

# **Optical detection of pigment contents in leaves: intercomparison of several handheld devices**

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## Abstract

Handheld devices have become popular to determine epidermal contents of UV-A absorbing compounds in intact leaves. For the comparability of the results between different labs, it is important that all instruments show the same or at least similar results. Therefore, the signals from seven different Dualex Scientific optical leaf clips from four laboratories were compared to each other, alongside a prototype of an LSA-2050 leaf state analyzer and a UV-A-PAM fluorometer. The values of the Flav index, which is used to quantify epidermal UV-A absorbing compounds such as flavonoids, corresponded well with each other ( $r^2 > 0.989$ ), except for one instrument. A higher variability among instruments was found for the chlorophyll (Chl) index, again with one instrument showing a significant offset. Although one Dualex instrument showed an excellent linear calibration for the anthocyanin (Anth) index relative to leaf extract concentrations, the Dualex instruments had all an offset against each other and at Chl index values below 30, erroneous Anth index values were observed. While Flav index results appeared quite reliable, it is recommended to calibrate a Dualex instrument against leaf extracts for exact determination of chlorophyll and anthocyanin contents. Simple tests for the functionality of a Dualex instrument are suggested.

## Introduction

Epidermal UV screening by the accumulation of compounds such as flavonoids and other polyphenolics is an important and widespread mechanism of plants for protection against the detrimental effects of UV radiation. The formation of flavonoids is induced by several different environmental factors, among them high photosynthetically active radiation (PAR), (low) temperature or nitrogen deficiency (for a review, see Pescheck and Bilger 2020). To follow this dynamic and highly variable response, especially under natural conditions, the repeated assessment of a large quantity of leaves is required. For this purpose, a non-destructive method based on measuring chlorophyll fluorescence was developed by Bilger et al. [2]. The basic principle of the measuring technique relies on the fact that chlorophyll fluorescence can be excited by a UV-A excitation beam as well as by a red beam. While the UV-A beam can be absorbed by other compounds absorbing in this wavelength range, this is not the case for the red beam, which, hence, serves as a reference. The ratio between the two fluorescence signals is related to the amount of all compounds which absorb UV-A radiation and optically interfere with the UV-A excitation beam. In later years, this originally laboratory-based method was transferred to portable devices [3-8], allowing the rapid testing of leaves under field conditions. Due to its ease of use, the Dualex Scientific instrument (formerly manufactured by ForceA, Orsay, France, currently by Pessl Instruments, Weiz, Austria) has become a popular device for determining epidermal UV-A absorbance via the so-called Flav index. Besides characterising the acclimation of leaves to UV radiation, the Dualex instrument

has also been introduced as an indicator of the demand for nitrogen fertilization in crop plants via the so-called nitrogen balance index (NBI), which is the quotient of the Flav index and the simultaneously determined chlorophyll content characterized by the Chl index (7, Cartelat et al. ). The rationale for this method is based on the fact that N deficiency leads to a reduction of chlorophyll and at the same time to an increase in flavonoid and phenylpropanoid formation (Jones and Hartley 1999). The Dualex scientific instrument has been widely used to study the effects of UV-B exposure to crop plants (Rodríguez-Calzada et al 2019), the influence of natural light exposure on understorey plants (Hartikainen et al. 2020), the annual time course of the Flav index in a *Vaccinium myrtillus* canopy (Solanki...) or as a tool to assess crop nitrogen status (Padilla et al 2019).

Given the wide application of the Dualex instrument, it is desirable to compare results among different investigations, i.e. among different instruments. There is no external reference standard existing, such that it is necessary to send the instrument to the manufacturer for renewed calibration. Therefore, we used the opportunity of a meeting of The International Association for Plant UV Research ("UV4Plants") at Göttingen in March 2024 to arrange an intercomparison of four Dualex 4 scientific and one Dualex 3. Since at Kiel university two further Dualex 4 instruments were available, these were also included in the overall comparison. In addition, two additional optical devices were included in the study, the established UV-A-PAM fluorometer [2, 4] and a prototype of a recently developed instrument, the LSA-2050 leaf state analyzer. While the primary aim was to assess the reliability and comparability among instruments of the Flav index, the two further indices determined by the Dualex 4 instrument, characterizing chlorophyll (Chl index) and anthocyanin (Anth index) content were also incorporated in the study. The measurement of the Anth index involves the same principle as that used for the Flav index, i.e. comparison of the fluorescence yields excited at two different wavelengths. Instead of using UV-A excitation, the measuring beam for anthocyanin measurement is centred around 530 nm (Pfündel et al, Nichelmann & Bilger). Anthocyanins are formed in young or senescent leaves and serve as photoprotectants. The Chl index is determined using a comparison of the transmittance of a red beam absorbed by chlorophyll and a beam in the near infrared, where chlorophyll no longer absorbs. Within a certain range, the chlorophyll content of a leaf is related to its photosynthetic capacity. The Chl index is also used in the assessment of N demand (see above). As measuring objects, a series of *Vicia faba* leaves was chosen, which had been grown at a broad range of irradiance inducing a strong variability in leaf optical properties. Since inconsistent results were obtained in the measurements of the Anth index, its reliability for the detection of anthocyanins was assessed in a separate experiment using leaves of a red hazelnut variety.

## **Material and methods**

Plants of *Vicia faba* L. cv. 'Fuego' were grown at Kiel University in a growth chamber (Johnson Controls, Mannheim, Germany) at a range of irradiances. The irradiance of photosynthetically active radiation (PAR) at the leaf level was manipulated by growing the plants at different heights in the chamber, thereby varying the distance from the lamps (CMT360LS WBH EYE Iwasaki Electric Co., Japan) and accordingly the photon irradiance (400-700 nm), which ranged from 25 to 450  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Environmental conditions in the chamber were 21°C air temperature, 60% relative humidity and a day/night cycle of 16/8 h. Varying irradiance was used as a tool to effectively manipulate the production of phenolic compounds in leaves, including accumulation of flavonoids that contribute to epidermal UV shielding. These changes can be assessed using the Dualex instrument through variations in the so-called "Flav" index. The plants were brought to Göttingen one day before the conference and kept in a growth chamber at a photon irradiance of 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 20°C. Right before the intercomparison, 20 leaf discs (diameter 2 cm) were punched from leaves of different plants and placed individually in a numbered Petri dish on filter paper moistened with tap water to secure the comparability of all measuring results.

On 21.03. 2024, within one hour, the participants did the series of measurements on these discs, always placing the Dualex instrument in the centre of each disc. In addition to the Dualex instruments, measurements were also done using a UV-A-PAM fluorometer (Gademann Instruments, Würzburg, Germany) in combination with a MINI-PAM-Fluorometer (Walz GmbH, Effeltrich, Germany). The latter served as a source for a red measuring beam, which is missing in the UV-A-PAM fluorometer. Data from this combination were normalized to the fluorescence values obtained from a blue plastic foil (Walz) after subtracting the values obtained with a non-fluorescing green PVC foil. As a reference for 100% transmittance, epidermis-free abaxial leaf sides of *V. faba* were used. From the obtained UV-A transmittance values, absorbance was calculated, which is equivalent to the Flav index provided by the Dualex instruments. For the latter instruments, their internal calibration provided by the manufacturer was used.

Measurements were also done using a prototype of an LSA-2050 instrument (Walz GmbH) using reference values from the abaxial side of a leaf from *Hylotelephium telephium* (L.) H. Ohba, provided in the instrument software. Further measurements were done at Kiel University on leaves from the same plants using two additional Dualex Scientific instruments.

In an additional experiment, leaves of a red-leaved variety of *Corylus avellana* L. cv. 'Rotblättrige Zellernuss' were collected in a local garden in Kiel. Discs with a diameter of 8 mm were punched from an area of the leaves with as little veins as possible using a cork borer. Dualex measurements were conducted from both sides, followed by snap freezing the disks in an Eppendorf cap in liquid nitrogen. The caps contained 2 large (diameter 4 mm) and 3 smaller (diameter 3 mm) glass beads

(Roth GmbH, Karlsruhe, Germany). The discs were ground in a Retsch MM2 vibration mill (Retsch, Haan, Germany) for 4 minutes at 90% power after addition of 800 µL cold methanol acidified with 1% concentrated HCl. After centrifugation in a cooled microcentrifuge (Fresco21, Thermo Fischer Scientific, Germany) at 11.6 x g, the supernatant was decanted and the pellet was resuspended in 350 µL acidified methanol. The suspension was then homogenized on a thermomixer (Biometra TSC ThermoShaker, Analytik Jena, Germany) and this step was repeated after a further centrifugation. From the unified supernatants absorbance spectra from 750 to 300 nm were determined in a Cary 60 UV-VIS spectrophotometer (Agilent, Waldbronn, Germany). As a measure for anthocyanin content absorbance at 532 nm was used, after correction for the pheophytin content in the extract according to the formula:

$$(A_{532} - A_{750}) - 0.225 * (A_{653} - A_{750})$$

This formula was based on Mancinelli et al. [16] and Zirngibl et al. [17] and adjusted to the situation in the extracts of *C. avellana*. The absorption spectra of the anthocyanic extracts had relative absorbance maxima at 653 and 532 nm. The factor of 0.225 for the subtraction of pheophytin absorption at 532 nm was determined in leaf extracts from a green variety of *C. avellana*.

Statistical analysis of the data was done using Sigmaplot 13 (Systat Software GmbH, Erkrath, Germany).

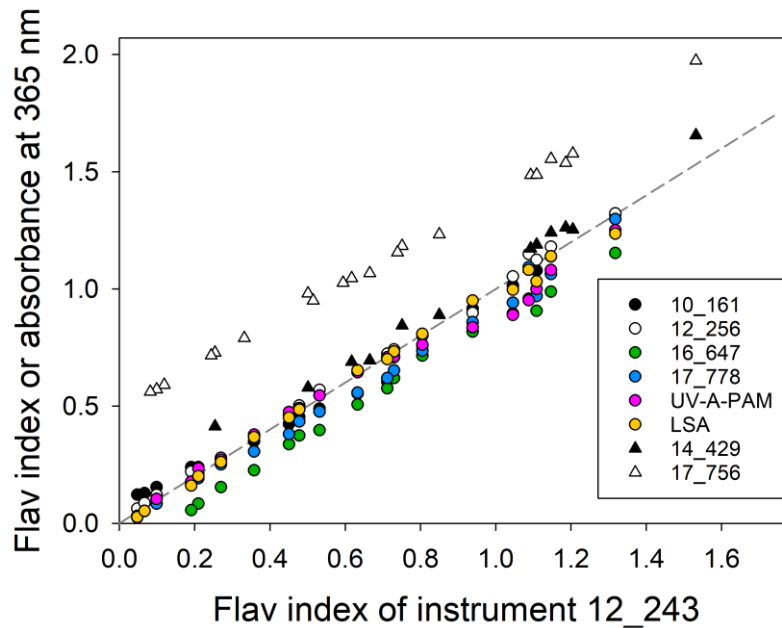


Fig. 1: Results from the measurements on 20 different leaf discs of *V. faba* grown at different irradiance as done with Dualex instruments identified by their serial numbers at Göttingen University (circles) and at Kiel (triangles). The data set includes also absorbance values determined by a UV-A-PAM fluorometer and an LSA-2050 leaf state analyzer (squares). All data are related to the values obtained by the Dualex instrument with serial number 12\_243. The dashed line represents identity between both axes.

The Flav values of the whole data set stretched over a wide range, from nearly 0 to over 1.2. Since the true value of epidermal UV-A absorbance is inherently unknown, data from all devices were plotted against the data of the Dualex instrument with the serial number 12\_243 (Fig. 1). By choosing this device as reference it was possible to include a second data set on corresponding leaves of the same plants using two additional Dualex instruments. It is apparent that all devices, including also the two non-Dualex ones, were linearly correlated with high regression coefficients ( $r^2 > 0.989$ ;  $p < 0.001$ ) to device no. 12\_243, and hence also to each other. The slopes were mostly close to 1, but only in three cases not significantly different from 1.0 (Tab. 1). The y-axis intercept was in four cases significantly different from zero, especially for 17\_756, which was far off (Tab. 1). In fact, this observation had inspired the call for the intercomparison and was related to an apparent malfunction.

Tab. 1: Results of the regression analysis for the flavonoid indices (Flav) between the indicated instruments and instrument 12\_243, as shown in Fig. 1. Values for the slope and the y-axis intersection of the linear equation  $y = ax + b$  are provided together with their respective standard errors. In addition, the regression coefficient  $r^2$  is shown. \* indicates a significant difference for the slope from 1.0 or for the intercept from 0.0, respectively, at an alpha error level of 5%.

|                 | slope a | stdd error a | intercept b | stdd error b | $r^2$  |
|-----------------|---------|--------------|-------------|--------------|--------|
| <b>10_161</b>   | 0.9370* | 0.0227       | 0.0287      | 0.0165       | 0.9896 |
| <b>12_256</b>   | 0.9974  | 0.0115       | 0.0158      | 0.0840       | 0.9976 |
| <b>14_429</b>   | 0.9890  | 0.0307       | 0.0913*     | 0.0299       | 0.9904 |
| <b>16_647</b>   | 0.9611* | 0.0162       | -0.1040*    | 0.0127       | 0.9958 |
| <b>17_756</b>   | 0.9277* | 0.0139       | 0.4787*     | 0.0111       | 0.9960 |
| <b>17_778</b>   | 0.9520* | 0.0191       | -0.0243     | 0.0139       | 0.9928 |
| <b>UV-A-PAM</b> | 0.8981* | 0.0223       | 0.0298      | 0.0162       | 0.9891 |
| <b>LSA-2050</b> | 0.9726  | 0.0162       | 0.0260*     | 0.0123       | 0.9956 |

The chlorophyll measurements were compared among the devices as well. Following the previous procedure, one device was chosen as a reference for the other 6. It is apparent that 4 instruments produced similar values, whereas one consistently showed approx. 10 units higher values than the others (Fig. 2). The regression coefficients were clearly lower than those obtained for the Flav index (Tables 1 and 2). The LSA instrument is not included in this analysis since at the time of the measurements, its chlorophyll indication was not yet finally calibrated.

From Tab. 2 it can be concluded that three instruments had a significantly different offset in comparison to instrument 12\_243, and two had a significantly lower slope, although at least for instrument 12\_256, the data points were scattering along the line indicating equal values for both instruments considered.

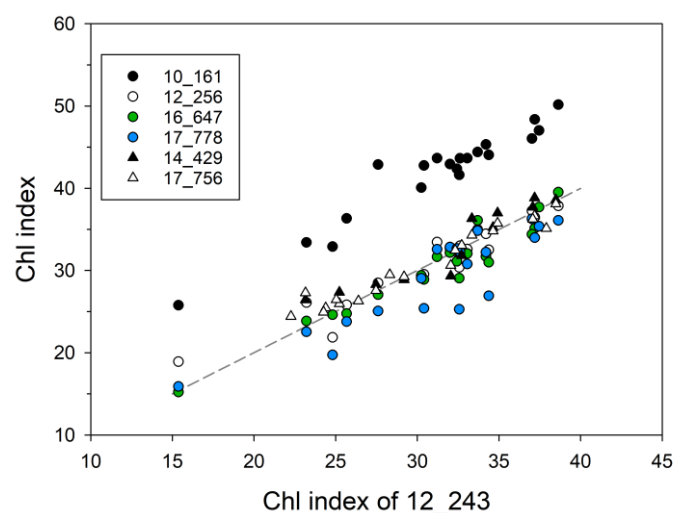


Fig. 2: The chlorophyll index from six different devices as a function of the chlorophyll index (Chl) determined with instrument 12\_243. The dashed line indicates the function for identical indices.

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Tab. 2: Regression analysis results for the chlorophyll index (Chl) between the indicated instruments and instrument 12\_243, as shown in Fig. 2. Values for the slope and the y-axis intersection of the linear equation  $y = ax + b$  are provided together with their respective standard errors. In addition, the regression coefficient  $r^2$  is shown. \* indicates a significant difference for the slope from 1.0 or for the intercept from 0.0, respectively, at an alpha error level of 5%.

|               | slope a | std error a | intercept b | std error b | $r^2$  |
|---------------|---------|-------------|-------------|-------------|--------|
| <b>10_161</b> | 0.9932  | 0.0642      | 10.8774*    | 2.0321      | 0.930  |
| <b>12_256</b> | 0.8755* | 0.0612      | 3.9488*     | 1.9372      | 0.919  |
| <b>14_429</b> | 0.9082  | 0.1064      | 3.8105      | 3.4544      | 0.8793 |
| <b>16_647</b> | 0.9445  | 0.0613      | 0.8969      | 1.9406      | 0.9296 |
| <b>17_756</b> | 0.8213* | 0.0441      | 5.8128*     | 1.3574      | 0.9506 |
| <b>17_778</b> | 0.9294  | 0.1068      | 0.1724      | 3.3832      | 0.8079 |

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158 In addition to the Flav index, most of the tested devices also determined an anthocyanin index  
 159 ("Anth") with the exception of instrument 10\_161 and the UV-A-PAM fluorometer. While the Flav  
 160 and related indices showed a satisfying correlation and uniformity, the same was not observed for  
 161 the Anth index. Since the leaves were evenly green with no red colouration obviously visible to the  
 162 human eye, it was already surprising that some instruments showed Anth values deviating from zero.

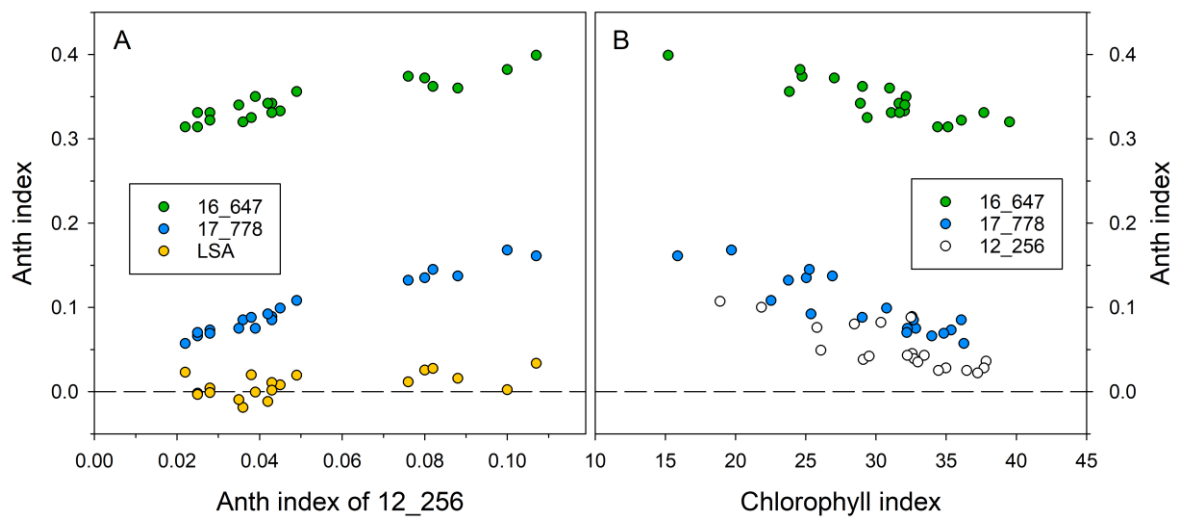


Fig. 3: Anth index as determined by three different instruments, respectively, as a function the Anth index determined by Dualex instrument 12\_256 (A) or as a function of the chlorophyll index determined with the same instrument (B).

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164 Fig. 3A shows the relationships of the detected Anth values among the instruments, using this time  
 165 instrument 12\_256 as reference. Dualex 12\_243 was excluded as it did not provide positive Anth  
 166 values. For each single instrument, the Anth values ranged within an interval of about 0.1 units in the  
 167 three Dualex instruments (Fig. 3B), whereas the corresponding index determined by the LSA-2050  
 168 displayed a smaller amplitude. However, the absolute levels of the detected Anth values varied  
 169 strongly over a range of 0.4 units, whereas the slopes of the mutual relationships were similar for the  
 170 Dualex instruments. The observed variation of the Anth values among the leaf samples was probably  
 171 related to the different chlorophyll content of the samples.

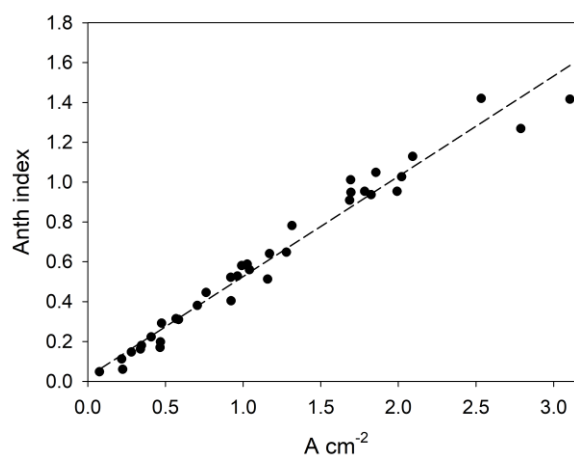


Fig. 4 Relationship of the sum of the Anth indices from the adaxial and abaxial sides of leaf discs of differently red leaves from *Corylus avellana* cv. 'Rotblättrige Zellernuss' and the anthocyanin absorbance at

532 nm of methanolic extracts from the same discs. The dashed line shows the regression line,  $r^2 = 0.969$ ;  $p < 0.001$ .

The variable performance of the Anth index of the Dualex instruments sparked an additional experiment to assess its general usefulness for anthocyanin detection, which was conducted with the Dualex device 12\_243. The hazel leaves used for this experiment varied strongly in their red colouration, with the youngest leaves showing the highest anthocyanin contents and the lowest chlorophyll index values. The latter index ranged from 20 to 42 units. Comparison of the Anth index, summarized over both leaf sides, and the absorbance of extracts from whole leaves showed a straight linear and proportional relationship, with a high correlation coefficient and a y-axis intercept not statistically different from 0 (Fig. 4). However, leaves with data points close to the origin still had a red hue visible to the naked eye, and when two seemingly green leaves from a non-anthocyanic variety were measured, the Dualex instrument showed 'NAN', indicating an index value of zero or in the negative range.

## Discussion

In general, the Dualex instruments showed a good or at least reasonable mutual agreement of the determined Flav values with one exception (17\_756). This instrument was subsequently sent to the manufacturer (Pessl Instruments) for repair. Although the two other types of instruments (UV-A-PAM fluorometer and LSA-2050) had a different technical design, their data agreed well with those of the Dualex instruments (Fig. 1). The non-Dualex instruments allow users to set the reference value for 100% transmittance using measurements on epidermis free leaves or chloroplast suspensions placed on filter paper [4]. This feature enables independent calibration against external standards. Although calibration of the Dualex instruments must be done at the factory, the usage of epidermis-free leaves allows everybody to check the quality of the instrument. From leaves of lamb's lettuce (*Valerianella locusta* (L.) Laterr.) or spinach (*Spinacia oleracea* L.) the epidermis of the abaxial leaf side can be easily removed on sufficiently large areas such that a test of zero screening is possible. Strong positive, but no negative deviations of the signal can be detected with this approach. The latter deviations can be assumed when even the intact abaxial sides of leaves of these species or from other shade grown plants show no numbers ('NAN'). The satisfying agreement among the various instruments indicates that published Flav index data [e.g., 11-14] are generally quantitatively comparable.

In contrast to the Flav indices, chlorophyll indices showed a larger variability among instruments (Fig. 2, Tab. 2). Part of this variability may be caused by the presence of veins in the leaf discs, which can

negatively affect the reproducibility of measurements. We recommend using several measurements per leaf to account for this potential variability. A simple test for a large offset of the Chl index can be performed using fully senesced leaves, which should contain only traces of chlorophyll. Since chlorophyll content is relatively easily accessible using standard protocols [18], every user should calibrate his own instrument for quantitative chlorophyll determination. Within a certain range, the relationship between the optical index and true chlorophyll content may be species specific. It has also been recommended to determine specific calibration coefficients for monocots and dicots [7].

A satisfying agreement among the various instruments was not observed with the Anth index. In principle, two different problems were detected. First, different instruments had an offset of the values against each other (Fig. 3A), varying from close to or below zero (indicated by “NAN” by the instrument) up to 0.4. This shift is presumably caused by varying baseline reference values among the different instruments.

Second, the different instruments showed a similar dependency of the Anth index over the samples, indicating consistently a slight increase in anthocyanin content along an unknown gradient over the leaves. However, in *V. faba* grown under benign conditions, we have never observed red colouration indicating anthocyanin accumulation and also the leaves investigated here were obviously green. A closer inspection of the data showed that the Anth values increased with decreasing chlorophyll content (Fig. 3B). Also, Nichelmann and Bilger [15] showed increasing  $F_{545}/F_{650}$  fluorescence excitation ratios with decreasing chlorophyll content of the sample, when suspensions of isolated chloroplasts were applied in varying concentration to filter paper. This behaviour can be explained by the fact that the measuring wavelengths are lying in regions of highly different extinction coefficients of chlorophyll. While the absorption of the red reference beam is still saturating even in the presence of reduced amounts of chlorophyll, the absorption in the green region, where the measuring beam for anthocyanins is located, begins to decline with decreasing chlorophyll concentration. The varying absorption ratio between these wavelength regions has previously been named as sieve or package effect [19]. It leads with declining chlorophyll content to a reduced  $F_{530}/F_{650}$  ratio, which is interpreted by the instruments as anthocyanin signal. Therefore, Anth values indicated by a Dualex instrument on visibly green leaves with a chlorophyll index below ca. 30 units should be considered with utmost caution. On the other hand, this does not mean that the Dualex instrument is not well suited to detect anthocyanin in an obviously red coloured leaf. The excellent linear relationship between the anthocyanin content and Anth values in red leaves of *C. avellana* cv. ‘Rotblättrige Zellernuss’ up to Anth values above 1.0 (Fig. 4) proves the suitability of the instrument. This was observed despite Chl index values down to 20 in the leaves with the highest anthocyanin contents (data not shown). Since anthocyanin absorption *in vivo* is dependent on the pH value and other

effects, such as complex formation and co-pigmentation with phenolic acids [20], the relationship between Anth index and anthocyanin content should be calibrated for the species used in the investigation.

This study profited from the parallel usage of several devices at identical objects. But how can a user who has only a single device available routinely check its quality? The results showed that the most critical difference among the devices was an offset in the signal. This is presumably due to a different reference value for the signal in the absence of pigments. In order to regularly check for this problem, we recommend a test of the signal in the absence of the pigments. This can be comparatively easily done with the visible pigments. Leaves with low chlorophyll content can be obtained by letting leaves senesce in darkness. Leaves without anthocyanins can be found everywhere. In contrast, leaves without UV-A screening compounds are very rare. In this case, epidermis-free shade grown leaves should be used. Commercially available lamb's lettuce (*Valerianella locusta* (L.) Laterr.) or spinach (*Spinacia oleracea* L.) leaves are well suited for the removal of sufficiently large areas of the abaxial epidermis. However, this method will not detect a negative offset and in this case no unambiguous test exists. Abaxial leaf sides, even when leaves are grown in the shade, should show low, but positive Flav values. When still no numbers are measured, a negative offset can be assumed. Such tests can help to detect developing signal deviations. Should they occur, a calibration at the manufacturer is recommended.

## Conclusions

With few exceptions, the instruments used in this study showed quite well comparable values, especially for the Flav index. This supports the role of this instrument as a workhorse in research characterizing leaf acclimation to various environmental conditions, among them nitrogen fertilization, high irradiance, low temperature and exposure to UV radiation, allowing a rapid orientation with large amounts of samples. Simple tests for instrument malfunctioning are described. When determining chlorophyll contents, repeated measurements on a leaf are recommended.

Measurements of the Anth index should always be carefully evaluated and artefacts caused by too low chlorophyll contents (Chl index <30) be taken into consideration. In our hands, visual inspection of the leaves for red colouration coincided well with Anth values when anthocyanins were indeed present. If there exists any doubt, the presence of anthocyanins should be verified by spectrophotometry on leaf extracts.

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