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# The use of chlorophyll fluorescence excitation spectra for the non-destructive *in situ* assessment of UV-absorbing compounds in leaves

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## ABSTRACT

**In this study a method was designed to assess non-destructively the type of UV-screening compounds present in the leaf epidermis. The method is based on the recording and calculation of the ratio of UV-excitation spectra of chlorophyll fluorescence (FER) from the adaxial and abaxial sides of bifacial leaves, or from older and younger segments of monocotyledonous leaves. The logarithm of this ratio (logFER) matched the absorption spectrum of the UV-absorbers present in the leaf, as confirmed by its overlap with the absorption spectrum of the methanolic extract of the leaf or of the isolated epidermis. By using the logFER approach, it was possible to demonstrate that the concentration but not the classes of compounds present in the epidermis that are responsible for UV-screening is affected by the side and the age of the leaves. In contrast, measurements from the leaves of seven dicots and one monocot indicated large difference in the classes of these compounds between species. Finally, it was shown that the logFER in the UV is independent of the emission wavelength, and that the method can be used for quantitative measurements. This method expands to the spectral domain the use of ChlF for the estimation of the leaf epidermal transmittance.**

*Key-words*: *Arabidopsis thaliana*; *Beta vulgaris*; *Hordeum vulgare*; *Nicotiana tabacum*; *Phaseolus vulgaris*; *Pisum sativum*; *Spinacia oleracea*; *Vitis vinifera*; biospectroscopy; phenolics; phenylpropanoids.

*Abbreviations*: BF, blue fluorescence; Chl, chlorophyll; ChlF, chlorophyll fluorescence; CSS, chloroplasts on solid support; FER, fluorescence excitation ratio; $F_o$, minimal ChlF level; FRF, far-red fluorescence; lidar, light detection and ranging; logFER, logarithm of the fluorescence excitation ratio; PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux density; RF, red fluorescence; QSEU, quinine sulphate-equivalent units.

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## INTRODUCTION

The leaf epidermis, in addition to its role as a mechanical and gas diffusion barrier, protects the underlying chlorophyllous mesophyll from the potentially deleterious effects of UV irradiation. In this article, the term 'epidermis' is used in its wider sense encompassing the cuticle, the proper epidermis and the hypodermis. The UV-sunscreen function of the epidermis is mainly fulfilled by phenolic compounds, flavonoids and hydroxycinnamic acids, which all have absorption maxima in the UV part of the spectrum (Table 1). The flavonoids can be present as glycosides in the epidermal vacuoles (Shimazaki, Igarashi & Kondo 1988; Koostra 1994; Strid, Chow & Anderson 1994), or dissolved as aglycones in epicuticular wax (Wollenweber & Dietz 1981). They have either an intense absorption band around 260 nm with a weaker band above 300 nm (isoflavones, flavanones), or two bands of about equal intensity, one around 260 nm and the other around 340 nm (flavones) or 360 nm (flavonols) (cf. Table 1). The hydroxycinnamic acids, with a pronounced absorption maximum around 320 nm (cf. Table 1), can be present in the vacuole as glycosides or more commonly esterified to the cutin of the cuticle, to the hemicellulose of the cell wall, or even to flavonoids (Furuya & Galston 1965). Nevertheless, the relative importance of the different classes of phenylpropanoids as the UV-sunscreen in various species remains an open question.

The comparison of absorbance spectra can provide an excellent guide to identification of the phenolic classes present in leaves, especially in the UV-A region in which the major classes, hydroxycinnamic acids, flavones and flavonols have absorption maxima separated by 20 nm (Table 1). Indeed, the measurements of transmittances of epidermal peels (e.g. Lautenschlager-Fleury 1955; Robberecht, Caldwell & Bilings 1980) and absorbances of total leaf extracts (e.g. He *et al*. 1993; Day, Howells & Rice 1994) have long been the major techniques for the analysis of the UV-absorbing properties of the leaf. A good negative correlation between the absorbances of total leaf extracts and epidermal transmittances obtained by these techniques (Day *et al*. 1994) confirmed that the epidermis is responsible for most of the UV-absorption of the leaf

**Table 1.** Spectral regions of absorption maxima of major classes of phenolics (aglycones in methanol). Compiled from Jurd (1957), Harborne (1989), Mabry, Markham & Thomas 1970) and from our own measurements. In most cases, glycosylation of these compounds shifts the Band I maximum towards shorter wavelength

| UV-absorbing compound | Absorption maxima (nm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Simple phenols | | | 266–295 | | | | | | |
| Phenolic acids | | 235–305 | | | | | | | |
| Hydroxycinnamic acids[a] | 227–245 | | | | 310–332 | | | | |
| Chlorogenic acid | 244 | | | | 329[b] | | | | |
| Caffeic acid | 244 | | | | 324[b] | | | | |
| Ferulic acid | 234 | | | | 320[b] | | | | |
| Sinapic acid | 235 | | | | 319 | | | | |
| Stilbenes | | | | 300–310 | 320–330 | | | | |
| Flavonoids | | Band II (benzoyl) | | | | Band I (hydroxycinnamoyl) | | | |
| Isoflavones | | 255–265 | | | (310–330)[c] | | | | |
| Flavanones | | | 275–290 | | (310–330)[c] | | | | |
| Flavones | | 250–270 | | | | 330–350 | | | |
| Apigenin | | 267 | | | | 336 | | | |
| Luteolin | | 267 | | | | 349 | | | |
| Flavonols | | 250–270 | | | | | 350–390 | | |
| Kaempferol | | 266 | | | | | 367 | | |
| Quercetin | | 255 | | | | | 370 | | |
| Chalcones | | 240–260 | | | | | | 365–390 | |
| Aurones | | 240–270 | | | | | | | 390–430 |
| Anthocyanins | | 267–275 | | | | | | | 475–545 |
| Spectral bands[d] | **240** | **260** | **280** | **300** | **320** | **340** | **360** | **380** | **Vis** |

[a]The spectral data for hydroxycinnamic acids are given for the E- (*trans*-) isoform of the acids, which is preponderant *in vivo*.
[b]Chlorogenic, caffeic and ferulic acid have a pronounced shoulder (inflection) at 299 nm.
[c]For isoflavones and flavanones band I is a minor band; it is of a much smaller amplitude than band II, or the band I of flavones or flavonols.
[d]The UV region is divided into 20-nm-wide spectral bands with the central wavelength indicated (Vis, visible).

(Lautenschlager-Fleury 1955). However, the influence of specific leaf structures on UV absorption and the local distribution of phenylpropanoids in leaves are lost by the extraction procedures. Therefore, the design of spectroscopic methods that would detect UV-absorbing compounds *in situ* is of particular importance.

Chlorophyll *a* (Chl) that is already present in leaves can be used as an endogenous fluorophore to assess epidermal transmittance. Despite the inherent variability of the chlorophyll fluorescence (ChlF) yield *in vivo*, UV-excited ChlF was applied to the screening of mutants with an impaired phenylpropanoid metabolism (Chapple *et al*. 1992). ChlF excitation spectra were also used to show the importance of sinapate esters as UV-absorbers in *Arabidopsis thaliana* leaves (Sheahan 1996). Bilger *et al*. (1997) further exploited the use of ChlF by introducing the ChlF excitation ratio (FER), namely the ratio of the ChlF yields induced by UV versus blue-green excitation, to estimate the leaf epidermal transmittance of UV radiation. Their use of a dedicated fluorometer, the Xe-PAM (Walz, Effeltrich, Germany), a pulse-amplitude-modulation fluorometer with a xenon lamp source that can measure ChlF at the $F_o$ level, allowed them to avoid the problem of light-induced variable ChlF, and to show that the UV transmittance of the abaxial epidermis is larger than that of the adaxial one in several plant species. The same approach and apparatus were applied to analyse the effect of UV-B-induced changes of epidermal transmittance (Barnes *et al*. 2000) and to estimate the role of hydroxycinnamates and flavonoids to epidermal shielding in rye primary leaves (Burchard, Bilger & Weissenbock 2000). Recently, Ounis *et al*. (2001) used a double-excitation lidar to apply the FER method to leaves and small canopies. These authors showed both theoretically and experimentally that the logarithm of the fluorescence excitation ratio (logFER) is a quantitative measure of epidermal absorbance (Ounis *et al*. 2001). This confirmed earlier observations in the domain of fluorescence remote sensing (Stober & Lichtenthaler 1993; Schweiger, Lang & Lichtenthaler 1996; Lichtenthaler & Schweiger 1998) that increased irradiance during plant growth decreases UV-excited ChlF, presumably due to an increase in UV-absorbing compounds in the epidermis.

In the present study we focus on the spectral aspects of the logFER method. We show that it can be used to assess the spectrum of leaf epidermal absorbance *in vivo* and therefore can be used for the non-destructive identification of the type of UV-absorbing compounds present in different plant species.

## MATERIALS AND METHODS

### Plant material

The species chosen for this study were the ones most often used in plant biochemistry, spinach and pea; those often used in plant physiology, pea, bean and tobacco; the standard in plant genetics, *Arabidopsis*; and the agronomic representatives for monocots, barley, for herbaceous dicots, sugar beet, and for ligneous dicots, grapevine.

Spinach leaves (*Spinacia oleracea* L. var. Wobli) were purchased at the local market. Pea (*Pisum sativum* L., cv. Petit Provençal or cv. Argenteum), bean (*Phaseolus vulgaris* L., cv. Carioca) and barley (*Hordeum vulgare* L., cv. Triumph) plants were grown in hydroponic culture under the controlled conditions of a growth chamber (PPFD = 350 $\mu$mol photons $m^{-2}$ $s^{-1}$, 16 h light/8 h dark, 21 °C day/15 °C night, 80% relative humidity). The seeds were germinated and plants grown in small pots (0·1 $dm^3$) filled with pure vermiculite immersed in tanks containing a nutrient solution equivalent to Hoagland (Hydrokani C2, 5/1000 diluted; Hydro Agri, Neuilly sur-Seine, France). The nutrient solution was changed twice a week. At the time of the experiments, plant ages were 6 weeks, 8 weeks and 8 d, for pea, bean and barley, respectively. Sugar beet (*Beta vulgaris* L. cv. Monohill) plants were grown in pots with soil in the same growth chamber. The first expanded leaf of a 6-week-old plant was used. *Arabidopsis* (*Arabidopsis thaliana* Heyn.) plants were crosses of a mixture of natural populations from 10 different sites. Plants were grown in pots with soil in the growth chamber for 2 weeks and then transferred in the greenhouse. Rosette leaves were used when the plants were 1 month old. Tobacco plants were grown in soil in the greenhouse. Leaves of 20-day-old *Nicotiana sylvestris* L. and 8-month-old *Nicotiana tabacum* cv. Burley plants were used. Grapevine (*Vitis vinifera* L., var. Cabernet Sauvignon) leaves were picked-up on a 3-year-old plant grown in large pots outdoors in summer, and in the greenhouse in winter.

### Chloroplast preparation

Isolated chloroplasts from young spinach and pea leaves were prepared according to (Cerovic & Plesnicar 1984), and trapped on cellulose filters (SM11301; Sartorius, Göttingen, Germany) as described in (Cerovic, Cheesbrough & Walker 1987). Chlorophyll concentration of chloroplast solutions and chloroplasts on solid support (CSS) was determined according to (Bruinsma 1961). All chemicals used were of pro-analysis grade, and were obtained from Sigma (St. Quentin Fallavier, France).

### Preparation of extracts and recording of absorption spectra

Leaves were sampled for pigment extraction using a cork borer with 1 $cm^2$ aperture. Samples were frozen in liquid nitrogen, and stored at –20 °C. Pigments were extracted by heating the crushed leaf samples for 30 min at 70 °C in 6 mL of methanol. The absorbance of cooled crude extracts was measured on a photodiode-array UV-Vis spectrophotometer (HP 8453; Hewlett-Packard, Les Ulis, France) from 190 to 1100 nm. Chl *a* and *b* contents were calculated using molar absorptivities and formulas from Lichtenthaler (1987).

All absorbance spectra of extracts were recalculated to take into account the dilution, and to make them correspond to the equivalent absorbance of compounds in the leaf *in vivo*. So, all presented spectra correspond to the extract of 1 $cm^2$ of leaf in 1 $cm^3$ of solution measured in a cell with 1 cm pathlength. In this context it is important to note that absorbance, defined as the logarithm of the reciprocal of transmittance, is unit-less.

### Spectroscopic separation of pigment contribution to extract absorbance

Chlorophyll-less absorption spectra of extracts were obtained by spectroscopic subtraction as described in Ounis *et al*. (2001). First, the concentration of Chl *a* and *b* and the a/*b* ratio was calculated from the red part of the spectrum using the extinction coefficients for methanol solutions from Lichtenthaler (1987). Based on these data and the known spectra for Chl *a* and *b* in methanol from 200 to 800 nm (Cerovic *et al*. 1999; Ounis *et al*. 2001), the total Chl spectrum was constructed and subtracted from the crude extract absorption spectrum. The same procedure was not attempted for carotenoids, because of their large number and variations in complement for different species; therefore the contribution of carotenoids is present in all chlorophyll-less absorption spectra of extracts (cf. Figs 4, 5 & 10).

### Fluorescence measurement

Fluorescence excitation spectra were measured on a custom-made fluorometer-set-up named FLU3, installed at the output of a storage ring bending magnet (SA4 beam-line of the Super-ACO synchrotron in Orsay, France), as described previously (Goulas, Moya & Schmuck 1990; Cerovic, Morales & Moya 1994; Latouche *et al*. 2000). Although this set-up was designed to acquire decay-associated spectra by time-correlated single-photon-counting using pulsed synchrotron radiation as the light source, it has several advantages for the present study on simple steady-state spectra. The high sensitivity of single photon counting, and high collection efficiency (emission monochromator with an f/2 aperture ratio) permitted us to use such low measuring light levels (less than 0·2 $\mu$mol photon $m^{-2}$ $s^{-1}$) that no noticeable variable ChlF was induced (non-actinic excitation). The excitation–detection geometry adapted to front-face measurements (excitation at 50°, emission detection at 0°) minimized the contribution of scattered light. The use of a double excitation monochromator and of a long-pass filter at emission (RG665, 3 mm; Schott, Clichy, France) completely eliminated the second-order light problem of diffraction gratings. The leaf or leaf piece holders had a light

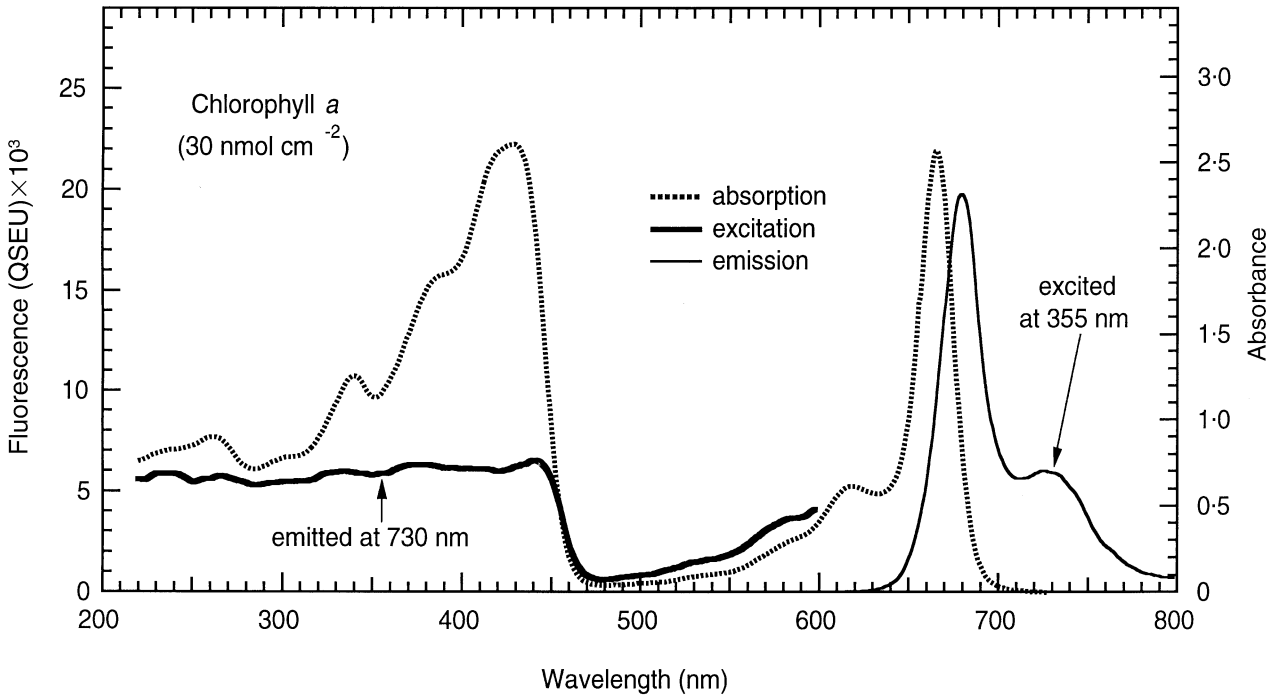



**Figure 1.** Comparison of the absorption spectrum and the fluorescence excitation and emission spectra of a concentrated Chl solution. Pure Chl *a* in 100% methanol. The chosen concentration of 150 $\mu$M in a 0·2 cm pathlength quartz-cell gives a surface content of 30 nmol cm$^{-2}$.The content of Chl in the measuring cell, expressed on area basis, was the one usually present in green leaves. Under these conditions Chl *a* behaves as a photon counter up to about 450 nm. Above this wavelength the absorbance of the solution decreases and some of the incoming light is lost by transmission.

trap behind the sample to avoid ChlF excited by reflected light (unless indicated otherwise).

Spectra of two forward and two backward scans of the excitation monochromator were averaged for each measurement. The emission was always set with the same bandpass of 9 nm, at 682 or 685 nm in the red, or 720 or 730 nm in the near infra-red. Spectra were corrected on-line for the variation of irradiance by using a part of the excitation beam deviated towards a cell of rhodamine B (3 g L$^{-1}$), whose fluorescence was continuously recorded. The lower limit of 220 nm for all excitation spectra was imposed by the absorptivity of rhodamine B. Spectra were in addition expressed in quinine sulphate-equivalent units (QSEU) (Cerovic *et al.* 1999). The use of a fluorescence standard common to all measurements allows a quantitative comparison among spectra obtained on different plant species (the presented results were acquired during a 5 year period). All fluorescence and absorption spectra are presented as obtained, but the logFER were smoothed using the Savitzky–Golay method (Savitzky & Golay 1964) to remove the noise introduced by the division of two spectra. The use of the fourth-order polynomial through 15 points permitted us to avoid any distortion of the spectrum by the smoothing process.

## Chlorophyll *a* as a photon counter

Even in the front-face configuration, which minimizes the inner filter effect (Eisinger & Flores 1979), the excitation spectrum of a fluorophore above a certain concentration no longer corresponds to the absorption spectrum; it becomes flattened. This is due to the total absorption of the exciting light. It is the case for Chl *a* in the UV and blue part of the spectrum, at a concentration present in leaves (Fig. 1). In an intact leaf, in addition, the fraction of incident irradiance that will be recovered as ChlF, will depend macroscopically on at least four other effects: (1) reflection and diffusion of light at the leaf surface; (2) light absorption by non-photosynthetic pigments before it reaches the chloroplast; (3) dissipation of absorbed light energy as heat by the non-photochemical quenching mechanisms of ChlF; (4) utilization of light energy for photochemical reactions in photosystem II, i.e. on 'photochemical quenching' of ChlF. By using a ratio of excitation, most of these fractional effects are eliminated. For instance, in the UV part of the spectrum, where carotenoids do not absorb any more (Lichtenthaler 1987), where reflectance remains constant (Gausman, Rodriguez & Escobar 1975), where Chl behaves as a photon counter (total absorption) (see Fig. 1), and for a non-actinic measuring beam, only the screening by UV-absorbing compound remains to define the ChlF excitation spectrum. At each excitation wavelength ($\lambda_{ex}$), the measured ChlF will then be:

$$\mathrm{ChlF_r}(\lambda_{ex}, \lambda_{em}) = I_r(\lambda_{ex})T_r(\lambda_{ex})E_r(\lambda_{ex}, \lambda_{em})D_r(\lambda_{em}) \quad (1)$$

and

$$\mathrm{ChlF_s}(\lambda_{ex}, \lambda_{em}) = I_s(\lambda_{ex})T_s(\lambda_{ex})E_s(\lambda_{ex}, \lambda_{em})D_s(\lambda_{em}), \quad (2)$$

where $I$ is incident irradiance, $T$ is epidermis transmittance, $E$ is absorption and emission efficiency and $D$ is detection sensitivity at the emission wavelength ($\lambda_{em}$), for the reference (r) and sample (s) spectrum, respectively.

If $T_{ad}$ is defined as the transmittance of a compound present in the sample and absent from the reference ($T_s = T_r\, T_{ad}$), then the ratio of the reference and the sample ChlF will be:

$$\frac{\mathrm{ChlF_r}(\lambda_{ex}, \lambda_{em})}{\mathrm{ChlF_s}(\lambda_{ex}, \lambda_{em})} = \frac{I_r(\lambda_{ex})T_r(\lambda_{ex})E_r(\lambda_{ex}, \lambda_{em})D_r(\lambda_{em})}{I_s(\lambda_{ex})T_r(\lambda_{ex})T_{ad}(\lambda_{ex})E_s(\lambda_{ex}, \lambda_{em})D_s(\lambda_{em})} = \frac{1}{T_{ad}(\lambda_{ex})} \quad (3)$$

when $I$, $E$ and $D$ are identical for the reference and sample measurements, conditions which are met for measurements at a fixed emission wavelength on the same spectrofluorometer. In addition, the logarithm of the ratio will be:

$$\log \frac{\mathrm{ChlF_r}(\lambda_{ex}, \lambda_{em})}{\mathrm{ChlF_s}(\lambda_{ex}, \lambda_{em})} = \log \frac{1}{T_{ad}(\lambda_{ex})} = A_{ad}(\lambda_{ex}) \quad (4)$$

where $A_{ad}$ is the absorbance of this additional (ad) compound. By scanning the excitation monochromator, and calculating the ratio of the obtained ChlF excitation spectrum for the reference and the sample (i.e. logFER) the absorption spectrum of the additional compound can be obtained.

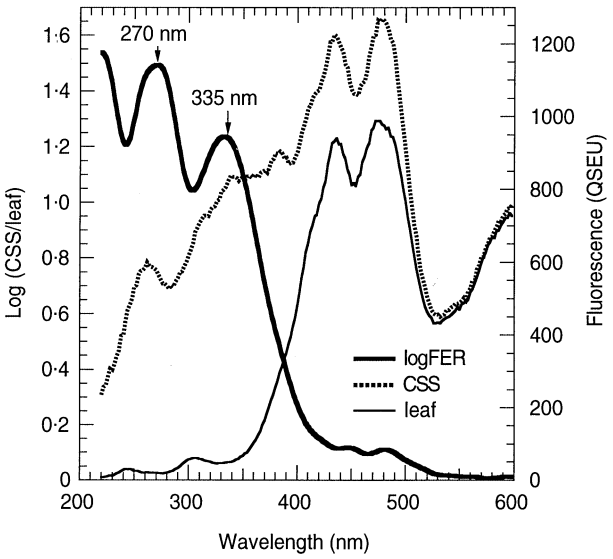



**Figure 2.** Comparison of the ChlF excitation spectrum of a spinach leaf and chloroplasts isolated from the same plant. The emission wavelength was set to 682 nm. The sample, a leaf with adaxial side towards the detector, or chloroplasts on solid support at 30 nmol Chl cm$^{-2}$, was positioned between two quartz windows.

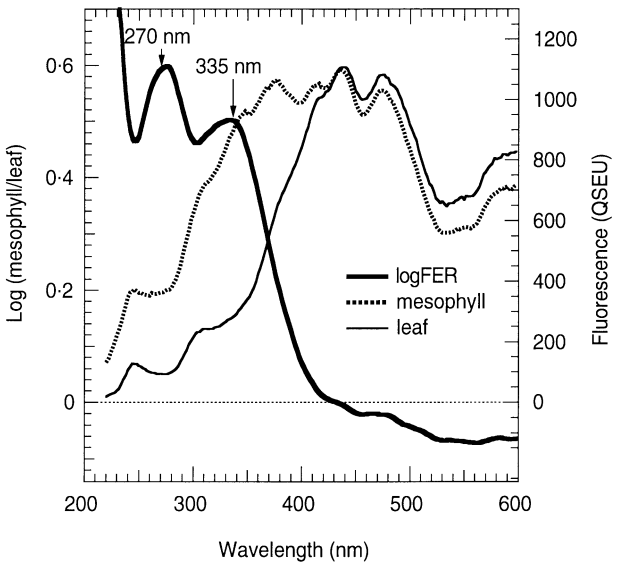



**Figure 3.** Comparison of the ChlF excitation spectra obtained from the abaxial side of a spinach leaf and the same leaf devoid of abaxial epidermis. The emission wavelength was set at 720 nm. The sample had a quartz window in front, and a light trap at the back.

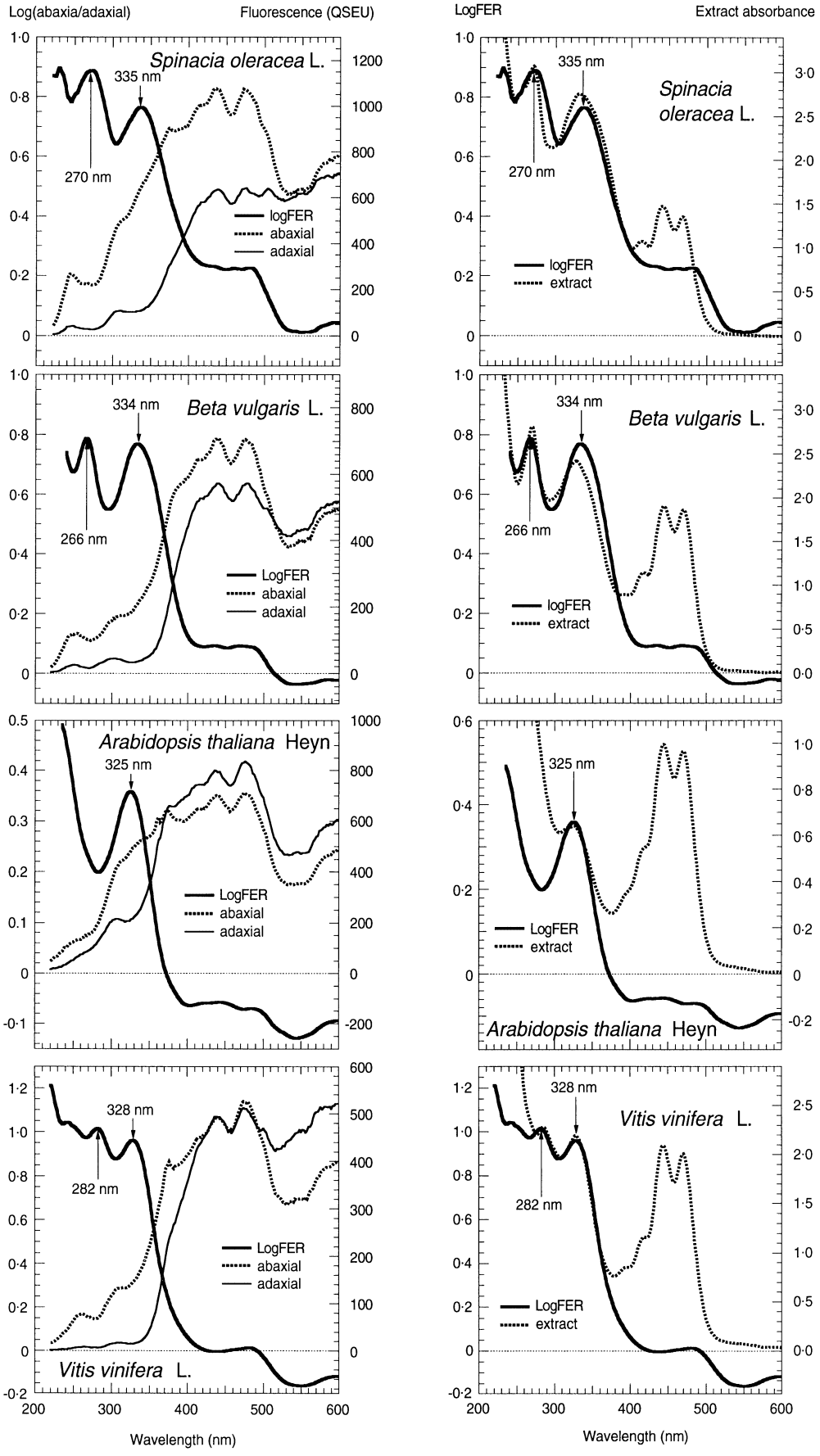
## RESULTS

### Leaves compared to chloroplasts

We tried our method initially for the assessment of epidermal UV-absorbing compounds using logFER on spinach, for two reasons. First, good isolated chloroplasts can be obtained from spinach leaves (Charriere-Ladreix & Tissut 1981; Walker, Cerovic & Robinson 1987) because they have a low content of polyphenols (Hrazdina, Alscher-Herman & Kish 1980); and second, the distribution (Tissut & Ravanel 1980; Charriere-Ladreix & Tissut 1981) and complement of flavonoids is well known in spinach (Hrazdina *et al.* 1980; Tissut & Ravanel 1980; Charriere-Ladreix & Tissut 1981; Aritomi & Kawasaki 1984). The logFER obtained by comparing ChlF excitation of the leaf with that of chloroplasts (Fig. 2) has two maxima in the UV, at 270 and 335 nm, which are characteristic for the absorption of spinach flavonoids. The latter are glycosides of the mono-, di- and tri-methylated derivative of quercetagetin (patuletin, spinacetin and 7-methoxyaxillarin) (Charriere-Ladreix & Tissut 1981; Aritomi & Kawasaki 1984). The logFER obtained by comparing a leaf to CSS with 30 or 50 nmol Chl cm$^{-2}$ were identical (data not shown).

In Fig. 3 the ChlF excitation spectra obtained from the abaxial (lower) side of a leaf and the same leaf devoid of abaxial epidermis were compared. The logFER yielded again a very similar spectrum, which now reveals the absorption spectrum of the abaxial epidermis. The differences seen in the blue and green part of the spectrum are due to the different emission wavelength used (see below).

Although they validate the spectral logFER method, both the comparison with chloroplast and the comparison with mesophyll were destructive. Therefore, knowing that


Log(abaxia/adaxial)
Fluorescence (QSEU)
LogFER
Extract absorbance
Spinacia oleracea L.
335 nm
270 nm
logFER
abaxial
adaxial
Spinacia oleracea L.
335 nm
270 nm
logFER
extract
Beta vulgaris L.
334 nm
266 nm
LogFER
abaxial
adaxial
Beta vulgaris L.
334 nm
266 nm
logFER
extract
Arabidopsis thaliana Heyn
325 nm
LogFER
abaxial
adaxial
Arabidopsis thaliana Heyn
325 nm
LogFER
extract
Vitis vinifera L.
328 nm
282 nm
LogFER
abaxial
adaxial
Vitis vinifera L.
328 nm
282 nm
LogFER
extract
Wavelength (nm)
Wavelength (nm)

the upper (adaxial) epidermis has a larger content of flavonoids than the mesophyll or the lower epidermis (Tissut & Ravanel 1980) we compared the ChlF excitation of the two sides of the same intact leaf (Fig. 4). Again, the logFER, which corresponds now to the difference in absorbance between the two epidermises, revealed the absorption spectrum of the same spinach flavonoids (compare Figs 2, 3 & 4). The amplitude of the logFER spectrum at 335 nm (0·76, Fig. 4) was almost identical to the value (0·74) obtained when subtracting the 335-nm absorbances of the abaxial epidermis 0·5 (Fig. 3, logFER mesophyll/leaf abaxial) from that of the adaxial epidermis 1·24 (Fig. 2, logFER CSS/leaf adaxial).

## Comparison of logFER to extract absorbance

If the sum of the 335-nm absorbances of the two epidermises, 1·74, is subtracted from the absorbance of the total leaf extract (2·75, Fig. 4), a value of 1·01 remains for the absorbance of the mesophyll. This distribution of flavonoids is in agreement with the value previously found for spinach (Tissut & Ravanel 1980). All this indicates that the flavonoids responsible for the protection of the adaxial side of the leaf are also the major UV-absorbing component of the leaf in spinach. As a consequence, in the UV region, there is a good agreement between the logFER spectrum obtained, corresponding to epidermal absorbance *in vivo*, and the absorbance spectrum of the crude extract of a whole spinach leaf (Fig. 4).

We also observed very good correspondence between the peaks of the extract absorbance and logFER spectra for a number of other dicots: *Arabidopsis*, sugar beet and grapevine (Fig. 4). A single maximum at 325 nm confirms the importance of hydroxycinnamates for UV-screening in *Arabidopsis* (Landry, Chapple & Last 1995; Sheahan 1996) as opposed to spinach and sugar beet in which flavonoids are more prominent (presence of two maxima). Grapevine showed a remarkably good agreement between the logFER and extract absorption maxima despite the complexity of its UV-absorbing compounds (Bate-Smith 1962; Ribéreau-Gayon 1968). The contribution of lipids to the absorbance of crude extracts below 250 nm precludes any comparison of extracts with logFER.

In comparison with the results presented in Fig. 4, the agreement between the *in vivo* logFER and the crude leaf extract was weaker in the case of bean and pea (Fig. 5), although a coarse coincidence of peak position is still present. Both bean and pea are member of the Papilionideae subfamily of Leguminosae, that is well known for the accumulation of isoflavonoid phytoalexins in addition to constitutive polyphenols (Williams & Harborne 1989). The logFER for bean shows typical maxima for glycosides of the flavonols quercetin and kaempferol (3-glucosides and 3-xylosylglucosides), which are known to be present in the epidermis (e.g. Hrazdina *et al*. 1980). As with bean, pea leaves contain kaempferol and quercetin glycosides (3-sophorotriosides) but the latter are largely acylated with *p*-coumaric and ferulic acid (e.g. Furuya & Galston 1965; Hrazdina *et al*. 1980; Peyron & Tissut 1981). This is why the absorption spectrum is flatter and the UV-A peak is shifted to 320 nm (Fig. 5).

Ideally the abaxial versus adaxial logFER should be compared to the extract of the adaxial epidermis only, which is usually very difficult to obtain. To this end, we used the *Argenteum* (*Arg*) pea mutant with peeling amiable leaves (Hoch, Pratt & Marx 1980) that allowed us to obtain a free adaxial epidermis (Fig. 6). The obtained spectrum of the extract was very close to the one published by Shimazaki *et al*. (1988) with a maximum at 315 nm, characteristic for pea flavonoid complexes (Furuya & Galston 1965). However, the correspondence with the logFER is still partial (Fig. 6). In the absorbance spectrum of the extract, a shoulder around 367 nm that corresponds to the band I peak of flavonoids is much more prominent than in the logFER spectrum. Note also the absence of carotenoids in the extract obtained from an epidermis, as expected (Fig. 6).

We consider that the logFER approach should yield a more realistic assessment of the *in vivo* epidermal UV-absorbance than the total leaf extract. So, in Fig. 7 we verified the possibility to use a young leaf as an alternative reference to assess the logFER spectrum. LogFER obtained by comparing leaves with CSS (Fig. 7a), by comparing the abaxial and adaxial epidermis (Fig. 7b) and by comparing leaves of different ages (Fig. 7c), yielded always the same differential epidermal absorption (logFER) spectrum. Differences between epidermal absorbances in leaves of different age (different node position) have been observed previously (Day, Howells & Ruhland 1996; Ounis *et al*. 2001), but interestingly, we show here that the absorbing compounds responsible for this difference are similar to those responsible for the differences of absorbances between the abaxial and the adaxial epidermis.

Furthermore, we applied the present spectral analysis to tobacco plants, in which the detailed quantitative analysis of UV-absorption of leaves of different ages has been performed at a single wavelength (Ounis *et al*. 2001). The logFER showed epidermal absorbances that were identical to the one seen in extracts presented in the previous work (Ounis *et al*. 2001). In tobacco, the preponderance of hydroxycinnamates (mainly chlorogenic acid) with its char-

**Figure 4.** Comparison of the absorbance spectra obtained from abaxial versus adaxial logFER and the absorbance spectra of total leaf extracts in methanol. Results for four broad-leaf dicot species are presented (the names of the species are indicated on the figures). The emission wavelength was set at 720 nm for all. For details of ChlF excitation spectra measurements (left side figures) and leaf extraction and absorbance measurement (right side figures) see Materials and methods. LogFER spectra are presented twice. Absorbance corresponds to the equivalent absorbance in the leaf, because it was recalculated to have the extract of 1 $cm^2$ of leaf in 1 $cm^3$ of solution measured in a 1-cm-pathlength cell.

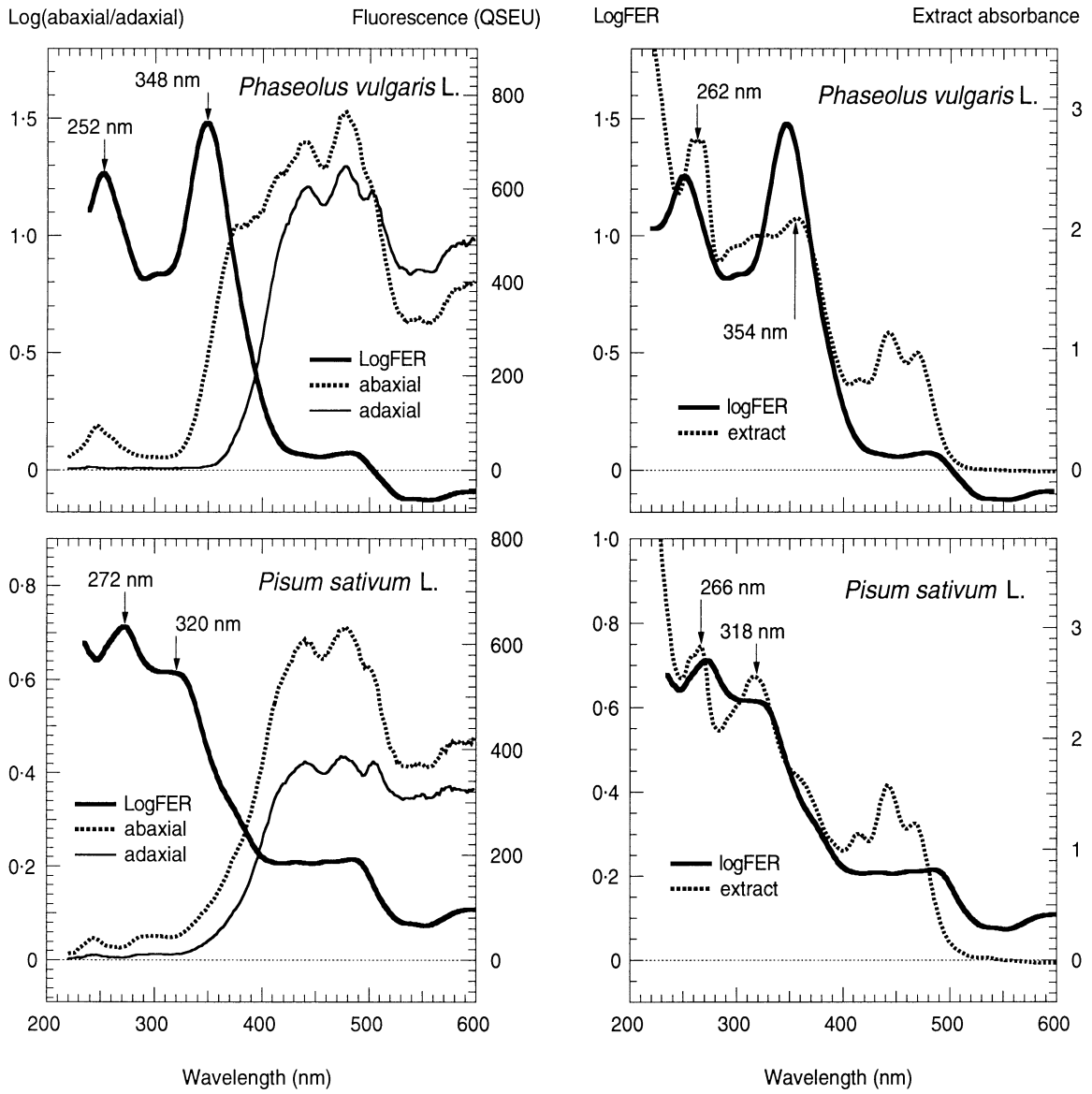



**Figure 5.** Comparison of the absorbance spectra obtained from abaxial versus adaxial logFER and the absorbance spectra of total leaf extracts for bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) leaves. Results are presented for the ninth leaf of a 6-week-old pea plant and the third leaf of an 8-week-old bean plant. The emission wavelength was set at 720 nm for both. For details of ChlF excitation spectra measurements (left side figures) and leaf extraction and absorbance measurement (right side figures), see Materials and methods. LogFER spectra are presented twice. Absorbance is unitless, and corresponds to the equivalent absorbance in the leaf, because it was recalculated to have the extract of 1 $cm^2$ of leaf in 1 $cm^3$ of solution measured in 1-cm pathlength cell.

acteristic maximum at 326 nm and shoulder at 290 nm, is prominent (Fig. 8).

## Emission wavelength

In Fig. 8 we also show that the wavelength at which the fluorescence emission is measured has no influence on the logFER in the UV part of the spectrum. However, because of the re-absorption effect, namely the overlap of the absorption and fluorescence emission spectrum of Chl *a* (cf. Fig. 1), the excitation spectra emitted either in the red (685 nm) or in the far-red (720 nm) are quite different in the blue-green part of the spectrum (Fig. 8a versus 8b) (compare also the adaxial side of the spinach leaf in Fig. 2 measured at 682 nm and at 720 nm in Fig. 4a). For the emission at 685 nm, there is a marked decrease in the excitation spectrum above 500 nm because the fluorescence coming from the absorbed green light is re-absorbed before it can leave the leaf (Ounis *et al*. 2001). At 685 nm, the effect is similar on both sides of the leaf, which yields a more or less flat logFER spectrum in the visible. However, when measuring ChlF emission at 720 nm, the logFER shows an increase in the apparent adaxial absorbance in the blue compared to the green part of the spectrum, implying that

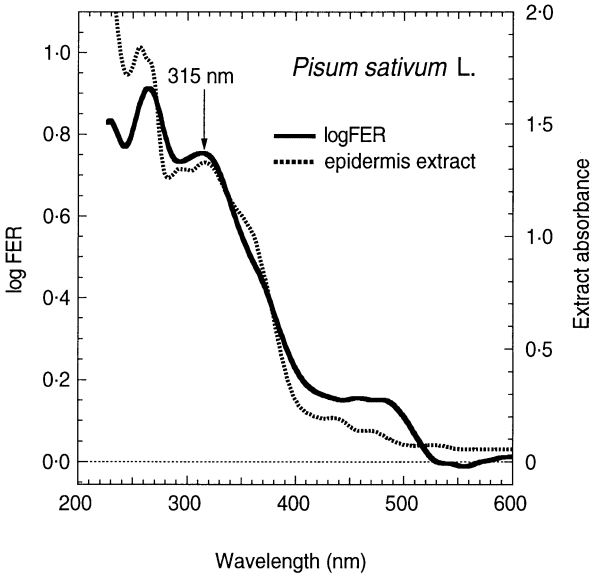



**Figure 6.** Comparison of the absorbance spectrum of the methanolic extract of the epidermis and the logFER of a pea (*Pisum sativum* L.) leaf. The methanolic extract of a peeled epidermis from an Argenteum mutant leaf (no. 5) is compared to the logFER obtained from fluorescence excitation of the abaxial and adaxial side of an adult pea leaf.

the optical properties of the two sides are different. This is repeatedly seen in abaxial versus adaxial logFERs (Figs 4, 5, 7b & 8a), indicating a decreased apparent efficiency in ChlF excitation by blue light coming from the adaxial side, or an increased efficiency when coming from the abaxial side. This effect is absent when logFERs are computed from spectra acquired from the same side of the leaf [leaf versus chloroplasts (Fig. 2) or leaf versus mesophyll (Fig. 3)].

## Monocots, longitudinal heterogeneity

As cereal leaves have a very small dorsiventral heterogeneity, the use of the abaxial side as the reference resulted only in logFER of small amplitude in barley leaves, a typical herbaceous monocot (data not shown). However, we could exploit the fact that, like pea leaves (Fig. 7), barley leaves accumulate UV-absorbing compounds during ageing (Blume & McClure 1979). Barley leaves are developed

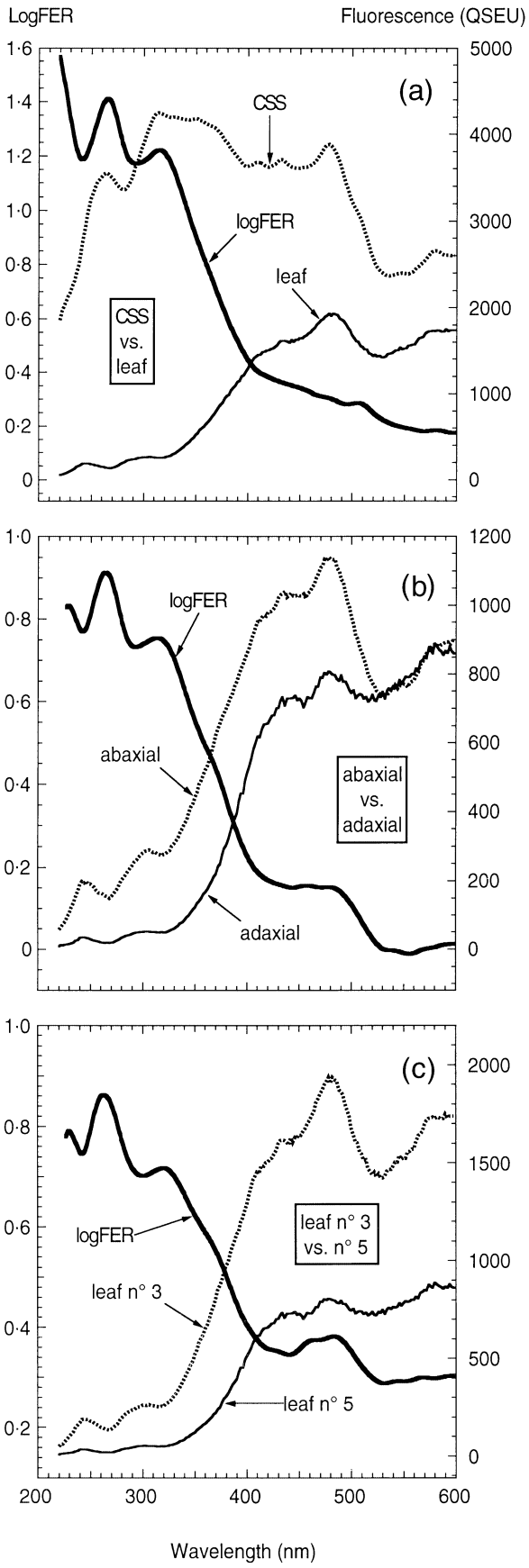



**Figure 7.** Three different means to obtain epidermal absorbance spectra using logFER of pea leaves. Like for spinach, logFER were obtained by comparing CSS to the adaxial side of a leaf (a) or the abaxial to the adaxial side of the same leaf (b). In addition, the ChlF excitation spectra of the adaxial side of two leaves of different age (older third leaf and younger fifth leaf) were compared, and the logFER calculated (c). The plant was 4 weeks old. Leaf fluorescence spectra were measured inside the chamber of the LI-COR 6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) at 23 °C, 400 p.p.m. $CO_2$, 20·7 mmol $mol^{-1}$ $H_2O$. In the case of CSS, the air flow was stopped to avoid desiccation. The emission wavelength was set at 730 nm.

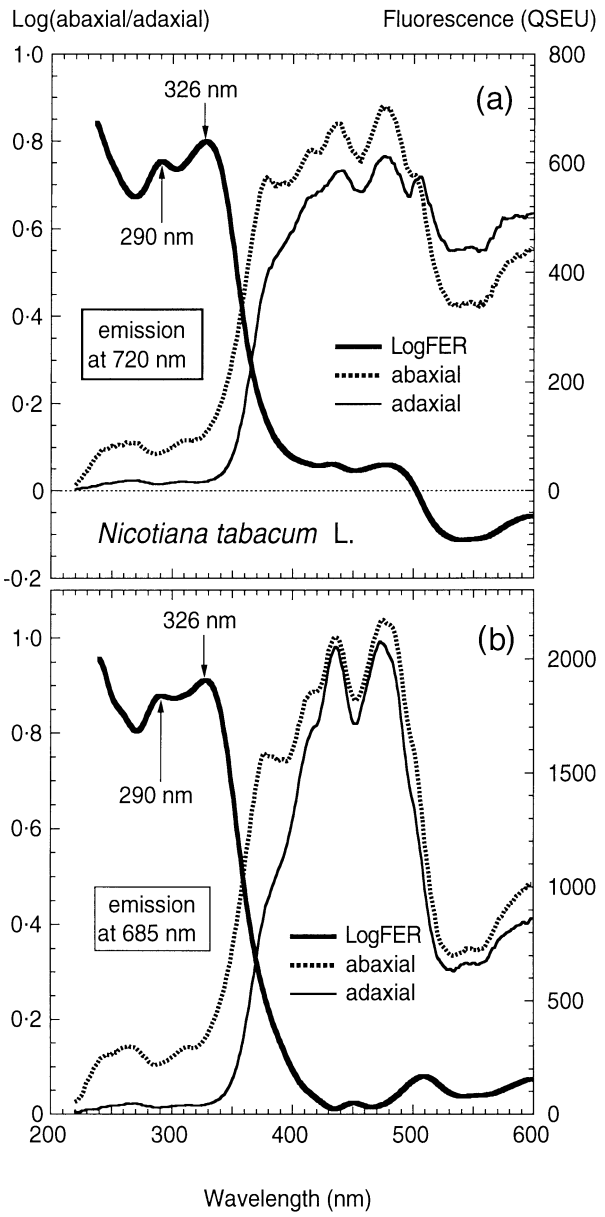



**Figure 8.** The influence of the emission wavelength. Fluorescence of a tobacco (*Nicotiana tabacum*, L. cv. Burley) leaf was measured on the standard holder (with light trap) without any window. The same leaf piece was first excited from the adaxial side then turned over and the excitation spectrum of the emission at 720 nm was recorded (a). The procedure was repeated on the same leaf but with an emission at 685 nm (b).

from an intercalary meristem at their base, therefore they have mature cells at their apex and progressively younger cells towards their base. The logFER of a young (4 cm from ligula) against an old (9 cm) segment yielded the absorption spectrum of the accumulated UV-screening compounds (Fig. 9). Furthermore, we peeled the epidermis from an 8-day-old leaf, the last stage at which it can still be peeled off (Liu, Gitz & McClure 1995), and therefore were able to compare its extract to the logFER (Fig. 9). The peak overlap was particularly good. The two peaks at 274 and 336 nm are characteristic of saponarin (an apigenin glucoside) the major flavonoid derivative in barley (Blume & McClure 1979; Liu *et al*. 1995). The large amount of hydroxycinnamic acids present in barley leaves (Liu *et al*. 1995) (mostly bound to cell walls) seems not to vary much with leaf segment age because it did not appear in the spectrum of logFER. Ferulic acid was only 10% of saponarin content (Liu *et al*. 1995).

Furthermore, the logFER method can benefit from the continuous developmental gradient as shown in Fig. 10. The logFER among barley segments of different ages were compared with the total segment extracts. The amplitudes of the 274 and 336 nm peaks increased as the difference of age of leaf segments increased. The insert in Fig. 10 shows that this type of logFER can be a quantitative measure of flavones content.

## DISCUSSION

We have demonstrated both theoretically and experimentally in a previous paper (Ounis *et al*. 2001) that the logarithm of the fluorescence excitation ratio (logFER) is equal to the UV-epidermal absorbance for the excitation of Chl at two wavelengths of equal molar absorptivity. We show here that this is also true when comparing two samples at the same wavelength (cf. Material and Methods). The logFER of two excitation spectra can therefore provide the absorbance spectrum of the compound responsible for the screening. We validated this spectral logFER approach on our set-up optimized for leaf samples, which uses synchrotron radiation and of a very sensitive time-correlated photon-counting detection. This allowed us to eliminate with confidence the potential pitfalls such as the choice of emission wavelength, the optical properties of the leaf, the influence of the Chl content and the effect of variable ChlF. We have also tested the method using several commercial spectrofluorimeters, and, provided some precautions were taken, satisfactory results could be obtained (data not shown). This was so because basically the method does not require corrected excitation spectra. The only reason why the first attempt of Sheahan (1996) to use the spectral FER method was not more conclusive was his use of the excessive normalization procedures to avoid problems of stray light and second-order Rayleigh scatter.

Our spectral logFER method can exploit the dorsiventral heterogeneity of bifacial leaves advantageously to identify UV-absorbers *in situ*. Indeed, frequently a larger proportion of flavonoids was found in the adaxial epidermis than the abaxial epidermis (Tronchet 1968; Tissut & Ravanel 1980; Weissenböck *et al*. 1984; Harborne 1988). For example, the absorbance of the methanol extract of the adaxial pea epidermis was twice as high as for the abaxial one (Shimazaki *et al*. 1988).

We also used the spectral logFER method to exploit the differences in UV-absorbing compounds related to the age of the leaf for their identification. By comparing leaves of different age the method revealed the same UV-absorbing

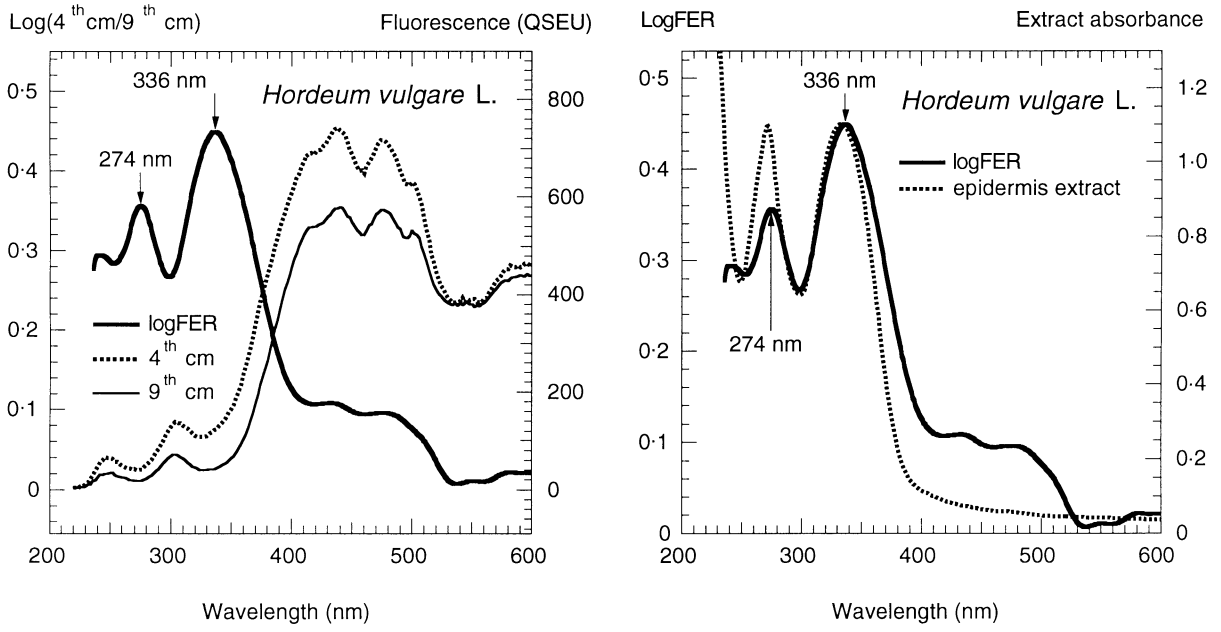



**Figure 9.** Comparison of the absorbance spectrum of the methanolic extract of the epidermis and the logFER of a barley (*Hordeum vulgare* L.) leaf. The logFER was obtained by comparing two segments of different age of a barley leaf, i.e. the younger segment 4 cm from the ligula, to the older segment 9 cm from the ligula and close to the tip of this 11-cm long leaf. Peels of the abaxial epidermis of this 8-day-old barley leaf could be obtained and extracted with methanol (right-hand figure). The logFER 4th cm versus 9th cm (left-hand figure) is compared with the absorbance spectrum of the extract. Absorbance corresponds to the equivalent absorbance in the leaf.

compounds as seen in the abaxial versus adaxial comparison, both in pea and tobacco. This confirms the assertion present in the literature (e.g. Markstädter *et al*. 2001) that the same UV-absorbing compounds are accumulated in both the adaxial and abaxial epidermis and with ageing of leaves. In monocots the leaf age approach is even more important because of the absence of notable dorsiventral heterogeneity in these leaves. In addition, our results show that the compounds responsible for UV-screening are very different in leaves of the seven dicotyledonous species that were tested. Tobacco and *Arabidopsis* could clearly be identified as having hydroxycinnamates for UV-absorbing compounds compared with spinach, sugar beet, bean and barley that have mainly flavonoids. Judging by the position of the UV-A maximum, which is shifted to 335 nm, we can even conclude that the screening in spinach is performed by heavily methylated and glycosylated forms of flavonols. The position of the UV-A logFER maximum for bean indicate that the responsible UV-absorbing compounds are flavonols. In pea and grapevine the hydroxycinnamates contribution seems preponderant again, but in a more complex mixture.

The good spectral correlation between the obtained logFER and extract absorbances in most species analysed in this study can be related to the finding of Day *et al*. (1994) that herbaceous dicots and grasses have a good complementarity between epidermis transmittance and total leaf extract absorbance. However, exceptions can be found, such as in pea and bean here, which show that the extracted UV-absorbers might not all be relevant for screening *in vivo*. The comparison of the logFER spectra to extracts spectra can be especially useful in the future for the detection of the contribution of bound UV-absorbers to the effective screening *in vivo*. Furthermore, fine changes of the environment of the absorbers, such as changes in the vacuolar pH for example, could be assessed using the logFER approach, but not by extraction.

In this article, we have concentrated on the UV part of the spectrum, but the availability of ChlF excitation spectra and logFER up to 600 nm revealed interesting observations also in the blue-green region of the spectrum. The decreased ChlF efficiency of the abaxial side in the green can be related to the smaller Chl content of the abaxial side (Cui, Vogelman & Smith 1991). In addition, because of the diffusive nature the spongy tissue, there is a lengthening of the lightpath by multiple reflections (Terashima & Saeki 1985) that leads to an increased ChlF compared to the adaxial side. It seems that these differences in leaf optical properties have a minor influence on the flatness of logFER in the UV part of the spectrum, thanks to the high extinction coefficient of Chl, which behaves like a photon counter (cf. Fig. 1). Also, we have shown here that even in the visible region the logFER spectra are flat and above zero when Chl concentration is the same in the sample and the reference (Figs 2, 7a & c), or when 685 nm fluorescence coming from the fist layers of the leaf is used (Figs 2 & 9b). Anyhow, the final interpretation of logFER spectra in the blue-green part of the spectrum awaits further studies.

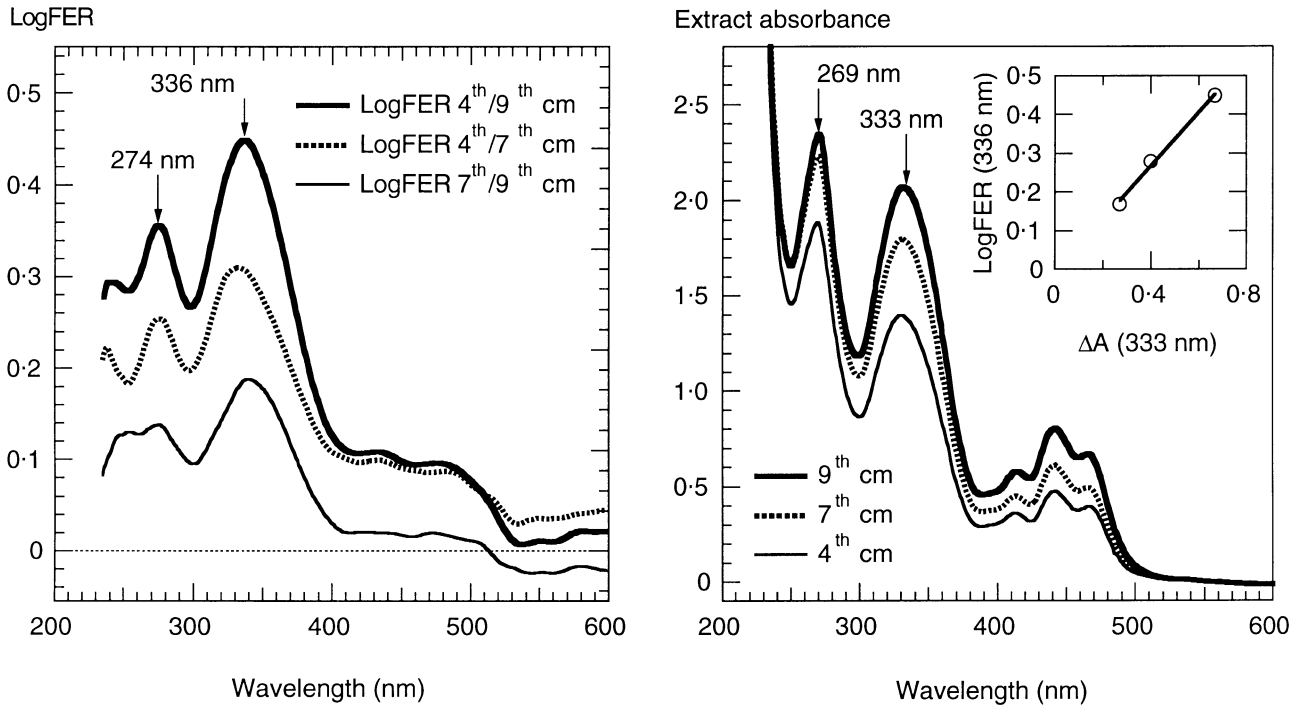



**Figure 10.** Fluorescence and absorbance along a primary leaf of barley. The use of logFER among different segments for the quantitative estimation of epidermal absorbance. ChlF excitation spectra of an 8-day-old leaf of barley (*Hordeum vulgare* L., cv Triumph) were recorded at the 4th, 7th and 9th cm from the ligula. The excited surface was 2 cm high and 4 mm wide. Leaf segments, 2 cm long around the nominal value, were then extracted in methanol as described in Materials and Methods (spectra in the right-hand figure). The logFER for the three combinations of segments were calculated (left-hand figure), and the values of the logFER maxima compared to the difference in absorbance of the 333 nm maximum among extracts (inserted figure). For example, the value at 336 nm of the logFER obtained by dividing the fluorescence of the 4-cm segment by the fluorescence of the 9-cm segment was plotted against the difference in absorbance of the 9-cm segment extract minus the 4-cm segment extract.

Although stabilized chloroplasts or mesophylls (cf. Bilger *et al*. 1997; Bilger, Johnsen & Schreiber 2001) could be further used as standards for logFER-derived absorption spectra of adaxial or abaxial epidermises, in general we have shown that an abaxial versus adaxial logFER or a young versus old epidermis are sufficient to identify the responsible UV-compounds. The logFER gives quantitative differences among samples, which is often sufficient to compare treated to untreated samples. Yet, for absolute quantitative purposes, there is also the possibility to use the two-wavelength logFER, which can measure independently the absorbance of epidermises on each side of the leaf and then calculate the sum of it (Ounis *et al*. 2001) (see also Bilger *et al*. 1997). Indeed, the spectroscopic logFER method will advantageously complement the recently developed devices capable of the two-wavelength FER measurements in the field (DUALEX, patent pending; Y. Goulas, Z. Cerovic and I. Moya) (see also Bilger *et al*. (2001) for another field device for FER assessment). DUALEX is a leaf-clip device designed to measure the UV-A transmittance of the epidermis on leaves still attached to the plant in less than a second. It is based on the alternated excitation of ChlF by a red and UV measuring beam.

Both the two-wavelength and spectral logFER method have numerous potential applications. By avoiding destruction of the sample, these methods will be used for rapid preliminary or for massive screening of the amount and type of UV-absorbers. They will also allow repeating measurements with time on the same sample. For example, they would be invaluable for the research on UV-B effects on plants because it has been shown in the laboratory (Wellmann 1975) and in the field (Flint, Jordan & Caldwell 1985) that UV-B induces the accumulation of flavonoids (for recent reviews on the subject cf. Rozema *et al*. 1997; Jansen, Gaba & Greenberg 1998). In addition, they will be used for the assessment of epidermal absorbance in research fields as varied as plant nutrition, plant development, plant defence against herbivores and pathogens, forage quality and mutant screening. As can be seen from our previous study (Ounis *et al*. 2001), the two-wavelength logFER method was designed originally for the purpose of remote sensing of vegetation using lidars. So, the spectral logFER method will also help to define the best excitation wavelength for the future fluorescence lidars adapted to different vegetation types.

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