Effect of anthocyanins, carotenoids, and flavonols on chlorophyll fluorescence excitation spectra in apple fruit: signature analysis, assessment, modelling, and relevance to photoprotection

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Abstract

Whole apple fruit (Malus domestica Borkh.) widely differing in pigment content and composition has been examined by recording its chlorophyll fluorescence excitation and diffuse reflection spectra in the visible and near UV regions. Spectral bands sensitive to the pigment concentration have been identified, and linear models for non-destructive assessment of anthocyanins, carotenoids, and flavonols via chlorophyll fluorescence measurements are put forward. The adaptation of apple fruit to high light stress involves accumulation of these protective pigments, which absorb solar radiation in broad spectral ranges extending from UV to the green and, in anthocyanin-containing cultivars, to the red regions of the spectrum. In ripening apples the protective effect in the blue region could be attributed to extrathylakoid carotenoids. A simple model, which allows the simulation of chlorophyll fluorescence excitation spectra in the visible range and a quantitative evaluation of competitive absorption by anthocyanins, carotenoids, and flavonols, is described. Evidence is presented to support the view that anthocyanins, carotenoids, and flavonols play, in fruit with low-to-moderate pigment content, the role of internal traps (insofar as they compete with chlorophylls for the absorption of incident light in specific spectral bands), affecting thereby the shape of the chlorophyll fluorescence excitation spectrum.

Key words: Anthocyanins, apple fruit, carotenoids, chlorophyll, flavonols, fluorescence, photoprotection.

Introduction

As an important defence mechanism against the deleterious effects of solar radiation, long-term adaptation of higher plants involves synthesis of relatively stable compounds capable of serving as light screens and/or internal traps (Day et al., 1993; Bilger et al., 1997, 2001; Smith and Markham, 1998; Cockell and Knowland, 1999; Barnes et al., 2000; Merzlyak and Chivkunova, 2000; Cerovic et al., 2002; Steyn et al., 2002; Merzlyak and Solovchenko, 2002; Pfundel et al., 2006). The build-up of such substances in specific cell and tissue structures reduces the fraction of radiation absorbed by potent photosensitizers, and thereby diminishes light-induced damage. It is helpful to distinguish between screening and competitive absorption. The term screening will be applied to systems in which the photosynthetic pigments can only absorb the light that emerges from a layer or compartment containing the protective pigments (Barnes et al., 2000; Cerovic et al., 2002); in other cases, where the protecting pigments must compete with photosynthetically active pigments for light absorption, the term ‘internal trapping’ is used. Several compounds absorbing in different spectral regions could provide photoprotection; vacuolar flavonoids (Bilger et al., 1997; Cockell and Knowland, 1999; Barnes et al., 2000; Cerovic et al., 2002; Pfundel et al., 2006) and...
Anthocyanins (AnC) (Merzlyak and Chivkunova, 2000; Steyn et al., 2002; Pfundel et al., 2006), absorbing mainly in the near UV and green regions of the spectrum, respectively, have been considered in the literature. The internal trapping role was also suggested for extrathylakoid carotenoid (Car) pigments in chloroplasts of stressed/senescing leaves and ripening fruit (Merzlyak and Solovchenko, 2002; Han et al., 2003; Merzlyak et al., 2005a).

Together with other approaches (Day et al., 1993; Cockell and Knowland, 1999; Barnes et al., 2000; Merzlyak and Chivkunova, 2000; Bilger et al., 2001; Solovchenko and Merzlyak, 2003; Solovchenko and Schmitz-Eiberger, 2003; Pfundel et al., 2006) the screening effect in green plant tissues was evaluated through the analysis of the excitation spectrum of chlorophyll (Chl) fluorescence (Bilger et al., 1997, 2001; Barnes et al., 2000; Cerovic et al., 2002; Barthod et al., 2007), which, at room temperature, is emitted from chlorophyll a (Chla) (Buschmann and Lichtenthaler, 1988; Lázár, 1999; Roháček, 2002). In leaves the ratio of Chl fluorescence excited by UV-B to that excited by blue-green light showed a negative correlation with the concentration of whole-leaf UV-B-absorbing pigments, and a positive correlation with the transmittance of isolated epidermal tissue, where flavonoids accumulate (Barnes et al., 2000). Cerovic et al., (2002) reported that UV-excitation spectra of Chl fluorescence from the adaxial and abaxial sides of bifacial leaves allowed identification of UV-absorbing screening pigments in the leaf epidermis. They found that, due to the strong absorption by Chl at wavelengths shorter than 450 nm, differences in leaf optical properties exert only a minor influence on the shape of UV-excitation spectra, and ‘Chl behaves as a photon counter’. In the commercial fruit industry, studies of the basic properties of light-screening substances and Chl fluorescence have been applied recently for non-destructive pigment assessment; methods allowing sensitive and rapid determination of flavonoids (including AnC) in the skin of apples (Hagen et al., 2006), marketable broccoli heads (Bengtsson et al., 2006), olive fruit (Agati et al., 2005), and grape berries (Agati et al., 2007) have been developed. In these studies, screening by flavonoids was quantified, on the leaf/fruit level, by using a Chl fluorescence excitation (CFE) ‘ratio’, for example, the ratio of the Chl fluorescence yields for different excitation wavelengths. Further progress can be made only by relating a CFE ‘spectrum’ to specific spectral features of Chl and individual light-screening and/or internally-trapping pigments in the specimen under examination.

Apples possess a fully functional photosynthetic apparatus (Blanke and Lenz, 1989) containing thylakoid-bound Chl and Car as well as vacuolar flavonoids as principal pigments absorbing in the visible range and near UV (Merzlyak et al., 2003, 2005b; Bengtsson et al., 2006; Merzlyak, 2006). As a result of development under strong solar radiation, apples accumulate phenolics with absorption maxima in the near UV, including flavonols (Flv) and other flavonoids (Solovchenko and Merzlyak, 2003; Solovchenko and Schmitz-Eiberger, 2003; Merzlyak et al., 2005b; Hagen et al., 2006; Merzlyak, 2006). Fruit of some apple cultivars under high-light conditions are able to accumulate AnC pigments, which absorb strongly in the green region of the spectrum and thus impart red colour to the fruit (Saure, 1990; Merzlyak and Chivkunova, 2000; Ma and Cheng, 2004). Ripening is accompanied by a progressive decline in the Chl content and the build-up of xanthophylls (Merzlyak and Solovchenko, 2002; Solovchenko et al., 2005). Due to its relatively low pigment content, apple fruit represents a simple natural system in which general plant ontogenetic and/or stress-induced pigment dynamics could be followed non-destructively and quantitatively, using measurements of diffuse reflectance (see for review Merzlyak, 2006).

In this work we have quantified competitive absorption by Chl, Flv, Car, and AnC by examining diffuse reflection spectra and CFE spectra of apple fruit differing in pigment content and composition, as a result of ripening and/or adaptation to strong sunlight. It is shown that a good approximation to CFE spectra in the visible range can be achieved by assuming simple relations between fluorescence, absorbed radiation, and internal trapping. The proposed model has enabled us to carry out a quantitative evaluation of internal trapping by AnC, Car, and Flv in apple fruit.

Materials and methods

Plant material

AnC-free Granny Smith and Golden Delicious, and AnC-accumulating Summer Red apple (Malus domestica Borkh.) fruit were purchased at local market of Trondheim in Autumn 2002 and Spring 2003. The fruits were selected on the basis of their colour. Sunlit surfaces of Granny Smith and Golden Delicious fruits were recognized by their paler and more yellowish colouration and specific features in the near UV part of the diffuse reflection spectrum, mainly due to Flv accumulation (Merzlyak and Chivkunova, 2000; Merzlyak et al., 2005b). Sunlit surfaces of Summer Red apples were pink-to-red in colour as a result of AnC pigmentation.

Spectral measurements

Whole fruit diffuse reflection spectra (against barium sulphate as a standard) were recorded by means of a 3010 Hitachi spectrophotometer equipped with a 150 mm diameter integrating sphere attachment. The reflectance data were sampled at 1 nm intervals in the 350–800 nm range.

Fluorescence excitation and emission spectra were measured using Fluorolog 3 (Jobin Yvon-Spix). Segments (c. 15×25 mm) sliced from the equatorial part of an apple, were placed in a sample holder made by removing two adjacent sides from a standard (1 cm²) plastic cuvette, which was itself placed in the sample compartment of the spectrophurometer. The excitation beam was at an angle of approximately 45° to the surface of the sample, and...
a right-angle arrangement was used for observing the emitted light. For all experiments, the band pass of the excitation and emission monochromators were 3 nm and 4 nm, respectively.

To minimize the effect of chlorophyll fluorescence induction kinetics (Buschmann and Lichtenthaler, 1988; Lazár, 1999; Roháček, 2002), the samples were pre-irradiated directly in the spectrophotometer for 550 s, using light of 430 nm for AnC-free apples (5.8 mW cm\(^{-2}\)) or 680 nm for AnC-containing apples (2.5 mW cm\(^{-2}\)). In the course of the pre-irradiation, the fluorescence intensity fell to nearly a half of its peak value, and reached a steady level within 450 s. Fluorescence spectra of the samples were acquired immediately after the termination of the pre-irradiation period; in order to prevent fluorescence recovery, an intense excitation beam was used, which had the additional advantage of providing a large signal-to-noise ratio. Under these conditions, two successive scans yielded spectra that were identical (within the limit set by noise) if the second scan was started within 1 min after the end of the first.

Fluorescence excitation spectra were recorded between 350 nm and 710 nm at a scan rate of 1 nm s\(^{-1}\) and emission set at 715 nm. To reduce the amount of exciting light scattered towards the detection system, a long pass filter transmitting above 700 nm (Oriel LP70) was placed before the emission monochromator; correction for the wavelength-dependence of the intensity of the excitation beam was made by using correction factors supplied by the manufacturer. For recording fluorescence emission spectra, the excitation wavelength was set at 440 nm. Since absolute measurements of fluorescence could not be performed with a standard spectrophotometer (where all the emitted light is not collected), the analysis was limited to a comparison of relative changes in the shapes of the recorded spectra. To minimize the influence of uncontrolled variations in the instrumental conditions (drift, possible variability of the fluorescence quantum yield), the spectra analysed here were all normalized (at the red Chl\(a\) maximum).

**Pigment analysis**

The pigment content in the peels of the apples examined here was determined with the aid of recently developed algorithms, which make use of values of \(R_{a}\), the diffuse reflectance for incident light of wavelength \(w\) \(\text{nm}\). The following indexes were used for quantification: \(R_{800}/[R_{570}/1-R_{800}]\) for Chl, \(R_{600}/[R_{520}-1-R_{620}]\) for Car, and \(R_{680}/[1-R_{520}-1-R_{700}]\) for AnC [Merzlyak et al., 2003], and \(R_{660}/[1-R_{520}-1-R_{700}]\) for Flv, whose content was expressed as that of rutin (Merzlyak et al., 2005b). Square brackets are used to denote the pigment concentration.

**Modelling of pigment contribution into spectral reflection**

The modelling of diffuse reflectance in the visible range was performed in accordance with a theory developed by Atherton (1955) for dealing with a dyed fabric: though similar to the better-known Kubelka–Munk approach, his analysis is more pertinent for spectral measurements, since it assumes the incident beam to be collimated (as in a spectrophotometer). One of his principal results, which forms the basis of our analysis, may be stated as follows: In a specimen containing many pigments (in concentrations \(c_1, c_2, \text{ etc.}\), and with absorption coefficients, \(e_1(\lambda)\), \(e_2(\lambda), \text{ etc.}\), where \(\lambda\) denotes the wavelength of the incident light), the diffuse reflectance \(R(\lambda)\) satisfies the following relation (Atherton, 1955; Alderson et al., 1961):

\[
1/R(\lambda) - 1/R^{(0)}(\lambda) = c_1 e_1(\lambda) + c_2 e_2(\lambda) + \ldots
\]

where \(R^{(0)}(\lambda)\) is the diffuse reflectance of the specimen in the absence of the pigments. On the basis of experience gained from light-induced bleaching experiments (Merzlyak, 2006), the reciprocal diffuse reflectance of apple fruit, \([R(\lambda)]^{-1} - \mathcal{R}(\lambda)\), may be visualized as the sum of two sub-spectra: that is, \(\mathcal{R}(\lambda) = \mathcal{R}^{(1)}(\lambda) + \mathcal{R}^{(0)}(\lambda)\), where the selective part \(\mathcal{R}^{(0)}(\lambda)\) contains features attributable to the various pigments, and the featureless non-selective part \(\mathcal{R}^{(0)}(\lambda)\) arises from factors other than absorption (mainly scattering within the fruit tissue). The simulated counterparts of \(\mathcal{R}(\lambda), \mathcal{R}^{(0)}(\lambda),\) and \(\mathcal{R}^{(0)}(\lambda)\), will be denoted by \(\mathcal{R}(\lambda), \mathcal{R}^{(0)}(\lambda),\) and \(\mathcal{R}^{(0)}(\lambda)\), respectively; our main task is to model \(\mathcal{R}^{(0)}(\lambda)\) so as to make their sum

\[
\mathcal{R}(\lambda) = \mathcal{R}^{(0)}(\lambda) + \mathcal{R}^{(0)}(\lambda)
\]

agree as closely as possible with \(\mathcal{R}(\lambda)\).

The first term in equation (2) was expressed as the sum

\[
\mathcal{R}^{(0)}(\lambda) = \sum_{\lambda} a_{\lambda} \mathcal{P}_{\lambda}(\lambda)
\]

agree as closely as possible with \(\mathcal{R}(\lambda)\).

While \(\mathcal{P}_{\lambda}(\lambda)\), \(\mathcal{P}_{\lambda}(\lambda),\) and \(\mathcal{P}_{\lambda}(\lambda)\) have been determined previously (Merzlyak, 2006), \(\mathcal{P}_{\lambda}(\lambda)\) was equated to the difference \(\mathcal{R}^{(0)}(\lambda)-\mathcal{R}^{(0)}(\lambda)\), where \(\mathcal{R}^{(0)}(\lambda)\) and \(\mathcal{R}^{(0)}(\lambda)\) now refer to Summer Red fruit with a moderate amount of AnC and with no AnC, respectively. A linear form \(a_{\lambda} \lambda + a_{\lambda}\), where \(a_{\lambda}\) and \(a_{\lambda}\) are constants, was used for representing \(\mathcal{R}^{(0)}(\lambda)\) (Merzlyak, 2006).

**Modelling chlorophyll fluorescence excitation spectra**

In an optically clear system containing only a single fluorophore, the fluorescence intensity is proportional to the quantity of absorbed radiation, \(1-T(\lambda)\), where \(T(\lambda)\) is the transmittance at wavelength \(\lambda\). Quantitatively, the relation between \(I_{\lambda}(\lambda)\), the intensity of the collimated incident beam, and \(I_{\lambda}(\lambda)\), the intensity of fluorescence emitted (in all directions) at wavelength \(\lambda\), can be expressed as:

\[
I_{\lambda}(\lambda) = \gamma \phi_{\lambda}(\lambda)[1 - T(\lambda)]
\]

where \(\gamma\) is an instrumental factor (that may depend on, among other things, \(\lambda\) and \(\lambda\)), and \(\phi\) is the fluorescence quantum yield; in most one-component systems, \(\phi\) is independent of the wavelength of excitation ( Bursteyn, 1968; Parker, 1968). If \(\gamma\) and \(\phi\) are constants and \(T\) vanishingly small (total absorption), \(I_{\lambda}\) becomes proportional to \(I_{\lambda}\), and the sample can be used as a ‘relative quantum counter’ (Browne, 1936).

In order to apply the foregoing reasoning to apple fruit, the following assumptions were made: (i) only the \(\mathcal{P}_{\lambda}(\lambda)\) pigment pool contributes to the fluorescence emission spectrum in the red (Buschmann and Lichtenthaler, 1988; Gitelson et al., 1998; Lazár, 1999; Roháček, 2002; Ramos and Lagorio, 2006), and (ii) the efficiency of energy transfer to Chl\(a\) is high for Chl\(a\) and the majority of Car which are in the thylakoids (Gradinaru et al., 2000; Croce et al., 2001) and zero for those Car which lie outside. Let \(\Gamma(\lambda) = \mathcal{H}(\lambda) / \mathcal{R}(\lambda)\) denote the fraction of light absorbed by the thylakoid-bound pigments, and let \(\phi_a\) denote the fluorescence quantum yield of Chl\(a\); the measured intensity of fluorescence \(I(\lambda)\) can then be expressed as

\[
I(\lambda) = \gamma \phi_{a}(\lambda)[1 - \mathcal{R}(\lambda)]\Gamma(\lambda)
\]

When we refer to the case \(\Gamma = 1\) (a fruit that is devoid of extrathylakoid pigments), an asterisk will be added, as shown below, where the form taken by equation (5) for \(\Gamma = 1\) is shown:
content of mature specimens was higher than their Car content, but ripening made Car the dominant pigment (Solovchenko et al., 2005); a reduction in [Chl] was accompanied by a remarkable increase in [Car]/[Chl] ratio reaching 2.4–2.5 in fruit with [Chl]<1 nmol cm$^{-2}$. Sunlit surfaces of the fruit also exhibited, in comparison with the shaded surfaces, a preferential increase in [Car] over [Chl] (see also Merzlyak et al., 2002; Ma and Cheng, 2004; Solovchenko et al., 2006) especially in Golden Delicious apples (not shown). The [Chl]-[Car] correlation was relatively high in Granny Smith apples ($r^2=0.80$), and much lower in Golden Delicious fruits ($r^2=0.49$). No [Chl]-[Flv] or [Car]-[Flv] correlation was found in AnC-free fruit; in Summer Red apples, [Chl] and [AnC] were uncorrelated. The Chl contents of the shaded and sunlit surfaces of Summer Red apples did not differ significantly, and on average equaled that in Golden Delicious apples. The AnC content of Summer Red apples grown under the shade was low ([AnC] <1.3 nmol cm$^{-2}$), but considerably higher in sunlit surfaces, with [AnC] reaching 40 nmol cm$^{-2}$.

**Anthocyanin-free fruit**

**Effect of chlorophyll content:** Representative diffuse reflection in the green-red region, where only Chl contributes to absorption, are presented in Fig. 1 for six samples of AnC-free apple fruit differing in Chl content. In this spectral range reflectance possessed features belonging to Chla (minimum at 678 nm) and Chlb (shoulder around 650 nm), and showed a monotonic decrease with increasing Chl content, without noticeable changes in the positions of the minima (Fig. 1A). The CFE spectra were more resolved and showed qualitative changes dependent on the Chl content. In fruit with low [Chl], the fluorescence excitation maximum was located around 678 nm and the fluorescence intensity near 650 nm was relatively small. An increase in [Chl] brought about pronounced changes in the shape, a considerable flattening of the spectra and a progressive shift of the maximum to shorter wavelengths. Simultaneously, a pronounced enhancement of the band 650 nm and intensification of fluorescence emission in the green and far-red ranges were observed. In a fruit with high [Chl] (8.06 nmol cm$^{-2}$) the amplitude of $F(\lambda)$ spectrum exceeded 0.5 in the green region, and the maximum was situated at 653 nm (Fig. 1B).

The shapes of Chl fluorescence emission spectra of apple fruit also showed a strong dependence on [Chl]. The fluorescence maxima in these spectra were located at 682 nm and 685 nm, in fruits with low (c. 1 nmol cm$^{-2}$) and high (c. 8.1 nmol cm$^{-2}$) Chl content, respectively. The latter spectrum was much broader with an intense band 700–740 nm (not shown) suggesting a strong effect of fluorescence re-absorption (Gitelson et al., 1998; Ramos and Lagorio, 2006).

**Sunlit versus shaded fruit:** To display the differences between the sunlit and shaded surfaces of AnC-free fruit,
apples were selected with a Chl content low enough to avoid distortion of the main Chl peak in the CFE spectra (Fig. 1). Averaged spectra for non-reflected radiation, $Q(\lambda) = 1 - R(\lambda)$, which represents light absorption by (the pigments) and scattering (by the tissue), are plotted in Fig. 2A for six shaded surfaces (curve 1) and six sunlit surfaces (curve 2); the corresponding plots of normalized CFE spectra appear in Fig. 2B. Compared with shaded surfaces, sunlit fruit surfaces were characterized by larger values of [Flv] and [Car], and lower values of $Q$ in the far red region. Typically for sunlit fruit surfaces (Merzlyak and Chivkunova, 2000; Merzlyak et al., 2005b), reflectance below 500 nm decreased and $Q(\lambda)$ increased, reaching a high and nearly constant level in the near UV (Fig. 2A).

Compared with their reflection counterparts, the CFE spectra of apple fruit (Fig. 2B) displayed narrower bands, associated with Chl $a$ (main maxima at 678 nm and 440 nm), Chl $b$ (shoulder at 650 nm, a band near 460 nm) and

![Fig. 1](https://academic.oup.com/jxb/article-abstract/59/2/349/539105)

**Fig. 1.** (A) Reflection spectra, $R(\lambda)$, and (B) normalized (at 678 nm) chlorophyll fluorescence excitation spectra, $F(\lambda)$, of Golden Delicious apples. The value of [Chl](mmol cm$^{-2}$) for each specimen is given in parentheses following the identification label: 1 (0.58), 2 (1.23), 3 (1.92), 4 (2.43), 5 (2.87), 6 (8.06).

![Fig. 2](https://academic.oup.com/jxb/article-abstract/59/2/349/539105)

**Fig. 2.** (A, B) Curves 1 and 2 show, respectively, the average spectrum for non-reflected radiation, $Q(\lambda) = 1 - R(\lambda)$, and the average normalized chlorophyll fluorescence excitation spectrum, $F(\lambda)$, of six AntC-free apples with low Chl content; error bars (± standard error) are also shown for each curve. Labels 1 and 2 identify shaded and sunlit surfaces, respectively. Key for the corresponding pigment contents (average ± SE, mmol cm$^{-2}$) (1): [Chl] = 0.81 ± 0.11, [Car] = 1.27 ± 0.31, [Flv] = 10.9 ± 5.4. (2): [Chl] = 0.82 ± 0.14, [Car] = 1.54 ± 0.57, [Flv] = 85.7 ± 20.3. The right vertical scale in (B) refers to curve 3, which is a plot of the ratio $F_1(\lambda)/F_2(\lambda)$, where the subscripts 1 and 2 indicate shaded and sunlit surfaces, respectively.
Car (peaks around 465–470 nm and 485–490 nm). In shaded fruit the ratio of Chl a fluorescence maximum in the Soret band to that in the red was, on average, c. 0.7, approximately 2-fold higher than that in sunlit fruit surfaces. In sunlit fruit surfaces below 520 nm, fluorescence intensity decreased considerably and two Chl a bands near 420 nm and 383 nm evident in the spectra of shaded fruits were reduced and absent, respectively. In these samples, fluorescence contained no spectral details between 350 nm and 400 nm.

**Signature analysis:** Correlation analysis was applied for investigating the influence of the principal pigments on the absorption and fluorescence spectra of apple fruit in the visible and near-UV range. In Fig. 3, which refers to AnC-free fruit, the solid curves in each panel show the spectra of correlation coefficient, $r$, of the linear relationship between the concentration of a pigment [Pgm], where Pgm stands for Chl, Car or Flv, and a $\lambda$-dependent quantity that is $Q(\lambda)$ for the upper row and $F(\lambda)$ for the lower row; the dotted curves in Fig. 3C and D display the $F(\lambda)-Q(\lambda)$ correlation. High correlation between $Q(\lambda)-[\text{Chl}]$ was observed in a narrow band around 700 nm and in a wider band, situated at 580±60 nm for Granny Smith and 610±30 nm for Golden Delicious apples. At shorter wavelengths the correlation for [Chl]

![Fig. 3](https://academic.oup.com/jxb/article-abstract/59/2/349/539105)
decreased with a concomitant increase in the correlation for [Car]. The maxima for [Car] were observed near 510–515, 470, and 440 nm, whereas the minima were situated around 480 nm and 450 nm; these spectral features were more pronounced in Golden Delicious apples. The $Q(\lambda)$-[Flv] correlation, weak in the orange-red region, increased significantly toward the violet, with peak positions opposite to those for the other two pigments. Between 360 nm and 420 nm the $F$-$Q$ correlation considerably exceeded the $F$-[Flv] correlation, and showed maxima near 360–370 nm and 400–410 nm for Granny Smith and Golden Delicious apples, respectively. The spectra of $F$-[Pgm] correlation contained fewer details (Fig. 3C, D). For [Chl] the correlation was relatively high near 700 nm and in the band 520–630 nm. In the green region, higher correlation was recorded over a broader spectral band for Granny Smith fruit (with a larger Chl content) than for Golden Delicious. At shorter wavelengths, the $Q$-[Chl] correlation as well as $F$-[Chl] correlation displayed a significant decline. Throughout the whole spectral range the shape of the correlation spectra for [Car] was similar to that for [Chl] but the correlation was much weaker (especially, in Golden Delicious fruit). The $F$-[Flv] correlation was low for $\lambda > 450$ nm, and the correlation spectrum contained few spectral details. At shorter wavelengths, the shape of the spectrum (for Granny Smith apples) resembled that for [Chl] approaching −0.7 near 375 nm, whereas in Golden Delicious apples the negative correlation in the broad band 380–420 nm was higher and reached −0.88 near 420 nm.

In Granny Smith apples, the $F$-$Q$ correlation spectrum contained the same features as the $F$-[Chl] correlation spectrum. In this fruit, positive and relatively high correlation was found near 700 nm and in the band 600 nm and 650 nm, whereas in Golden Delicious apples the latter band was much narrower with the maximum near 630 nm. Below 530–550 nm in both cultivars, $r$ decreased sharply with prominent minima near 440 nm and 480 nm, became negative at shorter wavelengths, and reached −0.7 in the 360–370 nm band.

**Anthocyanin-containing fruit**

The results for Summer Red apples, which are able to accumulate AnC pigments on their sunlit surfaces, are summarized in Fig. 4. The build-up of AnC brought about a considerable content-dependent increase in $Q(\lambda)$ at wavelengths shorter than 620 nm, along with the shift of absorption band from 510–520 nm to c. 550 nm. At high AnC content $Q(\lambda)$ exceeded 0.94 in the spectral range 350–575 nm, and Chl absorption in the red appeared on a large ‘AnC background’ (Fig. 4A). With increasing AnC content, $F(\lambda)$ suffered a noticeable decrease; the effect was particularly pronounced (relative fluorescence intensity of 4–5%) between 520 nm and 575 nm. As regards the blue region, Chl and Car spectral features, easily discernible in apples with moderate AnC, were suppressed in the sample with high [AnC] (Fig. 4B).

For the whole data set of Summer Red apples, the $Q$-[Chl] correlation was relatively high ($r > 0.9$) in 660–698 nm band (Fig. 4C); the $Q$-[AnC] correlation, very weak in this region, attained its maximum near 600 nm, and remained high down to 400 nm. In the entire spectral range, the $F$-[Chl] correlation showed a weak correlation.
with [Chl]; in contrast, the $F_{\text{corr}}$-[AnC] correlation had a large negative value, peaking near 580 nm, with additional bands around 500, 464, and 430 nm. The $F$-$Q$ correlation possessed weak positive bands around 645 nm and 700 nm and showed a negative correlation between 450 nm and 560 nm ($r > 0.9$) (not shown) resembling that for the $F_{\text{corr}}$-[Flv] correlation.

Spectral reconstruction of pigment absorption and modelling chlorophyll fluorescence excitation spectrum

Spectral reconstruction of the reflection spectra revealed that all samples contained a pool of extrathylakoid Car, which is characteristic of ripening (Merzlyak, 2006). Figure 5 demonstrates the reconstruction for fruit with the following pigment content and composition: (A) approximately equal amounts of Car and Chl, (B) low Chl content with elevated amounts of Flv and Car, and (C) AnC-accumulating Summer Red apple. The modelling provided a good simulation of CFE spectra in the spectral range 600–700 nm (cf. curves 1 and 3 in Fig. 5D–F). In general, in the blue-green region the modelling also succeeded in reconstructing the shapes of the spectra, the positions of the main maxima, and the ratios between the peaks. In addition, in Fig. 5 D–F, the model $F^\lambda(\lambda)$ spectra calculated for fruit ‘devoid’ of the internal-trapping $P_X$, $P_P$, and $P_A$ pigment pools (curves 2) and the spectral dependence of the trapping factors (curves 4) are presented.

Pigment assessment with chlorophyll fluorescence measurements

The results of correlation analysis were employed for assessment of Chl, Flv, and AnC content via Chl fluorescence measurements with linear models. For the fluorescence index in the form, $[\text{Chl}] = 0.109 + 0.046 \times F_{700}/F_{678}$, the determination coefficient, $r^2$, and the root mean square error (RMSE) of Chl determination were 0.86 and 0.03 nmol cm$^{-2}$, respectively. For the analysis of AnC in Summer Red apples, 580 nm was taken as the diagnostic wavelength for AnC, since the $F_{\text{corr}}$-[AnC] correlation reaches its minimum here (Fig. 4, curve 4); for normalization, 700 nm was chosen, since this is the wavelength at which the $F_{\text{corr}}$-[Chl] correlation becomes large for AnC-free fruit (Fig. 3C, D, curve 1) and low for Summer Red apples (Fig. 4C, curve 3). With the fluorescence index in the form $[\text{Anth}] = -7.10 + 15.7 \times F_{700}/F_{580}$, $r^2$ and RMSE for AnC estimation turned out to be 0.88, and 5.0 nmol cm$^{-2}$, respectively. For estimating [Flv], the diagnostic wavelength was taken as 400 nm, and the normalizing wavelength as 440 nm (where the $F_{\text{corr}}$-[Fig] correlation was low for Chl, Car, and Flv). For AnC-free fruit with [Flv]$ < 100$ nmol cm$^{-2}$, values of 0.89 nmol cm$^{-2}$ and 9.2 nmol cm$^{-2}$ were found for $r^2$ and RMSE, respectively, by using the fluorescence index $[\text{Flv}] = -27.5 + 24.0 \times F_{440}$.

$F_{400}$. In this Flv content range with the power model the determination coefficient of 0.91 was achieved. Attempts to employ the fluorescence technique for the assessment of total [Car] in the spectral band 450–500 nm were unsuccessful.

Discussion

In line with previous investigations (Barnes et al., 2000; Bilger et al., 2001), this study was undertaken to consolidate the employment of Chl as an intrinsic probe for evaluating light screening efficiency in plants; analysis of whole apple fruit reflectance and Chl fluorescence was applied to samples widely differing in Chl, Car, Flv, and AnC content, due to differences in the degree of fruit ripeness and illumination during fruit development.
Although precise fruit prehistory was unknown, spectral reconstruction revealed a pool of extrathyaloid Car in each sample, characteristic of the ripening process (Merzlyak and Solovchenko, 2002; Solovchenko et al., 2005, 2006; Merzlyak, 2006). Furthermore, our previous observations make it plausible that the specimens with a high [Chl]/[Car] ratio represent mature fruit at the early stages of ripening whereas those with lower [Chl] but with enhanced [Car] are characteristic of advanced stages of ripening (Merzlyak and Solovchenko, 2002; Merzlyak et al., 2002; Solovchenko et al., 2005, 2006). A rich Flv content and a higher [Car]/[Chl] ratio is inherent to Granny Smith and Golden Delicious apples exposed to strong solar radiation during their growth as compared with shaded fruit (Merzlyak et al., 2002, 2005b; Solovchenko et al., 2006). As a response to strong sunlight (Saure, 1990), Summer Red apples accumulated considerable amounts of AnC on their sunlit surfaces. It should be noted that in sunlit fruit surfaces Flv (Granny Smith and Golden Delicious) and AnC (Summer Red) content exceeded those of Chl and Car by a factor of 10–100.

CFE spectra of apple fruit were structured and contained details which to a lesser degree were expressed in reflection spectra. In AnC-free apples in the green-red region where only Chla and b participate in light absorption, the CFE spectra were highly resolved. In fruit with low [Chl], fluorescence excitation maximum coincided with the main Chla absorption maximum at 678 nm and between 550 nm and 700 nm the shapes of the measured fluorescence spectra were close to the model spectra. The growth of Chl content was accompanied by remarkable changes in the shape of the spectra, the increase in fluorescence excitation ratio $F_{650}/F_{678}$ and the shift of maximum position towards shorter wavelengths in CFE spectrum reaching as large as 15–18 nm in apples with high Chl. Evidently together with an increase of normalized fluorescence in the green-orange and far-red regions, along with an increase in Chl, the spectral changes in the red involve re-absorption of Chl fluorescence also manifested in emission spectra of apple fruit (Ramos and Lagorio, 2006). Below 550 nm CFE, spectra of AnC-free apple fruit, in addition to the sharp maximum near 440 nm, showed Chla bands around 386 nm and 418 nm as shoulders not apparent in reflection spectra.

To determine spectral bands in CFE spectra sensitive to individual pigments, correlation analysis was carried out. It should be noted that apart from absorption of incident radiation by Flv and Car, the decrease in the $F_{650}/F_{678}$ correlation in AnC-free fruit results from scattering and could also be associated with a non-linear relationship between light absorption and pigment content. In particular, this effect appears as the gap near 678 nm in the correlation spectra between spectral reflection and Chl in apple fruit (Merzlyak et al., 2003). In addition, in fruit with high [Chl], fluorescence re-absorption brought about an apparent shift of the fluorescence maximum. These reasons account for the low $F_{650}/F_{678}$ correlation observed in the 650–690 nm band. With the exception of this band, spectral regions correlated linearly with Chl content were found in the narrow band near 700 nm, and in the green up to approximately 500 nm. Below 500 nm, where the $Q_{22}/$[Chl] correlation increased, the $Q_{22}/$[Chl] correlation underwent a considerable decline, as did the $F_{650}/$[Chl]. The $F_{650}/$[Car] correlation was consistently low, and with the cultivars studied here, the lower the [Chl]–[Car] correlation, the lower was the $F_{650}/$[Car] correlation. A further decrease of the $F_{650}/$[Chl] correlation occurred at wavelengths at which Flv exerted a dominant contribution on the reflection spectra and the negative $F_{650}/$[Flv] correlation was strong. In the 380–420 nm band, especially high $Q_{22}/$[Flv] correlation and $F_{650}/$[Flv] correlation, reaching the maximum near 410 nm, was found for Golden Delicious fruit, characteristic of sun-exposed fruit accumulating high amounts of vacuolar Flv (mainly quercetin-3-glycosides) (Solovchenko and Merzlyak, 2003; Merzlyak et al., 2005b; Hagen et al., 2006).

The CFE of sunlit and shaded fruit surfaces of Golden Delicious apples contrasting in Flv displayed a remarkable difference below 500 nm. Notably, in these fruit the influence of the Flv was pronounced even in the blue range of the spectrum. In sunlit versus shaded fruit Chl fluorescence intensity was markedly lower, especially between 360 nm and 400 nm, resulting in obliteration of Chla spectral features. In addition, the ‘shaded-to-sunlit’ ratio spectrum (Fig. 2B) revealed two bands near 455 nm and 485 nm attributable to Car (Gradinaru et al., 2000; Croce et al., 2001; Merzlyak and Solovchenko, 2002; Merzlyak et al., 2003), whose content was reported (Merzlyak et al., 2002; Ma and Cheng, 2004; Solovchenko et al., 2006) to increase in sun-exposed apple fruit. The modelling of CFE spectrum of a Golden Delicious fruit with an increased Flv content indicates that near 400 nm internal trapping by Flv (and, to some extent, also by Car) are responsible for an almost 8-fold decrease of Chl fluorescence.

Although the photoprotective role of AnC in apple fruit has been questioned (Ma and Cheng, 2004), the results of the present study show that these pigments exert an unmistakable effect on Chl fluorescence. The increase in [AnC] (mainly, cyanidin-3-galactoside) (Hagen et al., 2006) in Summer Red fruit was accompanied by a content-dependent decrease of Chl fluorescence in a broad band up to 650 nm, and at high AnC content only a weak Chla peak at 440 nm was detected (Fig. 4A, B). The effect of AnC was so strong that the correlation between Chl fluorescence and its content was very weak throughout the whole visible range. Our modelling of the CFE spectrum of a Summer Red fruit with a moderate content of AnC has revealed that the pigment exerts a large influence on spectral features between 450 nm and 650
nm, peaking near 530 nm where $\Psi(\lambda)$ approached 8. It is also remarkable that vacuolar Flv and AnC which at high concentrations govern optical properties in the near UV (Merzlyak et al., 2005b) and green (Merzlyak et al., 2003) spectral ranges, respectively, displayed similar (but with opposite sign) correlation with $Q(\lambda)$ and $F(\lambda)$.

In ripening fruit, Car (presumably those localizing in chloroplast plastoglobuli) (Merzlyak and Solovchenko, 2002) exerted a significant effect on CFE that was manifested by a reduction in the $F\text{-}[\text{Car}]$ correlation between 400 nm and 500 nm. According to our estimates, light trapping attributable to the extrathylakoid Car in the band 440–490 nm in unripe apples with high [Chl] was small (not shown), increased significantly at advanced stages of fruit ripening (Fig. 5A, D) and in sunlit fruit (Fig. 5B, E) when $\Psi(\lambda)$ was close to 2 and 3, respectively. It is noteworthy that model $F(\lambda)$ spectra contained poorly resolved and overlapping bands of Chlb and Car absorption between 450 nm and 490 nm inherent in the spectra of higher plant light harvesting pigment–protein complexes (Croce et al., 2000; Gradinaru et al., 2000) and $P_T(\lambda)$ pool. By contrast, recorded CFE spectra contained distinct maxima near 464–472 nm and 485–490 nm (Figs 2, 5). The modelling of the spectra of AnC-free fruit strongly suggests that the fluorescence in this band resulted mainly from the overlapping absorption by $P_X(\lambda)$ and $P_T(\lambda)$ pigment pools. As could be seen from Fig. 5, the shapes of the spectra, the ratios between maxima and the positions of maxima in the bands of combined Chl and Car absorption were, to a large extent, reproduced in the model spectra.

Together with reflection spectroscopy, Chl fluorescence is a promising tool for rapid and non-destructive analysis of pigments able to compete with Chl for light absorption (Cerovic et al., 2002; Agati et al., 2005; Bengtsson et al., 2005; Hagen et al., 2006; Barthod et al., 2007). The analysis carried out in this study showed that, in AnC-free apples, Chl content itself could be determined from CFE analysis carried out in this study showed that, in AnC-free cultivars, to the red regions of the visible spectrum. In extending from UV to the green and, in AnC-accumulating cultivars, to the red regions of the visible spectrum. In conclusion, it should also be noted that spectrophotometric analysis and the possibilities of reconstruction of pigment contributions in total spectral absorption provide opportunities for quantitative evaluation of efficiency of the photoprotective pigment in intact tissues.

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**References**


