Leaf Chlorophyll Fluorescence Corrected for Re-absorption by Means of Absorption and Reflectance Measurements

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Summary

The \textit{in vivo} emission spectra of chlorophyll (Chl) fluorescence of green leaves, taken at room temperature, show two maxima near 685 nm and 735 nm. The shape of the spectra is modified by re-absorption processes and depends on the Chl content of leaves. By performing reflectance, transmission and fluorescence measurements, we investigated the reason for the repeatedly reported increase of the fluorescence ratio F685/F735 during stress or damage-induced breakdown of chlorophyll in plants. The \textit{in vivo} spectra of Chl fluorescence (F), transmittance (T) and reflectance (R) were taken at room temperature at the same leaf spot. Leaves with a wide range of green colour (Chl content varied from 70 to 670 mg m\textsuperscript{-2}) were chosen from a beech (\textit{Fagus sylvatica} L.) and an elm tree (\textit{Ulmus minor} Miller) as well as from a wild vine shrub (\textit{Parthenocissus tricuspidata} L.). Strict linear correlations (with a determination coefficient of $r^2 > 0.92$) were found between the ratio F685/F735 on the one hand, and (i) the ratio of the non-absorbed radiation (R685 + T685)/(R735 + T735), (ii) the reflectance ratio R685/R735, and (iii) the reflectance at 685 nm, on the other hand. The results demonstrate that 92 % or more of the ratio F685/F735 variation in leaves during development or at damage and stress events is determined by the variation in Chl content and corresponding changes of the optical properties of leaves.

The Chl fluorescence emission spectrum has been corrected at each wavelength for re-absorption by means of non-absorbed radiation ($R + T$) and reflectance yielding the actually emitted \textit{retrieved} Chl fluorescence. The retrieved Chl fluorescence at 685 and 735 nm is found to linearily increase with the Chl content. The shape of the retrieved Chl fluorescence spectrum was very similar to that of strongly diluted Chl $a$ in solution, only the position of the emission maxima is different (673 nm of Chl $a$ in ethanol solution, and 685 nm in leaves).

Studies on the actually emitted «true» Chl fluorescence, as retrieved from room temperature and outdoor fluorescence measurements, may show new ways of plant stress detection.

\textbf{Key words}: corrected or retrieved chlorophyll fluorescence spectrum, \textit{Fagus sylvatica}, \textit{in vivo} absorption of leaves, \textit{in vivo} chlorophyll fluorescence, \textit{Parthenocissus tricuspidata}, re-absorption of chlorophyll fluorescence, reflectance, \textit{Ulmus minor}.

\textbf{Abbreviations}: a + b = chlorophyll $a$ and chlorophyll $b$; A = absorption; Chl = chlorophyll; F685 and F735 = measured chlorophyll fluorescence at 685 and 735 nm, respectively; F685/F735 = ratio of the measured Chl fluorescence emission; f685 and f735 = retrieved chlorophyll fluorescence at 685 and 735 nm, respectively; f685/f735 = ratio of the retrieved fluorescence; NIR = near infra-red range; R685 and R735 = reflectance at 685 and 735 nm, respectively; T685 and T735 = transmittance at 685 and 735 nm, respectively; x + c = total carotenoids (xanthophylls and carotenenes).

\textsuperscript{*} Dedicated to Prof. Dr. Martin Bopp, Heidelberg, on the occasion of his 75th birthday.
Introduction

At room temperature the red and far-red chlorophyll (Chl) fluorescence, as induced by UV-A, blue, green or red light, is emitted from the antenna and reaction center Chl a of the photosynthetic photosystem II (Papageorgiou, 1975; Lichtenhaler and Rinderle, 1988a; Govindjee, 1995). At room and field temperatures of plants, the Chl fluorescence of photosystem I is extremely low and can be neglected, since it does not really contribute to the overall fluorescence emission of illuminated leaves. This is in contrast to liquid nitrogen temperatures, where photosystem I, besides photosystem II, essentially contributes to the Chl fluorescence emission (Butler, 1977; Siffl and Sesták, 1988). These basic differences in Chl fluorescence emission at room temperature and liquid nitrogen temperatures have to be kept in mind when evaluating Chl fluorescence spectra or kinetics of plant leaves.

The in vivo emission spectrum of the chlorophyll fluorescence of leaves taken at room temperature is characterized by a maxima in the red and far-red region near 685 nm and 735 nm which are termed F685 and F735. The ratio of the two fluorescence peaks F685/F735 usually ranges from ca. 0.4 to 5, whereby the absolute value also depends on the excitation wavelength (Lichtenhaler, 1987; Lichtenhaler and Rinderle, 1988a; Lichtenhaler et al., 1990; Rinderle and Lichtenhaler 1988). The Chl fluorescence ratio F685/F735 is Chl dependent and decreases with increasing Chl content. Therefore it can be used to monitor changes in the Chl content during leaf development (Buschmann, 1981; D’Ambrosio et al., 1992; Tuba et al., 1993), autumnal Chl breakdown (Lichtenhaler, 1987a; Hák et al., 1990), the course of a year (Rinderle et al., 1991), and also as a result of natural and anthropogenic stress and/or damage events (Lichtenhaler et al., 1986; Rinderle and Lichtenhaler, 1988a; Buschmann et al., 1996). The ratio F685/F735 increases by 25 to 30% during inhibition of the photosynthetic electron transport by the herbicide diuron (Lichtenhaler, 1986; Lichtenhaler and Rinderle, 1988a; Rinderle and Lichtenhaler, 1988a; Edner et al., 1995; Buschmann et al., 1996; Lichtenhaler et al., 1997). During the chlorophyll fluorescence induction kinetics (Kautsky effect) the ratio F685/F735 decreases by ca. 30% from the maximum (Fm) to the steady state fluorescence Fs (Buschmann and Lichtenhaler, 1988; Kocsanyi et al., 1988). The maximum fluorescence Fm, measured in pre-darkened leaves shortly after the onset of illumination, represents also a non-functional state of the photosynthetic apparatus, which is similar to the diuron-inhibited state. Short-term stress to the photosynthetic apparatus, which inactivates the photosynthetic electron transport but does not affect the Chl content, can thus be detected by an up to 30% increase of the fluorescence ratio F685/F735.

Long-term stress, however, always induces a lower Chl content in leaves. For this reason, larger rises of the fluorescence ratio F685/F735 are a general indicator of the decline of the Chl content as induced by long-term stress and damage to the photosynthetic apparatus. Changes in the Chl fluorescence ratio F685/F735 at room temperature are, however, not due to variations of Chl fluorescence emission from photosystem I, although this has been speculated by some authors (e.g. Agati et al., 1993). At room temperature the photosynthetic photosystem I contributes hardly anything to the Chl fluorescence emission of leaves (Butler, 1977; Siffl and Sesták, 1988). Since a leaf represents an inhomogeneous light scattering sample (e.g. Fukshansky, 1981; Baret et al., 1988; Fukshansky et al., 1993; Vogelmann, 1993) the Chl fluorescence ratio F685/F735 might, however, be influenced by the structure of the leaf tissue. Thus, in an early study (Virgin, 1954) it had been stated that the shape of the emission spectrum of Chl fluorescence depends on the light scattering of the leaf tissue. Yet in sun and shade leaves of the beechn and other trees, which considerably differ in their morphology, thickness, cell size and cell arrangement (Lichtenhaler et al., 1981; 1983; 1984), the values of the Chl fluorescence ratio F685/F735 predominantly depend on the leaves’ Chl content indicating that the leaf structure contributes very little to changes in the ratio F685/F735 (Lichtenhaler and Rinderle, 1988a).

The correct use of the ratio F685/F735 for physiological and photosynthetic studies and for monitoring stress or damage-induced changes in Chl content requires a detailed knowledge of the information contained in the Chl fluorescence ratio F685/F735. In two recent papers (Dahn et al., 1992; Agati et al., 1993) simple models to remove Chl re-absorption from measured Chl fluorescence emission spectra have been suggested. Nevertheless, the exact quantitative contribution of Chl re-absorption and the contribution of morphological leaf characteristics to the measurable Chl fluorescence ratio F685/F735 have not yet been determined.

For this reason, the dependence of the ratio F685/F735 on the Chl content and optical characteristics of the leaf (i.e. absorption, scattering and reflectance) was studied by measuring the in vivo fluorescence, reflectance and transmittance spectra in the range of the Chl fluorescence (600 to 800 nm) at the same leaf spots. Leaves within a wide range of Chl levels were chosen from beechn trees (Fagus sylvatica L.), an elm tree (Ulmus minor Miller) and a wild vine shrub (Parthenocissus tricuspidata L.). The measured spectrum of Chl fluorescence was corrected for re-absorption of the emitted Chl fluorescence inside the leaf as deduced from absorption and/or reflectance spectra of the same leaf sample. This correction yielded the actually emitted original in situ Chl fluorescence which has been termed here retrieved or «true» Chl fluorescence.

It had been shown earlier that the Chl content of leaves can be deduced from reflectance data remotely sensed from a large distance (e.g. Horler et al., 1983; Guyot and Major, 1988; Gitelson and Merzlyak, 1996; Gitelson et al., 1996; Lichtenhaler et al., 1996). Therefore special regard was given to the application of the results for remote sensing of vegetation by combined reflectance and Chl fluorescence measurements which are more and more becoming a promising tool for a fast and wide-spread analysis of vegetation and its state of health.

Materials and Methods

Plants

Leaves of beechn trees (Fagus sylvatica L.), an elm tree (Ulmus minor Miller) and a wild vine shrub (Parthenocissus tricuspidata L.) growing on the University of Karlsruhe campus have been taken in
August and September 1996. Leaves were selected visually according to their difference in green colour and Chl content. Small branches were cut at different heights and the spectra of the leaves were measured immediately afterwards. Three sets of beech leaves (28 leaves altogether), two sets of wild vine leaves (16 leaves) and a set of elm leaves (nine leaves) were measured. In the case of beech and elm, the leaves investigated also included the light-green, yellowish-green and white-green leaves of the second flush (Johannistrieb) which had developed during the end of June to mid-July and which do not turn fully green.

**Chlorophyll a solution**

Green leaves were extracted with 100% acetone using a mortar and sand. The water in the extract was removed by transferring the acetone extract into petrol ether and by subsequent drying with NaSO₄. The extract was concentrated by mild evaporation, then the pigments were separated by thin-layer chromatography (Lichtenthaler, 1987b). Chlorophyll a was eluted with 95% aqueous ethanol.

**Chl fluorescence emission spectra of leaves**

Fluorescence emission spectra were taken at room temperature from intact leaves between 600 and 800 nm using the Fluorescence Spectrometer LS52, Perkin-Elmer, Überlingen/Germany. The excitation wavelength was set to 430, 550 or 630 nm as indicated in the text. The slit of the excitation monochromator was set to 15 nm, the slit of the emission monochromator to 10 nm. Chl fluorescence was excited in angle of 30° to the perpendicular leaf axis and sensed in angle of 60°. The fluorescence emission spectra of different concentrations of Chl a in 95% ethanol were measured using the same instrument.

**Transmittance, reflectance and absorption spectra**

Reflectance (R) and transmittance (T) spectra were measured in percent in the spectral range between 400 and 800 nm with data points every 2 nm using a spectrometer with integrating sphere (UV-2101 PC, Shimadzu, Duisburg/Germany). Care was taken to measure the transmittance and reflectance spectra at the same spot of the leaf where the Chl fluorescence had been sensed. The slit width of the monochromator was set to 1 nm which corresponds to a spectral resolution of 0.8 nm minimum. The absorption (A) of leaves was calculated in percent as A = 100 − T − R.

The spectra of fluorescence, reflectance and transmittance were stored in a PC and calculated using a spreadsheet program (QUATTRO PRO).

**Determination of leaf pigments**

The leaf pigments were quantitatively determined from the same leaf sample used for taking the spectra. Circular pieces of a 10 mm diameter were pierced from the leaves and extracted with 100% acetone using a mortar. The green pigment extract was centrifuged for 5 min at 5000g in order to make the extract fully transparent. The level of Chl a and b as well as of total carotenoids x+c was determined spectrophotometrically (UV-VIS Scanning Spectro-photometer W-2101PC, Shimadzu, Kyoto/Japan) and calculated using the re-evaluated equations of Lichtenthaler (1987b). The colour of leaves varied from yellowish-green to green for wild vine, and from white-green to dark-green for elm and beech. Among the leaves studied, the pigment content varied in a very wide range (Table 1).

<table>
<thead>
<tr>
<th>plant</th>
<th>Chl a</th>
<th>Chl b</th>
<th>a+b</th>
<th>x+c</th>
<th>ab</th>
<th>(a+b)/(x+c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Elm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>43.2</td>
<td>19.6</td>
<td>62.8</td>
<td>27.6</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Maximum</td>
<td>474.8</td>
<td>163.2</td>
<td>638.0</td>
<td>132.5</td>
<td>3.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Average</td>
<td>213.6</td>
<td>70.8</td>
<td>284.4</td>
<td>73</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Median</td>
<td>169.3</td>
<td>51.5</td>
<td>220.6</td>
<td>62.3</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>b) Beech</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>63.5</td>
<td>21.9</td>
<td>85.5</td>
<td>30.1</td>
<td>2.2</td>
<td>1.3</td>
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<tr>
<td>Maximum</td>
<td>541.4</td>
<td>192.0</td>
<td>750.0</td>
<td>137.2</td>
<td>3.2</td>
<td>6.7</td>
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<tr>
<td>Average</td>
<td>287.6</td>
<td>106.5</td>
<td>391.1</td>
<td>92.6</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Median</td>
<td>317.6</td>
<td>103.3</td>
<td>431.6</td>
<td>94.6</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>c) Wild Vine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>30.9</td>
<td>10.5</td>
<td>41.6</td>
<td>12.0</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Maximum</td>
<td>348.7</td>
<td>122.8</td>
<td>471.5</td>
<td>102.9</td>
<td>4.5</td>
<td>4.8</td>
</tr>
<tr>
<td>Average</td>
<td>164.5</td>
<td>53.9</td>
<td>218.4</td>
<td>52.9</td>
<td>3.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Median</td>
<td>161.4</td>
<td>56.9</td>
<td>223.0</td>
<td>48.8</td>
<td>3.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**Determination of leaf thickness**

The thickness of the leaves was determined using a micrometer with an accuracy of 0.001 mm (Digimatic Indicator IDC series 543, Mitutoyo, Tokyo/Japan). The thickness of the elm and beech leaves varied from 0.085 mm (shade beech leaf) to 0.2 mm (sun beech leaf) with an average near 0.15 mm. Wild vine leaves were much thicker, from 0.2 mm to 0.35 mm with an average near 0.25 mm.

**Results**

**Reflectance spectra**

The reflectance spectra of leaves of an elm tree with different chlorophyll content (Fig. 1A) showed a minimum in the range of 670 nm to 685 nm, and a steep rise towards the near infra-red (NIR) reaching about 45% reflectance at 750 nm. The absorption spectrum, in turn, always was inverse to the reflectance spectrum: showing an absorption maximum near 680 nm and a sharp decrease towards the NIR range (Fig. 1B). With increasing Chl content, the absorption at 685 nm increased from 50% (for white-green and yellowish-green leaves) to more than 90% for green to dark-green leaves (Fig. 2). The changes of absorption at 685 nm were highest when the Chl content increased from 50 to 200 mg m⁻² (white-green to green leaves). Then the absorption leveled off and remained almost constant when the Chl content increased from 250 to 700 mg m⁻². The leaf absorp-
tion at 735 nm exhibited at the higher Chl content a fairly constant value of only 16% of the incident light with a low variation (5.5%), but at lower chlorophyll levels the 735 nm absorption slightly increased with increasing Chl content (Fig. 2).

**Spectra of non-absorbed radiation**

The spectrum of the non-absorbed radiation, which is the sum of transmittance and reflectance (T+R) (Fig. 1C), resembled that of the reflectance, however, the spectral features were more pronounced. At 685 nm, even at the lowest Chl content (white-green leaves), the non-absorbed radiation R+T did not exceed 25%. At a high Chl content the non-absorbed radiation R+T at 685 nm was less than 10%, indicating that in this range of the spectrum the green leaf absorbed more than 90% of the incident radiation. In the NIR range the transmitted and reflected radiation R+T was high, reaching 80% at 735 nm for white-green leaves and ca. 70% for dark-green leaves (Fig. 1C).

**Measured Chl fluorescence spectra**

The Chl fluorescence emission spectra of green leaves have always been showing maxima in the red and far-red regions near 685 nm and 735 nm (Fig. 1D). A low Chl content of a leaf could be recognized by a relatively high yield of the measured red fluorescence F685 which declined with increasing Chl content of leaves. The magnitude of the far-red fluorescence peak F735, in turn, was much less influenced by the Chl content. The ratio of the Chl fluorescence yield in the maxima at 685 nm and 735 nm, ratio F685/F735, is curvilinearly related to the Chl content of leaves (Fig. 3A). The same type of relation with Chl content as for the fluorescence ratio F685/F735 has also been observed for the ratio of non-absorbed radiation (R+T) at 685 nm to that at 735 nm, (R685 + T685)/(R735 + T735), as shown in Fig. 3A, and also for the reflectance ratio at 685 nm and 735 nm, R685/R735, and for the reflectance at 685 nm, R685, as presented in Fig. 3B.

The relationships between the Chl content and the above mentioned spectral leaf characteristics X (fluorescence, reflec-
Fig. 2: Light absorption at 685 nm and 735 nm of white-green to dark-green leaves of an elm tree (*Ulmus minor* Miller) and a beech tree (*Fagus sylvatica* L.) versus total chlorophyll content given in mg m⁻² leaf area. The absorption at 685 nm significantly increases with the increase in chlorophyll content reaching a percentage of 90–95 at a chlorophyll content >250 mg m⁻². The leaf absorption at 735 nm is quite small (average value 15%) and its variation did not exceed 5.5%. Although being located quite far from the maximum of leaf chlorophyll absorption at 675 nm, the increase in leaf chlorophyll content also led to a clear increase in the leaf absorption at 735 nm.

Figure text, non-absorbed radiation ratios and R(685), shown in Fig. 3A and B, can be described by the following most best fit suitable function:

$$X = k_1 \exp(-\text{Chl}/k_2) + k_3$$

where $k_1$, $k_2$, and $k_3$ are constants and Chl is the chlorophyll content.

The coefficients and statistics of equation (1) are presented in Table 2. For all optical characteristics X of leaves, the values of the coefficient $k_2$ were almost the same.

Table 2: Coefficients of the relationships of the variable X upon the Chl content as given by the equation: $X = k_1 \exp(-\text{Chl}/k_2) + k_3$ (equation 1) and its statistics. The term X represents the variables contained in Fig. 3A and B (values and ratios of the fluorescence t; transmittance T or reflectance R at 685 and 735 nm). $k_1$, $k_2$, and $k_3$ are coefficients of the above equation. STD = standard deviation, $r^2$ = determination coefficient, Coef. Var. = coefficient of variation in %.

<table>
<thead>
<tr>
<th>X</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_3$</th>
<th>STD</th>
<th>$r^2$</th>
<th>Coef. Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F685/F735</td>
<td>3.205</td>
<td>104.0</td>
<td>1.055</td>
<td>0.125</td>
<td>0.92</td>
<td>9</td>
</tr>
<tr>
<td>T(685 + R685)</td>
<td>0.654</td>
<td>104.0</td>
<td>0.121</td>
<td>0.022</td>
<td>0.94</td>
<td>12</td>
</tr>
<tr>
<td>T(735 + R735)</td>
<td>0.629</td>
<td>101.7</td>
<td>0.127</td>
<td>0.029</td>
<td>0.89</td>
<td>15.6</td>
</tr>
<tr>
<td>R685/R735</td>
<td>30.7</td>
<td>102.0</td>
<td>5.4</td>
<td>1.36</td>
<td>0.90</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Fig. 3: Curvi-linear dependence of the measured Chl fluorescence and reflectance (part A and B) on the chlorophyll content of differently pigmented leaves of beech (*Fagus sylvatica* L.) and an elm tree (*Ulmus minor* Miller). Against the chlorophyll content of the leaves are plotted A) the values of the measured chlorophyll fluorescence ratio F685/F735 and the ratio of non-absorbed radiation (R685 + T685)/(R735 + T735), B) the reflectance ratio R685/R735, and the reflectance at 685 nm. In C) the ratio of the retrieved fluorescence F685/T735 (corrected for chlorophyll re-absorption) is plotted against the leaves’ Chl content. The correction for re-absorption almost totally removed the dependence of the fluorescence ratio, here F685/T735, on chlorophyll content. The coefficient of variation of the retrieved Chl fluorescence ratio F685/T735 for all leaves was as low as 14.6%.
The ratio of the measured Chl fluorescence F685/F735 was found to be very closely related to the following spectral variables (Fig. 4):

- ratio of the non-absorbed radiation at 685 nm and 735 nm, (R685 + T685)/(R735 + T735) (Fig. 4 A),
- ratio of the reflectance at 685 nm and 735 nm, R685/R735 (Fig. 4 B), and to
- reflectance of the leaf at 685 nm, R685 (Fig. 4 C).

The lowest, but still very high determination coefficient ($r^2 = 0.91$) was found for the relationship between F685/F735 and R685 in leaves of wild vine (data not shown), and that of elm and beech leaves range from 0.92 to 0.95 (Fig. 4).

Very close relationships between F685/F735 and all other spectral variables (X) were found to be independent of the wavelength of Chl fluorescence excitation. The shape of the Chl fluorescence emission spectra depends upon the excitation wavelength. The relative Chl fluorescence yield at 685 nm is higher for blue than for green or red excitation light, as has been shown before (Lichtenthaler and Rinderle, 1988 a and 1988 b; Rinderle and Lichtenthaler, 1988). Thus, the absolute values for the fluorescence ratio F685/F735 are higher at blue (430 nm) than at green (550 nm) or red excitation light (630 nm), as shown in Fig. 5. In the latter, the correlation between F685/F735 and the ratio of the non-absorbed radiation (R685 + T685)/(R735 + T735) is shown for excitation at 430 nm, 550 nm and 630 nm. For beech leaves, the minimal value of $r^2$ of this relationship was as high as 0.95 and the coefficient of variation was less than 5.6%.

**Corrected Chl fluorescence ratio**

The Chl fluorescence ratio F685/F735 can directly be corrected to the ratio f685/f735 in three different ways. The effect of the partial re-absorption of the emitted Chl fluorescence on the ratio F685/F735 was removed by dividing the ratio of the measured Chl fluorescence by the ratio of the non-absorbed radiation R + T at 685 nm and 735 nm. Thus, the corrected ratio of the retrieved Chl fluorescence f685/f735 was determined according to the following equation:

$$f685/f735 = k \frac{(F685/F735)\cdot (R685 + T685))/(R735 + T735)}{(R685/R735)}$$

where $f_3$ is the retrieved Chl fluorescence, $F_3$ is the measured Chl fluorescence and $k$ is a constant.

Taking into account the extremely close relationships between the ratios F685/F735, R685/R735 (Fig. 4 B) and R685 (Fig. 4 C), the correction of the ratio F685/F735 for Chl fluorescence re-absorption in the leaf can also be carried out in the two following ways:

$$\frac{f685}{f735} = m \frac{(F685/F735)\cdot (R685/R735)}{(R685/R735)}$$

$$\frac{f685}{f735} = n \frac{(F685/F735)\cdot (R685/R735)}{(R685)$$

where $m$ and $n$ are constants.

The correction of the F685/F735 ratio for Chl fluorescence re-absorption almost completely removed its dependence on Chl content. Fig. 3C demonstrates very small coefficient of variation (less than 15%) of the retrieved fluo-
Corrected leaf chlorophyll fluorescence spectra

The effect of re-absorption of the emitted Chl fluorescence by leaf Chl was removed from the measured Chl fluorescence emission spectrum at a given wavelength by dividing the measured fluorescence $F_\lambda$ by the non-absorbed radiation at the same wavelength $(R + T)_\lambda$. This yielded the retrieved fluorescence $f_\lambda$:

$$f_\lambda = F_\lambda / (R + T)_\lambda \tag{5}$$

where $l$ is constant.

In the retrieved Chl fluorescence emission spectra, corrected for re-absorption by Chl, the intensity of the fluorescence peak at 685 nm (f685) was at least eight times higher than the measured fluorescence F685, whereas the magnitude of the peak at 735 nm (f735) changed only slightly (Fig. 6A). The shape of the retrieved leaf Chl fluorescence spectrum became almost the same as that for a strongly diluted Chl $a$ solution (Chl concentration of less than 0.2 µg mL$^{-1}$), where re-absorption of fluorescence can practically be excluded (Fig. 6B). As compared to the fluorescence emission spectrum of the diluted Chl $a$ solution, the position of the main fluorescence peak of leaf Chl $a$, which in vivo is bound to particular chlorophyll-carotenoid-proteins of the thylakoids of chloroplasts (Lichtenthaler et al., 1981), was shifted by 10 nm to 12 nm from 673 nm towards longer wavelengths.

Fluorescence spectra of Chl $a$ solutions

With the increase of Chl concentration, the re-absorption processes also proceeded in Chl $a$ solutions (Fig. 7), whereby the fluorescence emission maximum at 673 nm of the diluted Chl $a$ solution in ethanol continuously shifted to longer wavelengths of ca. 685 nm (Fig. 8 B). The overall yield of Chl $a$ fluorescence in solution increased up to 7 µg Chl per mL (Fig. 7). Then the fluorescence yield declined in the whole spectrum, but to a much higher degree in the red fluorescence maximum near 680 nm (Fmax) than near 735 nm (F735) (Fig. 8A). Due to the differential re-absorption of the Fmax and the F735 Chl $a$ fluorescence, the fluorescence ratio Fmax/F735 decreased from values of 10 (at 0.2 µg Chl $a$ per mL) to 2.8 (at 20 µg Chl $a$ per mL) as shown in Fig. 8 B.

Chl fluorescence spectra of different plants

Figs. 9 and 10 show the measured and retrieved fluorescence spectra for beech and elm leaves. While the shape of the measured Chl fluorescence spectrum ($F$) was strongly dependent on the Chl content of the leaves (Figs. 9 A and 10 A), the shape of the retrieved Chl fluorescence spectra virtually did not change with Chl content (Figs. 9 B and 10 B). The yield of the retrieved fluorescence f685 and f735 increased linearly with increasing Chl content as also shown in Fig. 11. Moreover, the retrieved fluorescence f685 varied at increasing Chl content nearly in synchrony with the retrieved fluores-

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Fig. 5: Plot of the measured chlorophyll fluorescence ratio F685/F735 versus the ratio of the non-absorbed radiation at 685 nm and 735 nm, i.e. the ratio of the sum of reflectance and transmittance at the same wavelengths (R685 + T685)/(R735 + T735). The values are given here for differently pigmented beech leaves (Fagus sylvatica L.). The wavelength of Chl fluorescence excitation was A) 430 nm, B) 550 nm, and C) 630 nm.
Fig. 6: A) Spectra of the measured chlorophyll fluorescence emission, the retrieved chlorophyll fluorescence (corrected for chlorophyll re-absorption), and of non-absorbed radiation (transmitted and remitted) of a green beech leaf (Fagus sylvatica L.). The excitation wavelength for the Chl fluorescence measurement was 430 nm. The measured Chl fluorescence at 685 nm (F685) was at least eight times lower than the actually emitted retrieved fluorescence, F685.

B) Spectrum of retrieved fluorescence (corrected for chlorophyll re-absorption) of a beech leaf, and fluorescence emission spectrum of pure chlorophyll a in solution (0.2 μg Chl a mL⁻¹, solvent: 95% ethanol). Note that the shape of the fluorescence emission spectrum of chlorophyll a in solution and of the retrieved chlorophyll fluorescence spectrum of the intact leaf are almost the same, except for the position of the fluorescence maximum which is at a shorter wavelength in case of the Chl a solution.

cene F735. This is in contrast to the measured Chl fluorescence, where F685 varied independently from F735 (Figs. 9A and 10A).

Chl fluorescence spectra of sun and shade leaves

Differences in Chl fluorescence emission of leaves from the same species but with different leaf structure and cell arrangement were tested for sun and shade leaves of the beech. As compared to shade leaves, the sun leaves analysed in this investigation exhibited an about equal Chl content on a leaf area basis. Since sun leaves possess, however, a thicker leaf blade (average: 0.2 mm for sun leaves, 0.1 mm for shade leaves), they had a much lower Chl concentration, given here in g m⁻² (Fig. 12 C). The two leaf types could be distinguished by their difference in the yield of retrieved Chl fluorescence at 685 nm which reflects their difference in Chl concentrations (Fig. 12 C).

Fig. 7: Fluorescence emission spectra of different concentrations of isolated chlorophyll a dissolved in 95% aqueous ethanol. The numbers given in the figure indicate the concentration of Chl a in μg mL⁻¹.

Fig. 8: Variation of parameters of the fluorescence emission spectrum of isolated chlorophyll a in 95% aqueous ethanol with the concentration of chlorophyll a. Upper part: Magnitude of the chlorophyll a fluorescence in the red maximum (Fmax) and at 735 nm (F735). Lower part: Peak position of the Chl a fluorescence emission spectrum (Fmax) and ratio of the fluorescence signals of Fmax to F735. The parameters are based on the Chl a fluorescence spectra shown in Fig. 7. The ratio Fmax/F735 exhibits an inverse curvilinear relationship to the chlorophyll concentration with r² = 0.987.
Discussion

Spectra of measured chlorophyll fluorescence

The \emph{in vivo} emission spectrum of the leaf Chl fluorescence in the red maximum near 685 nm strongly overlaps with the maximum of the leaf absorption spectrum near 680 nm (Figs. 1B and D). As a result, a significant and large part of the red Chl fluorescence F685 is re-absorbed by the Chl molecules inside the leaf before it can be detected outside of the leaf. Thus, the intensity of the measurable Chl fluorescence F685 is much lower for a fully green leaf than for a light-green leaf as has been pointed out before (Buschmann, 1981; Lichtenhaler et al., 1986; Lichtenhaler and Rinderle, 1988a and b; Babani and Lichtenhaler, 1996).

By means of fiber optic measurements inside a green leaf Borrman et al. (1991) found that the maximum amount of detected Chl fluorescence emanates from near the boundary between the palisade and spongy mesophyll. They discovered that the chloroplasts near the leaf surface of the upper leaf side in the palisade parenchyma layer emit twice as much as...
those near the spongy parenchyma layer. 50% of the 685 nm Chl fluorescence signal arose from the initial 18 μm part of the palisade parenchyma cells, and 90% of the 685 nm fluorescence originated from the first 47 μm of the palisade layer. Thus, the Chl fluorescence signal is mainly emitted from the near surface part of the leaf. Before this fluorescence signal can be measured outside of the leaf, it passes through a part of the Chl and chloroplast containing palisade cells.

The absorption by the leaf at 735 nm (at the long wavelength far-red Chl fluorescence maximum) is small and correspondingly changes with the Chl content of leaves, but much less than the absorption at 685 nm (Fig. 2). Thus, with an increase in Chl content, the red Chl fluorescence F685 decreases considerably, whereas the far-red fluorescence F735 changes only slightly. As a result, the ratio F685/F735 declines with increasing Chl content as had been demonstrated before. The sensitivity of the F685/F735 ratio to changes in Chl content is high at the levels <250 mg m⁻² (in yellowish-green and white-green leaves, Figs. 3A and B). At higher Chl levels (>300 mg m⁻²), the sensitivity of the ratio F685/F735

![Graphs showing fluorescence emission spectra](image)

**Fig. 11:** Dependence of the retrieved chlorophyll fluorescence at 685 nm and 735 nm on the total chlorophyll content. **A** leaves from a beech tree (*Fagus sylvatica* L.) and **B** leaves from an elm tree (*Ulmus minor* Miller). The data are taken from the spectra shown in Figs. 9 and 10, respectively. The variation of retrieved fluorescence at 685 nm (F685) with chlorophyll content was nearly in synchrony with that of the retrieved chlorophyll fluorescence at 735 nm (F735).

![Graph showing fluorescence emission spectra](image)

**Fig. 12:** Measured and retrieved chlorophyll fluorescence emission spectra of sun and shade leaves of a beech tree (*Fagus sylvatica* L.). **A** Measured fluorescence emission spectra taken with an excitation at 430 nm. **B** Retrieved chlorophyll fluorescence emission spectra (corrected for re-absorption by chlorophyll). **C** The retrieved chlorophyll fluorescence at 685 nm plotted versus the chlorophyll concentration of sun and shade leaves. In contrast to the other figures, the chlorophyll is given here as concentration in g m⁻³. The sun leaves, which are thicker than shade leaves, exhibited on a volume basis a significantly lower Chl concentration than shade leaves, and consequently a lower retrieved Chl fluorescence.
drops and differences at high Chl levels, e.g. between fully green to dark-green leaves, can no longer be clearly distinguished by means of this fluorescence ratio. The reason for this can be found in the saturation of the Chl absorption near 680 nm, which can clearly be seen in Fig. 2 and in other references (Gitelson and Merzlyak, 1996; Gitelson et al., 1996).

Thus, a larger portion of the measurable red Chl fluorescence F685 nm (with a high probability of re-absorption) emanates mostly from the upper cell layers, whereas the fluorescence at 735 nm (with a much lower re-absorption probability) also emanates from cells deeper inside the leaf. Re-absorption processes are the explanation for the rise of the ratio F685/F735 when photosynthesis is inhibited by the herbicide diuron (Lichtenthaler, 1987 b; Buschmann et al., 1996; Lichtenthaler et al., 1997). Then, a larger portion of the light energy, absorbed in the upper palisade cells and usually transferred into photosynthesis, is converted into Chl fluorescence, and the rise of the total Chl fluorescence can mainly be seen in an increase of F685. Therefore, the effect of inhibition of photosynthesis on the ratio F685/F735 at maximum fluorescence Fm (a non-functional state of photosynthesis), shown by Buschmann and Lichtenthaler, 1988, and Kosanyi et al., 1988, as compared to the lower values at steady state fluorescence Fs, in which case the photosynthetic apparatus and electron transport are fully functional.

The shape of the Chl fluorescence emission spectrum depends upon the wavelength of the excitation light. Blue excitation light yields a much higher red Chl fluorescence yield F685 than red excitation light (Rinderle and Lichtenthaler, 1988 b), and this is demonstrated here for green excitation light as well, whereas the far-red Chl fluorescence F735 is much less affected. As a consequence, the Chl fluorescence ratio F685/F735 decreases for the same leaf spot when excited by blue as compared to green or red light (Fig. 5). This again can be explained by a partial re-absorption of the emitted measurable Chl fluorescence. In fully green leaves, the blue excitation light (λ 430–450 nm) is readily absorbed by chlorophylls and carotenoids already by the chloroplasts of the upper outer part of the palisade parenchyma cells. Hence, the major part of the blue-light induced Chl fluorescence emanates from these upper outer part of the palisade cell chloroplasts. Thus, the red fluorescence F685 is re-absorbed only to a relatively low extent. In contrast, green excitation light (550 nm), and also orange-red excitation light (e.g. He-Ne laser 632.8 nm) are only absorbed by chlorophylls (and not by carotenoids) and, in addition, to a much lower degree than blue light. As a consequence, the green and orange-red light induced Chl fluorescence comes from much deeper leaf layers, and its red form F685 is more heavily re-absorbed by the mass of chlorophylls of the densely packed palisade parenchyma cells than the blue light induced fluorescence F685. Hence, the Chl fluorescence ratio F685/F735 exhibits considerably lower values at green (550 nm) and red-light (630 nm) excitation as compared to blue-light (430 nm) excitation (cf. Fig. 5).

It is of interest in this respect, that the same Chl fluorescence re-absorption processes also take place in diluted Chl a solutions with increasing concentration of Chl a. Also in this case, the red fluorescence emission maximum (Fmax) is re-absorbed to a much higher extent than the far-red Chl fluorescence F735 (Fig. 7). These re-absorption processes are similar to the situation of in vivo Chl, which is bound to the chlorophyll/carotenoid-protein complexes in the photosynthetically active thylakoids of chloroplasts (Lichtenthaler et al., 1981). Thus, the red maximum of the Chl a fluorescence of Chl a in solution shifts from 673 nm to 685 nm with increasing Chl concentrations (Fig. 8), whereas in leaves the measured red Chl fluorescence maximum near 685 nm (white-green leaves) shifts to 692 nm (green leaves), and up to 696 nm in dark-green leaves. From the fact that in Chl a solutions of very high Chl a concentration (>160 mg/mL; not shown) practically all emitted Chl fluorescence is re-absorbed, one can conclude that also in dark-green, and especially in thicker leaves with a high Chl density; very little or almost no Chl fluorescence is detectable.

Reflectance spectra

The reflectance spectrum and the spectrum of non-absorbed radiation \(R + T\) (sum of reflectance and transmittance) of a leaf show a shape inverse to its absorption spectrum in the spectral range of the Chl fluorescence (cf. Fig. 1A and C with Fig. 1B). Thus, the shapes of reflectance spectra are mainly determined by leaf absorption. The absorption spectrum, as transformed from the transmittance spectrum \(A = 100-T-R\), shows the identical inverse shape to the measured transmittance. In this study each spectrum was taken using an integrating sphere, therefore the reflectance included the backward scattered light, and the transmittance included the forward scattered light not kept inside the leaf. In the NIR range, absorption of photosynthetic pigments drops, and the spectral behaviour of the leaf is governed by the internal leaf structure and its scattering.

Chl absorption contributes significantly to reflectance of the leaf in the visible range of the spectrum; it implies that the reflected light is mainly sensed from the Chl-containing tissue below the epidermis. In fact, Fukhansky et al. (1993) showed that the strong peaks for reflected radiation arise in light passages through the leaf at very short path lengths (below 0.5 of the adaxial palisade layer thickness). Thus, the radiation that is not reflected at the beginning of the passage, will very likely be transmitted and absorbed. Therefore, reflected radiation is mainly formed in the beginning of the light passage and has a path length of about 0.5 of the adaxial palisade layer thickness. One can thus conclude that Chl fluorescence and reflectance arise approximately from the same leaf depth near the surface. Thus, the optical characteristics of the palisade layer are mainly responsible for re-absorption of Chl fluorescence as well as for the amount of reflected radiation. This explains why light absorbed in this layer (which is proportional to absorption and inversely proportional to reflectance) is closely inversely related to the emitted Chl fluorescence.

When the Chl fluorescence emission spectra are measured from the abaxial, lower leaf side of a bifacial leaf, where the spongy parenchyma layer contains a much lower Chl content, and where cells are separated by large aerial interspaces,
the Chl fluorescence ratio F685/F735 exhibits much higher values than measured at the upper leaf side with its densely packed palisade cells (Lichtenthaler and Rinderle, 1988 a). Again, this observation can be explained by a lower re-absorption of the emitted Chl fluorescence due to the lower Chl content of the abaxial leaf side. This also demonstrates that the Chl fluorescence primarily emanates from that leaf half and leaf side which is irradiated.

**Influence of chlorophyll absorption on the measured chlorophyll fluorescence**

Spectral characteristics of a leaf, which are of major importance for the re-absorption of chlorophyll fluorescence, are non-absorbed radiation (R + T) and the reflectance. With increase in Chl content of the leaves, non-absorbed radiation (R685 + T685) and R685 decrease, whereas (R735 + T735) and R735 almost do not change. The measured Chl fluorescence has the same pattern with Chl increase; F685 decreases and F735 virtually does not change. Thus, the relationships between Chl content and spectral ratios and variables, such as F685/F735, (R685 + T685)/(R735 + T735), R685/R735 and R685 (Fig. 3) are almost the same. Therefore, the Chl content governs the behaviour of non-absorbed radiation and reflectance in the same way as fluorescence. This can clearly be seen in Fig. 4, where relationships between fluorescence ratio F685/F735 and spectral characteristics of the leaves (R685 + T685)/(R735 + T735), R685/R735 and R685 are shown. The variation in the F685/F735 ratio is almost completely determined by spectral characteristics of the leaf as (R685 + T685)/(R735 + T735), R685/R735 and R685, which are controlled by Chl content (see also Gitelson and Merzlyak, 1996; Lichtenthaler et al., 1996). Thus, the ratio F685/F735 is nearly exclusively dominated by the Chl absorption of the leaf, and re-absorption of Chl fluorescence is responsible for at least 92% or more of the variations in the measured fluorescence ratio F685/F735 in leaves with different Chl content. The influence of Chl re-absorption on the F685/F735 ratio can even be higher: in beech leaves at least 95% of the variation in the F685/F735 ratio (with excitation at 430 nm, 550 nm, and 630 nm) is due to re-absorption by Chl (Fig. 5). These facts clearly demonstrate that the leaves' chlorophyll fluorescence, emitted at room temperature, emanates practically exclusively from photosystem II, which possesses a fluorescence maximum near 685 nm. A distinct 735 nm fluorescence band/peak of photosystem I could not be detected, but should have shown up if it existed.

As a consequence, when spectral characteristics of the leaf (absorption or/and reflectance) are known, one can correct the measured Chl fluorescence (F) for re-absorption by Chl. and the retrieved fluorescence (f) thus determined represents the actual true fluorescence, emitted by the Chl a molecules at the site of light absorption inside the leaf. Almost the same relationships of fluorescence ratio F685/F735 versus Chl content and of spectral characteristics of the leaf (R685 + T685)/(R735 + T735), R685/R735 and R685 versus Chl content (Fig. 3 and Table 2) confirm that the effect of Chl re-absorption can practically completely be removed from the measured Chl fluorescence.

**Spectra of retrieved chlorophyll fluorescence**

For green leaves, the measured fluorescence at 685 nm (F685) amounts to less than 10% of the retrieved fluorescence F685, while the measured fluorescence at 735 nm (F735) is as high as approximately 90% of the retrieved fluorescence F735 (Fig. 6A). This is due to the fairly small leaf absorption at 735 nm as compared to 685 nm. Thus, even a small variation in internal leaf structure and thickness (not related to Chl absorption, but leading to changes in scattering and absorption at 735 nm) results in a significant variation of the measured fluorescence ratio F685/F735. Moreover, a notable variation in the originally emitted retrieved fluorescence at 685 (F685) is strongly reduced by leaf re-absorption (more than 90%) and, therefore, the measured ratio F685/F735 is more sensitive to the fluorescence at 735 nm than at 685 nm.

If the variation in the ratio of the measured fluorescence F685/F735 depends on the variation of Chl content to a great extent, one can conclude that the ratio of the retrieved fluorescence F685/F735 virtually should not depend on Chl content. In fact, this is demonstrated for the ratio of the retrieved fluorescence F685/F735 which is almost constant over a wide range of Chl content variation (Fig. 3 C). The small variation around a value of 8 shows that the fluorescence yield at 685 nm and 735 nm varies almost synchronously with the Chl content of leaves. For all leaves studied from several plants with extremely differing Chl content, thickness, and environmental conditions, the variation in the retrieved fluorescence ratio F685/F735 did not exceed 15%.

The shape of the retrieved Chl fluorescence spectra of leaves resembled that of a strongly diluted solution of Chl a (Fig. 6 B). This is in agreement with the classical knowledge that the in vivo Chl fluorescence emanates only from Chl a (e.g. Rabinowitch and Govindjee, 1969; Lichtenthaler, 1986). As compared to the fluorescence of a diluted Chl a solution in ethanol (λ max at 673 nm), the fluorescence of in vivo Chl a in a leaf has a red maximum shifted by ca. 12 nm towards longer wavelengths. It is known that the maximum of absorption and fluorescence emission of free Chl a in solution strongly depends on the polarity of the solution (for absorption, see Lichtenthaler, 1987 b). Furthermore, Chl a in leaves is located in chlorophyll/carotenoid-protein-complexes which absorb at longer wavelengths than extracted Chl a (French et al., 1972; Lichtenthaler et al., 1982).

One very essential point of our results is the fact that re-absorption effects can be retrieved from the measured Chl fluorescence spectra using reflectance data (the reflectance ratio R685/R735 and the reflectance R685) which can also remotely be detected from larger. Thus, the correction of Chl fluorescence spectra measured at a larger distance from the plant is possible from remotely sensed reflectance data. This opens new possibilities for the remote sensing of terrestrial vegetation.

**Conclusions**

The general conclusions pertaining to determination of the relationships between optical properties of leaves and Chl
fluorescence as well as the deconvolution of the measured Chl fluorescence by absorption and reflectance can be summarized as follows:

- The spectral behaviour of the measured Chl fluorescence depends to a very high extent on the absorption properties of the leaves. The values of the fluorescence ratio F685/F735 are determined by the absorption of leaves at 685 nm and 735 nm. Re-absorption of the emitted Chl fluorescence is responsible for more than 95% of the variation in the measured Chl fluorescence ratio F685/F735.
- The ratio of the retrieved Chl fluorescence f685/f735 virtually does not depend on Chl content.
- Transmission and reflectance of the leaves can be used to retrieve the actually emitted Chl fluorescence from the measured Chl fluorescence signal. To remove re-absorption effects from the measured Chl fluorescence, one can also apply the remotely measured leaf reflectance.
- The spectral behaviour of the retrieved Chl fluorescence of leaves was found to be very similar to that of low concentrations of Chl in solution.
- For leaves in different stages of development and pigment content, the retrieved Chl fluorescence at 685 nm and 735 nm was found to be virtually linearly proportional to the chlorophyll content.

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