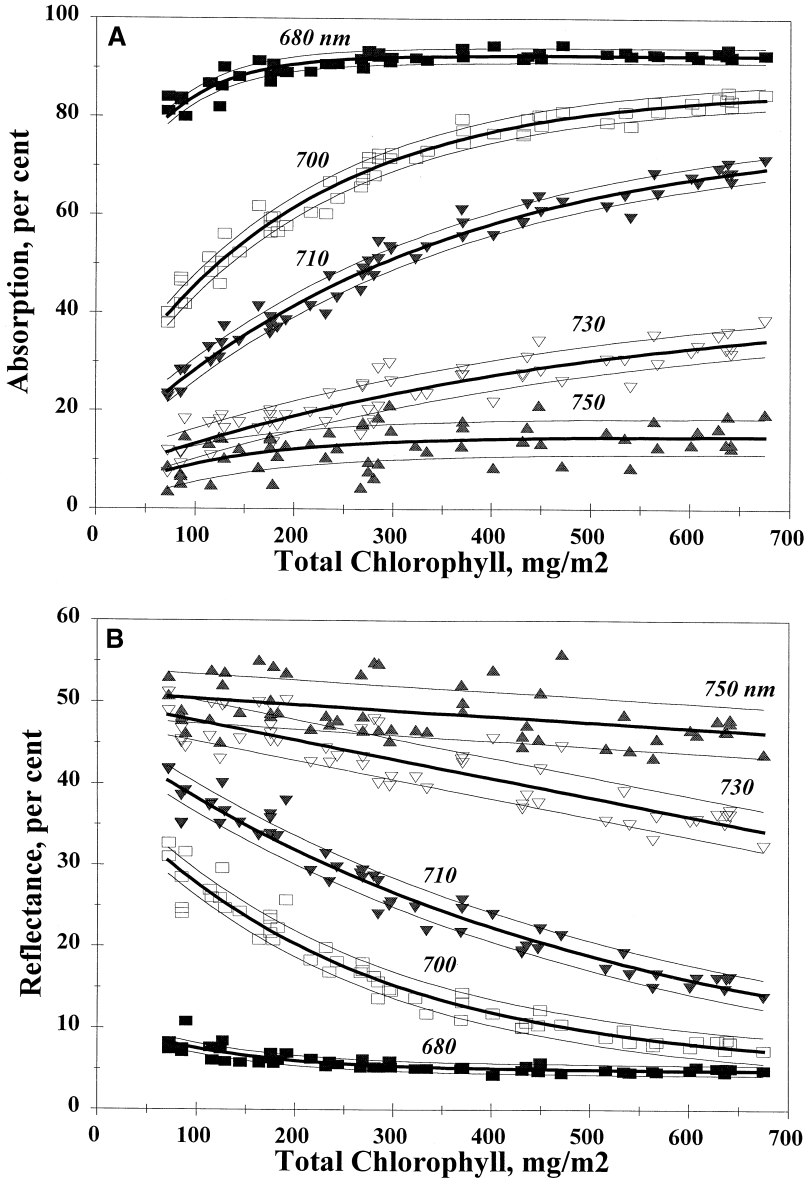

Figure 1. Absorption (A) and reflectance (B) in the red and near-infrared range of the spectrum (680–750 nm) versus total chlorophyll content for elm, beech, and wild vine leaves. Best fit functions for each wavelength are presented by solid lines; standard deviation from the regression curves showed by thin lines on either side of the fitted lines. Absorption and reflectance in the range 700–710 nm were found to be the most sensitive to Chl content throughout 50–675 mg m^{-2} range.

and at 690 nm for Chl around 150–200 mg m^{-2} (data not shown). In the range 700–710 nm, the reflectance did not reach saturation level even for very high Chl content (above 670 mg m^{-2}) and the sensitivity of the reflectance to Chl remained high across a wide range of leaf greenness from slightly green to dark green. In the range 720–730 nm, sensitivity of the reflectance to Chl became much smaller, and near 750 nm, the reflectance virtually did not depend on Chl content.

The relationship [Eq. (1)] between R_{700} and the Chl content was found to be hyperbolic:

$$R_{700}=6450/(\text{Chl} + 121), \quad (1)$$

with determination coefficient $r^2=0.95$. Thus, the relationship of Chl content with the reciprocal of reflectance at 700 nm, $(R_{700})^{-1}$ was linear, with $r^2>0.96$ for all species studied (Fig. 2).

As was mentioned (see Materials and Methods), when reflectance and transmittance of the leaves were measured, leaves were exposed to light spectrum ranged not more than 1 nm from wavelength measured. Thus, the variables measured (reflectance and transmittance) did not include fluoresced light. When leaves are exposed to a full light spectrum, Chl fluorescence can contribute to leaf reflectance in the red range of the spectrum. The contribution of Chl fluorescence was found to be maximum at wavelengths near 685 nm, reaching 23% of the reflectance for moderate (25 mg m^{-2}) Chl content (Kim et al., 1993). However, in the region between 700 nm and 735 nm, the contribution of Chl fluorescence decreased sharply and was kept small and almost invariable (about 4% for high Chl and less than 2% for low Chl). This suggests that Chl fluorescence would markedly change neither ratio $1/R_{700}$ no the reflectance in the

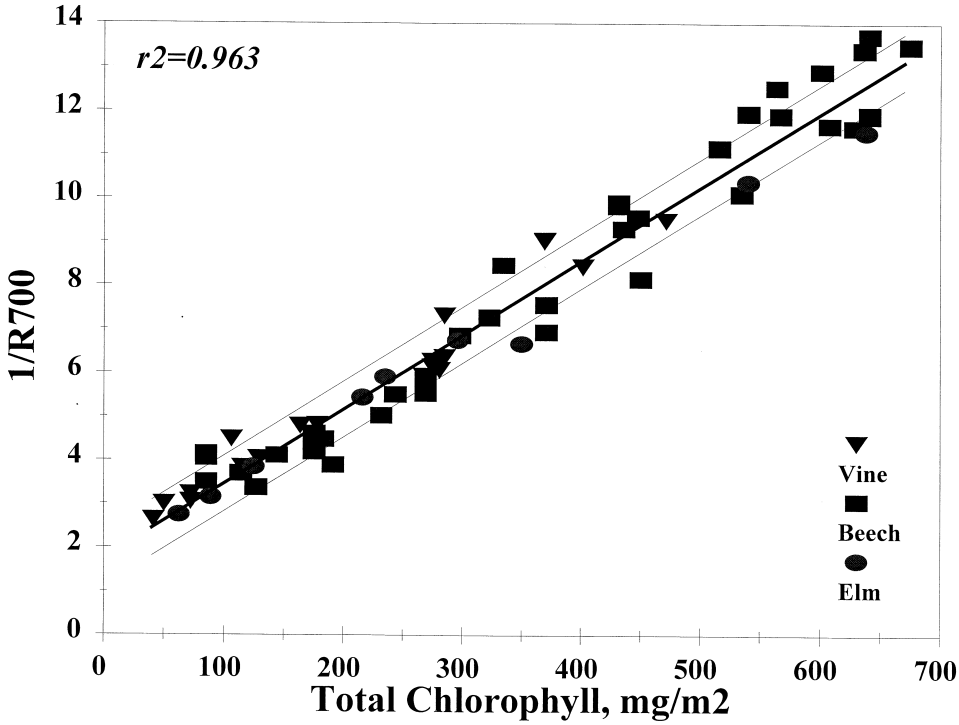

Figure 2. The reciprocal of the reflectance at 700 nm, $(R_{700})^{-1}$ versus total chlorophyll content for beech, elm, and wild vine leaves. This relation is linear, with a determination coefficient $r^2>0.96$ for all species studied. The solid line represents best fit function; the standard deviation from the regression line is shown by thin lines.

range 700–735 nm. The decrease of the ratio $1/R_{700}$ due to Chl fluorescence would be about 4% for high Chl content, and about 2% for Chl<10 mg m^{-2}. It could lead to the variation in the slope of the relationship $1/R_{700}$ vs. Chl (Fig. 2) of not more than 0.02% (Gitelson and Merzlyak, 1994).

The ratio of the Chl fluorescence, F_i, in the range i from 685 nm to 720 nm to that at 735 nm (F_i/F_{735}) showed a very close linear relationship with reflectance R_i (Fig. 3). The minimal determination coefficient for this relationship, $r^2=0.91$ was found at 720 nm, and the maximal one ($r^2>0.96$) at 700 nm (insert in Fig. 3). An increase in reflectance, R_i, is associated with a decrease in the absorption by Chl, which, in turn, causes a de-

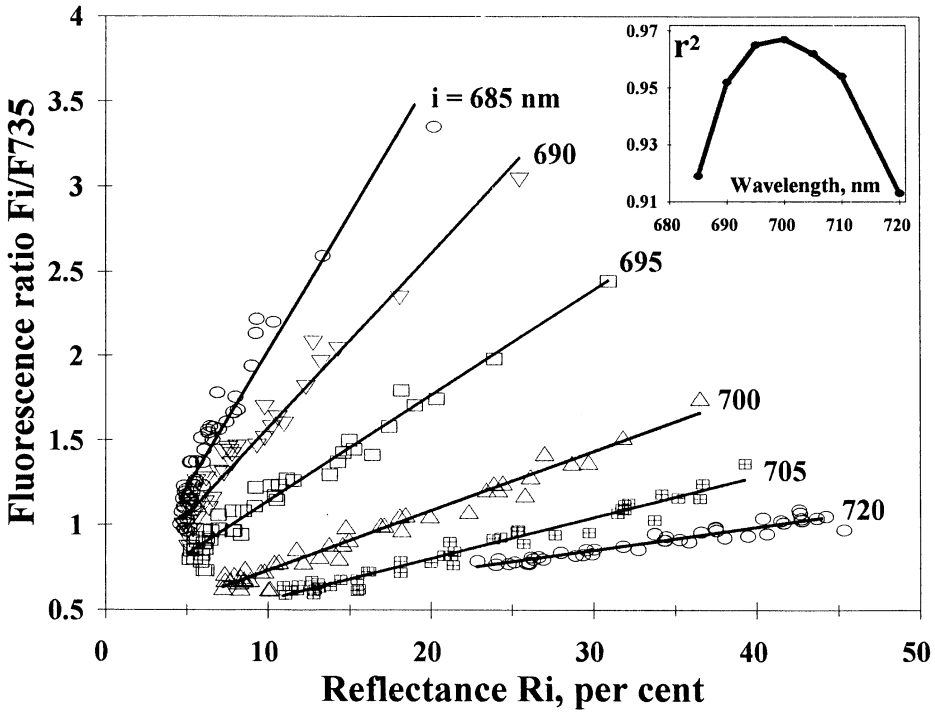

Figure 3. Emitted fluorescence in the range i from 685 nm to 720 nm, F_i, normalized to that at 735 nm (F_i/F_{735}) plotted against reflectance in the range 680–720 nm, R_i. The wavelength of Chl fluorescence excitation was 430 nm. In insert: determination coefficient r^2 for the relationships F_i/F_{735} vs. R_i at different wavelengths. An increase in reflectance is associated with a decrease in Chl absorption in leaves and a decrease in the reabsorption of Chl fluorescence. As a result, the fluorescence ratio increases with an increase in reflectance.

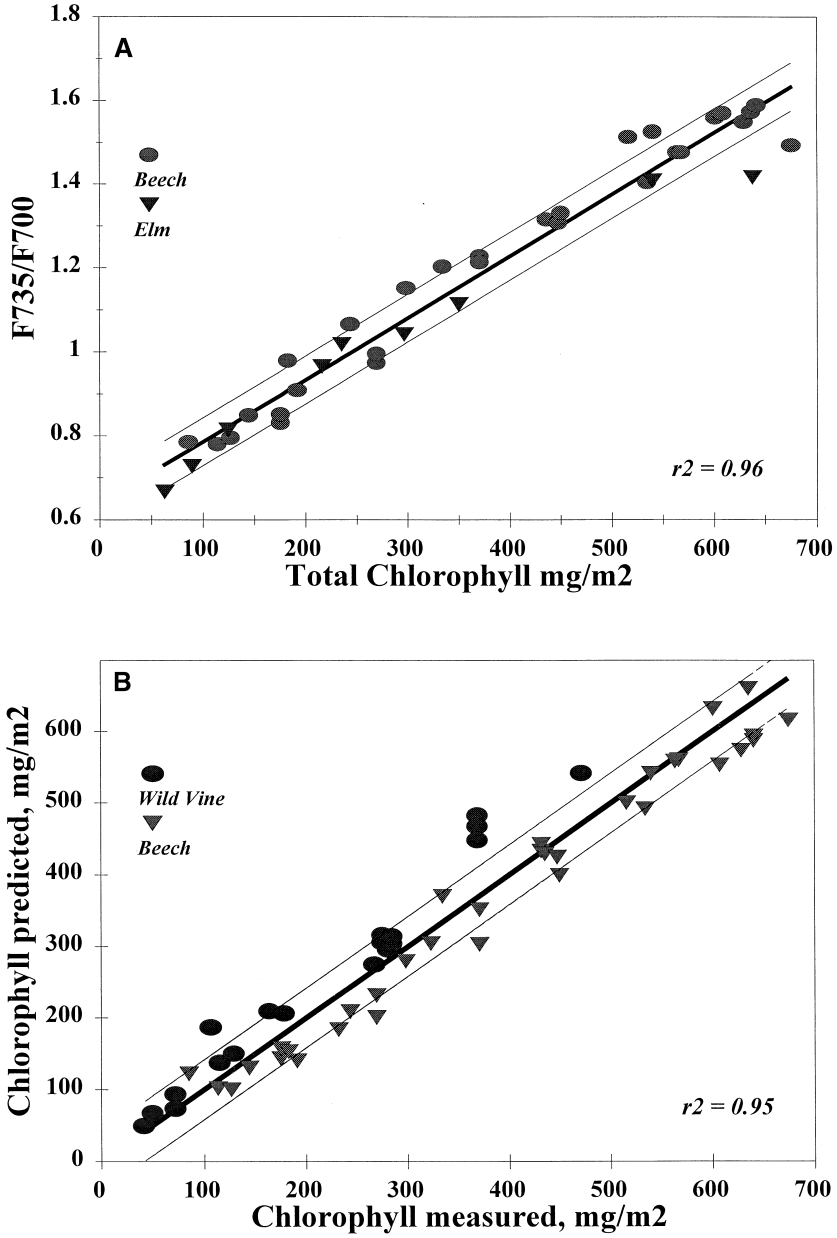

Figure 4. A) Ratio of measured fluorescence F_{735}/F_{700} versus total chlorophyll content. The wavelength of Chl fluorescence excitation was 430 nm. The solid line represents best fit function [Eq. (2)]; standard deviation from the regression line is shown by thin lines. The fluorescence ratio was linearly proportional to Chl content throughout the 50–675 mg m^{-2} range; determination coefficient for this relationship r^2 was found to be as high as 0.96. B) The results of validation of the ratio F_{735}/F_{700} as a predictor of Chl content: Chl content, predicted by fluorescence ratio F_{735}/F_{700} plotted versus analytically measured Chl. The predicted Chl content in mg m^{-2} was calculated via Eq. (2) for the model-development subset, with fluorescence ratio F_{735}/F_{700}, measured in the leaves of the model-testing subset. The wavelength of Chl fluorescence excitation was 430 nm. In the range of Chl content from 41 mg m^{-2} to 675 mg m^{-2}, an error in Chl prediction was less than 42 mg m^{-2} and the determination coefficient r^2= 0.95. The solid line represents the function $Chl_{predicted}=Chl_{measured}$; the standard deviation of predicted Chl values from the regression line is shown by thin lines.

crease in the reabsorption of Chl fluorescence and increase of F_i. The effect of reabsorption of fluorescence at 735 nm is much smaller than that at 700–710 nm (Fig. 1a); thus, with increase of F_i, the fluorescence ratio F_i/F_{735} increases. Therefore, in the range from 680 nm to 720 nm for the species studied, the ratio of Chl fluorescence F_i/F_{735} and reflectance R_i have almost the same information content, and the fluorescence ratio F_i/F_{735} is, nearly exclusively, determined by the reabsorption of Chl fluorescence (see also Gitelson et al., 1998).

In Figure 3, ratio of fluorescence excited at wavelength 430 nm was presented. Although the different excitation wavelengths penetrate a leaf differently (green radiation penetrates maximum while blue and red penetrate the least), very strong correlation (r^2>0.94) was found also between reflectance R_i and fluorescence ratio F_i/F_{735} when fluorescence was excited at 550 nm and 630 nm (data not shown). Relationships between nonabsorbed radiation at 685 nm (reflectance and transmittance) and the ratio F_{685}/F_{735} of fluorescence excited at 430 nm, 550 nm, and 630 nm were also found to be linear with r^2=0.95 (Gitelson et al., 1998, Fig. 5).

Taking into account the fact that $(R_{700})^{-1}$ is directly proportional to the Chl content (Fig. 2), and that fluorescence ratio F_{700}/F_{735} is directly proportional to reflectance at 700 nm, the ratio F_{735}/F_{700} was expected to be proportional to the Chl content throughout a wide range of Chl. In order to check it for the species investigated here, the ratio F_{735}/F_{700} was compared to the analytically measured total Chl content (Fig. 4a, the wavelength of Chl fluores-

cence excitation was 430 nm). Linear relationship with standard deviation of less than 40 mg m^{-2} was found between the F_{735}/F_{700} ratio and Chl content ranged from 41 mg m^{-2} to 675 mg m^{-2}. When Chl fluorescence was excited at 550 nm and 630 nm, standard deviation from the fitted line did not exceed 42 mg m^{-2} (data not shown).

The accuracy of the Chl prediction via the fluorescence ratio F_{735}/F_{700} was determined. To retrieve the Chl content from the fluorescence ratio, a regression equation obtained for model-development subset (nine elm leaves) was employed:

$$\text{Chl} = 634 * F_{735}/F_{700} - 391. \quad (2)$$

For this equation, $r^2=0.97$ and standard deviation from the regression line was 29 mg m^{-2}.

The predicted Chl content in mg m^{-2} was calculated via Eq. (2), using the fluorescence ratio F_{735}/F_{700} measured in model-testing subset—55 leaves of beech trees and wild vine shrubs. The predicted by Eq. (2) Chl values were compared to the analytically measured Chl content (Fig. 4b). In the range of Chl from 41 mg m^{-2} to 675 mg m^{-2}, the determination coefficient of the relationship between predicted and measured Chl content was $r^2=0.95$, with an error in Chl prediction of less than 42 mg m^{-2}. An error of Chl prediction by fluorescence ratio F_{735}/F_{700} when fluorescence was excited at the wavelengths 550 nm and 630 nm did not exceed 45 mg m^{-2} (data not shown).

The relationship between the reflectance at 700 nm and 710 nm and Chl content showed a similar behavior (Fig. 1B). Relationship of the ratio F_{735}/F_{710} with Chl content was found to be linear with $r^2=0.96$ (data not shown). We also checked the accuracy of chlorophyll prediction by fluorescence ratio $F_{735}/F_{700-710}$ where $F_{700-710}$ is fluorescence averaged over the range 700–710 nm. The error of Chl prediction of less than 46 mg m^{-2} was achieved. Thus, the ratio of emitted fluorescence at 735 nm to fluorescence in the range 700–710 nm can be used for a precise and nondestructive determination of the Chl content.

This opens up interesting possibilities for the remote assessment of Chl fluorescence and Chl content via a combined measurement of reflectance and of laser-induced Chl fluorescence. True fluorescence can be retrieved from measurement of emitted fluorescence and the reflectance at 685 nm and 735 nm (Gitelson et al., 1998). As we have shown in this article, Chl content can be accurately estimated by the fluorescence ratio $F_{735}/F_{700-710}$. Thus, a necessary condition for remote assessment of true Chl fluorescence and Chl content is the measurement of fluorescence at wavelengths 685 nm, 700–710 nm, and 735 nm, as well as reflectance at 685 nm and 735 nm. Recently an Nd:YAG laser-induced fluorescence imaging system was designed (e.g., Lichtenthaler et al., 1996) allowing the remote sensing of fluorescence signals. Thus, a combined remote sensing of reflectance and chlorophyll fluorescence may, in the future, provide a promising tool.

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