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Reflectance spectral features and non-destructive estimation of chlorophyll, carotenoid and anthocyanin content in apple fruit

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Abstract

Fruit reflectance spectra of five apple (*Malus domestica* Borkh.) cultivars (Zhigulevskoe, Antonovka, Granny Smith, Golden Delicious and Renet Simirenko) with a wide range of peel pigment (chlorophylls *a* and *b*, carotenoids and anthocyanins) content have been studied to develop non-destructive techniques for pigment assessment. In addition to chlorophylls, positions of in vivo absorption maxima were established for carotenoids (480, 455 and a shoulder at 425 nm) and for anthocyanins (near 550 nm). In anthocyanin-free fruit, a close relationship between reflectance at 550 nm (R_{550}) and 700 nm (R_{700}) has been found ($r^2 > 0.95$). In fruit with chlorophyll content more than 5 nmol/cm^2, the reflectance near 678 nm was insensitive to a variation in chlorophylls, whereas, reflectance in the bands 550–650 nm and 690–705 nm remained sensitive to chlorophyll content in a wide range of its variation. The reflectance ratios, R_{800}/R_{700} and R_{800}/R_{640}, were directly proportional to total chlorophyll content ranging from 0.4 to 11 nmol/cm^2 ($r^2 > 0.93$). The reflectance in the band 520–530 nm was found to be dependent mostly on carotenoids absorption. The index $R_{800}(1/R_{520} - 1/R_{700})$ was suggested for estimation of carotenoid content in the range 0.6–4.5 nmol/cm^2. The index for assessment of a carotenoid/chlorophyll ratio was proposed in the form, $(R_{480} - R_{678})/R_{800}$. Reflectance in the green region of the spectrum proved to be sensitive to anthocyanin content. The index $R_{800}(1/R_{550} - 1/R_{700})$ was developed to estimate anthocyanin content in peel ranging from 2.5 to 50 nmol/cm^2; the determination coefficient of the index with anthocyanin content was higher than 0.93.

Keywords: Anthocyanins; Apple fruit; Carotenoids; Chlorophyll; Non-destructive determination; Reflectance

1. Introduction

The content of chlorophylls, carotenoids and anthocyanins as well as their proportions determine fruit color and appearance (Saure, 1990; Abbott, 1999) and serve as markers of quality. Pigment changes occur during ripening, storage and as a result of various stresses (Knee, 1972, 1988; Chuma et al., 1981; Merzlyak et al., 1997, 1999; Abbott, 1999; Merzlyak and Chivkunova, 2000; Merzlyak et al., 2002). The pigments in fruit fulfil several important roles. In apples, peel

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chloroplasts, containing the main part of fruit chlorophyll and carotenoids, are competent in photosynthesis with efficacy comparable with that in leaves (Blanke and Lenz, 1989). Carotenoids participate in light harvesting and are recognized as powerful antioxidants, and excited states and singlet oxygen quenchers are involved in photoprotection (Palett and Young, 1993; Edge et al., 1997). Anthocyanins are responsible for the red color of apples; such fruit are more attractive for consumers and usually preferable at the market (Saure, 1990; Lancaster et al., 1994; Reay, 1999). Some lines of evidence suggest that anthocyanins are also involved in the protection of fruit against harmful UV and excessive sun irradiation (Chalker-Scott, 1999; Smillie and Hetherington, 1999; Merzlyak and Chivkunova, 2000; Merzlyak et al., 2002).

An obvious advantage of non-destructive optical techniques is that they allow one to perform successive measurements of a sample, providing valuable information about the pattern of pigment changes and their state in plant tissues (Knee, 1980; Williams and Norris, 1987; Gitelson and Merzlyak, 1996, 1998; Penuelas and Filella, 1998; Abbott, 1999; Gamon and Surfus, 1999; Gitelson et al., 2001, 2002). The studies performed in this laboratory with apples indicated remarkable changes in spectral reflectance occurring during ripening on the tree, maturation and storage (Merzlyak et al., 1997, 1999), acclimation to high-light stress (Merzlyak and Chivkunova, 2000; Merzlyak et al., 1998, 2002), sun scald development (Merzlyak et al., 2002) and superficial scald-induced browning (Chivkunova et al., 2001). In addition, color and spectral reflectance changes are the basis for fruit sorting in commercial grades (Chuma et al., 1981; Abbott, 1999). Further progress in these fields requires a comprehensive understanding of fruit tissue optical features to obtain quantitative information from spectral measurements.

The chemical methods routinely in use for chlorophyll, carotenoid and anthocyanin analysis in fruit, presuming the loss of the material under study, are expensive and time-consuming, involve some artifacts related to pigment instability and those arising during extraction and possess low sensitivity (see Solovchenko et al., 2001). The application of optical spectroscopy of intact plant tissues is a potent alternative to chemical analysis (Gitelson and Merzlyak, 1997; Penuelas and Filella, 1998; Gamon and Surfus, 1999; Merzlyak et al., 1999; Gitelson et al., 2001, 2002). However, to the best of our knowledge, very little information on the use of reflectance spectroscopy for pigment assessment in fruit is available in the literature. Knee (1980) suggested employing reflectance at 675 nm to estimate peel chlorophyll content in Cox Orange Pippin apple. Chuma et al. (1981) found a correlation between the magnitude of reflectance minimum in the red and chlorophyll content and used log R_{680} for chlorophyll content estimation as well as for ripeness and internal quality assessment in orange fruit.

The objective of the present study was to investigate reflectance spectral properties of apple and to develop techniques for non-destructive pigment assessment in the wide ranges of their content in apple fruit. In order to find the spectral signatures of chlorophyll, carotenoid and anthocyanins in apple fruit reflectance, we employed the approaches developed in our previous studies with plant leaves (Gitelson and Merzlyak, 1994, 1996; Gitelson and Merzlyak, 1998; Merzlyak et al., 1999; Gitelson et al., 2001, 2002). As a result, in vivo absorption maxima of chlorophyll, carotenoids and anthocyanins in apple fruit were established, specific wavebands sensitive to pigment contents were found and spectral reflectance indices for non-destructive determination of the pigment content were suggested.

2. Materials and methods

2.1. Fruit

Healthy fruit without symptoms of damage of five apple (*Malus domestica* Borkh.) cultivars were used to cover a wide range of peel pigment content and composition. The cultivars used were Antonovka, n (the number of fruit) = 50, Zhigulevskoe ($n = 82$), Granny Smith ($n = 12$), Golden Delicious

($n = 11$) and Renet Simirenko ($n = 12$). The fruit were grown at the Botanical Garden of Moscow State University (Antonovka-2000) or obtained from Michurinsk, in the Tambov region, Russia (Antonovka, Zhigulevskoe in 1999–2000). Mature fruit were studied immediately after picking or stored for 1–4 months at 4 °C in common air atmosphere prior to examination.

Granny Smith, Golden Delicious and Renet Simirenko apples were delivered from the Krasnodar region, Russia, (August 2000) and studied within 2–3 weeks of storage at 4 °C. Granny Smith and Renet Simirenko fruit possessed green coloration; Antonovka, Golden Delicious and the shaded sides of Zhigulevskoe fruit were pale green or yellowish. Sunlit (exposed to sun throughout the growth period) sides of Zhigulevskoe fruit possessed red pigmentation due to accumulation of anthocyanins.

2.2. Spectral reflectance measurements

Diffuse reflectance spectra of whole apple fruit were recorded in a range of 400–800 nm with a 150–20 Hitachi (Japan) spectrophotometer equipped with an integrating sphere attachment (internal diameter 150 mm, part 150-0901) against barium sulfate as a standard. Spectral data were interfaced to a personal computer for further processing.

2.3. Pigments analysis

The pigments were quantitatively determined in the peel from the same fruit surfaces used for taking the spectra. An extraction procedure providing both removal of interfering impurities and a high sensitivity of carotenoid and chlorophyll analysis was used (Solovchenko et al., 2001). A total of 4–10 disks (0.55 cm in diameter and ca. 0.1 cm thick) were cut from the apple peel, rinsed twice in chloroform for 1 min to remove epicuticular lipids and then ground with a pestle and mortar in approximately 5 ml chloroform–methanol (2:1 v/v) in the presence of 100 mg of MgO (200 mg in the case of immature fruit) to prevent pheophytization. Water then was added to the filtered extract (up to one fifth of the total extract volume). After 10 min centrifugation at $3000 \times g$, chlorophyll *a*, chlorophyll *b* and carotenoids were determined in the chloroform phase of the extracts using absorption coefficients reported by Wellburn (1994). A molecular weight of 570 for carotenoid was assumed. Anthocyanins were assayed in the water–methanol phase of the extract after acidification with concentrated HCl, final concentration of 0.1%. To complete anthocyanin extraction, dry material obtained after Folch's extraction was reextracted with methanol containing 0.1% HCl. Total anthocyanin content of the sample was calculated as a sum of pigment concentrations in the water–methanol phase of the extract and in the acidic methanol extract. For anthocyanin quantification, an absorption coefficient of 30 l/mmol per cm at 530 nm (Strack and Wray, 1989) was accepted.

3. Results

3.1. Peel pigment content

In the apple fruit studied, chlorophyll content varied from 0.2 nmol/cm^2 in nearly yellow fruit to 11 nmol/cm^2 in green fruit. Granny Smith fruit possessed the highest chlorophyll content. Renet Simirenko fruit peel also contained a relatively high amount of chlorophyll (see Fig. 1A). Mature Antonovka and Golden Delicious fruit possessed approximately three times lower chlorophyll content, and the pigment content decreased significantly during ripening and storage.

Synchronous changes of chlorophyll *a* and *b* content ($r^2 = 0.99$) were found to be characteristic for all apple fruit studied. For the data set, a molar chlorophyll *a* to *b* ratio was 2.35 ± 0.25. The presence of anthocyanins in Zhigulevskoe apples did not affect this relationship (Fig. 1A).

In fruit with anthocyanin content < 1 nmol/cm^2, the highest total carotenoid content was recorded in Granny Smith and Renet Simirenko fruit, but those cultivars also had the smallest carotenoid to chlorophyll ratios, 0.45 ± 0.09 and 0.46 ± 0.04, respectively. Antonovka and Golden

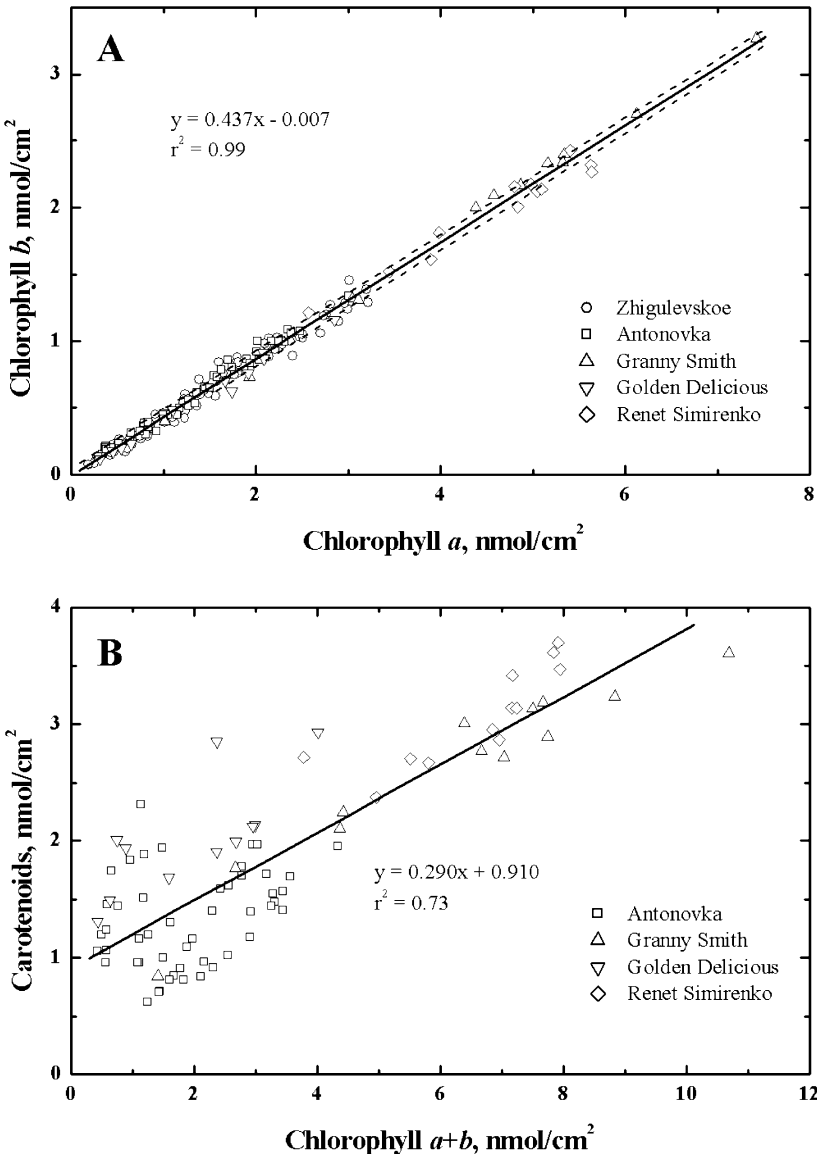

Fig. 1. Relationships between chlorophyll *b* and chlorophyll *a* (A), carotenoids and total chlorophyll in the peel of anthocyanin-free apple fruit (B) of the different cultivars studied.

Delicious peel possessed approximately two times lower chlorophyll content but higher carotenoid to chlorophyll ratios, 0.94 ± 0.68 and 1.48 ± 0.87, respectively. When chlorophyll decreased from 11 to 3 nmol/cm^2, a gradual decrease in carotenoid content was observed in the fruit. However, for chlorophyll content below 3 nmol/cm^2, the carotenoid level remained relatively high (Fig. 1B). Such a relationship between pigment contents reflects the phenomenon of carotenoid retention and/or accumulation in the progress of apple fruit ripening.

In red Zhigulevskoe apple, anthocyanin content varied from 1 to 50 nmol/cm^2 in dark-red fruit, whereas, chlorophyll content was in the range 0.4–4.6 nmol/cm^2.

3.2. Fruit reflectance spectra

Apple fruit with anthocyanin content below 1 nmol/cm^2 (referred to as anthocyanin-free fruit) and anthocyanin-containing fruit (sunlit sides of cv. Zhigulevskoe), which appeared as pink-to-red with higher anthocyanin content, were analyzed separately (Figs. 1–3). The average spectra calculated for fruit subgroups with variable chlorophyll and anthocyanin content are presented in Fig. 2A and B, respectively.

For all apple cultivars (Figs. 2 and 3A), maximal and slightly variable reflectance was observed in the near infra red (NIR) region of the spectrum (79.4 ± 3.52% at 800 nm). In the red region, a broad band of chlorophyll *a* (a distinct minimum of reflectance near 678 nm) and chlorophyll *b* absorption (a shoulder near 650 nm) were presented in both anthocyanin-free and anthocyanin-containing fruit (Fig. 2, see also Merzlyak et al., 1999; Merzlyak and Chivkunova, 2000). As can be seen from Fig. 2A, Fig. 3A, with an increase of chlorophyll content of up to 3–5 nmol/cm^2, reflectance at 678 nm dropped sharply and remained almost invariant at higher chlorophyll content (see also Knee, 1980). In contrast, reflectance at 700 nm underwent a gradual monotonous decrease with an increase in chlorophyll content.

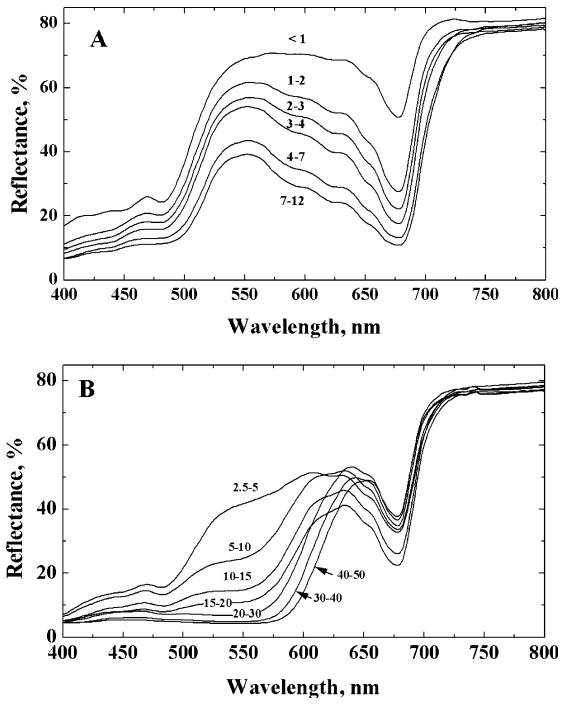

Fig. 2. Average reflectance spectra of anthocyanin-free green to yellow–green apple fruit (A) and Zhigulevskoe apples accumulating anthocyanins on sun-exposed sides of the fruit (B). Numbers indicate the ranges of chlorophyll (Fig. 2A) and anthocyanin (Fig. 2B) content (nmol/cm^2) in the peel of the fruit.

In the blue, where both chlorophylls and carotenoids absorb, reflectance of green-to-yellow apple fruit was low, especially at high chlorophyll content (Fig. 2A). In fruit with extremely low chlorophyll content (upper curve on the Fig. 2A), some spectral details could be attributed to carotenoids and, to a less extent, to chlorophyll *b* absorption (see also Merzlyak et al., 1999; Merzlyak and Chivkunova, 2000).

As a result of the accumulation of low amounts of anthocyanins (2.5–15 nmol/cm^2) in Zhigulevskoe fruit, spectral reflectance in the green showed a gradual decrease with the appearance of a shoulder near 550 nm (Fig. 2B). A further increase in anthocyanin content brought about a substantial fall of reflectance in the range between 675 and 400 nm reaching 4.5–5%; for anthocyanin > 30 nmol/cm^2, reflectance was even less than in anthocyanin-free fruit with high chlorophyll content (cf. panels A and B in Fig. 2) and without noticeable spectral details. The increase in anthocyanin content also resulted in a progressive broadening of the band with minimal reflectance and the shift of the green edge position toward longer wavelengths (Fig. 2B). In spite of dramatic changes induced by anthocyanin in the range 625–400 nm, reflectance in the red and NIR regions was not changed and remained close to that in green-to-yellow fruit.

In anthocyanin-free fruit, as in leaves of diverse plants (Gitelson and Merzlyak, 1998), reflectances at 550 and 700 nm correlated closely ($r^2 = 0.95$, Fig. 3B) regardless of chlorophyll content. In contrast, in anthocyanin-containing apple fruit, reflectance at 550 nm was considerably lower compared with that at 700 nm. As a result of anthocyanin absorption, a strong correlation of R_{550} and R_{700} was lost in red fruit (Fig. 3B).

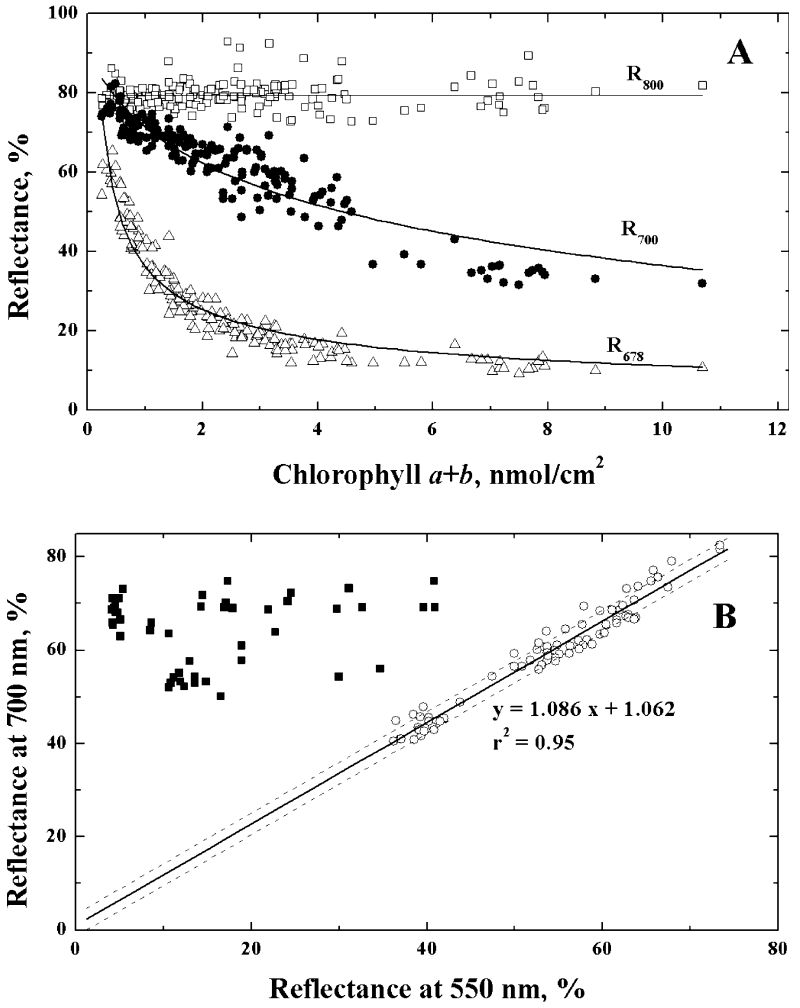

Fig. 3. Reflectances at 678, 700 and 800 nm vs. chlorophyll content (A), and reflectance at 700 nm versus reflectance at 550 nm (B) in apple fruit. (B) Circles and squares are anthocyanin-free and anthocyanin-containing fruit, respectively. Solid lines represent the best-fit functions; dashed lines represent the standard deviation (STD). For green to green–yellow fruit, R_{550} vs. R_{700} is linear with a determination coefficient higher than 0.95, whereas, for anthocyanin-containing fruit, $R_{550} < R_{700}$ and the linear relationship between them was disturbed.

3.3. Signature analysis of reflectance spectra and indices for pigment assessment

3.3.1. Chlorophylls

We found in our previous studies that reciprocal reflectances at wavelengths out of the pigment absorption maxima could be successfully used for assessment of chlorophyll content in leaves of a number plant species (Gitelson and Merzlyak, 1996; Gitelson and Merzlyak, 1998). We tested these techniques for apple fruit.

Spectra of the determination coefficient (r^2) of the relationship between reciprocal reflectance, $R_{800}/R(\lambda)$, and chlorophyll content in apple peel are shown in Fig. 4A for yellow-to-green apples and in Fig. 4B for reddish apples (Gitelson and Merzlyak, 1994, 1996, 1997). Very low correlation was observed in the NIR (between 750 and 800 nm) for all cultivars studied. For chlorophyll > 3

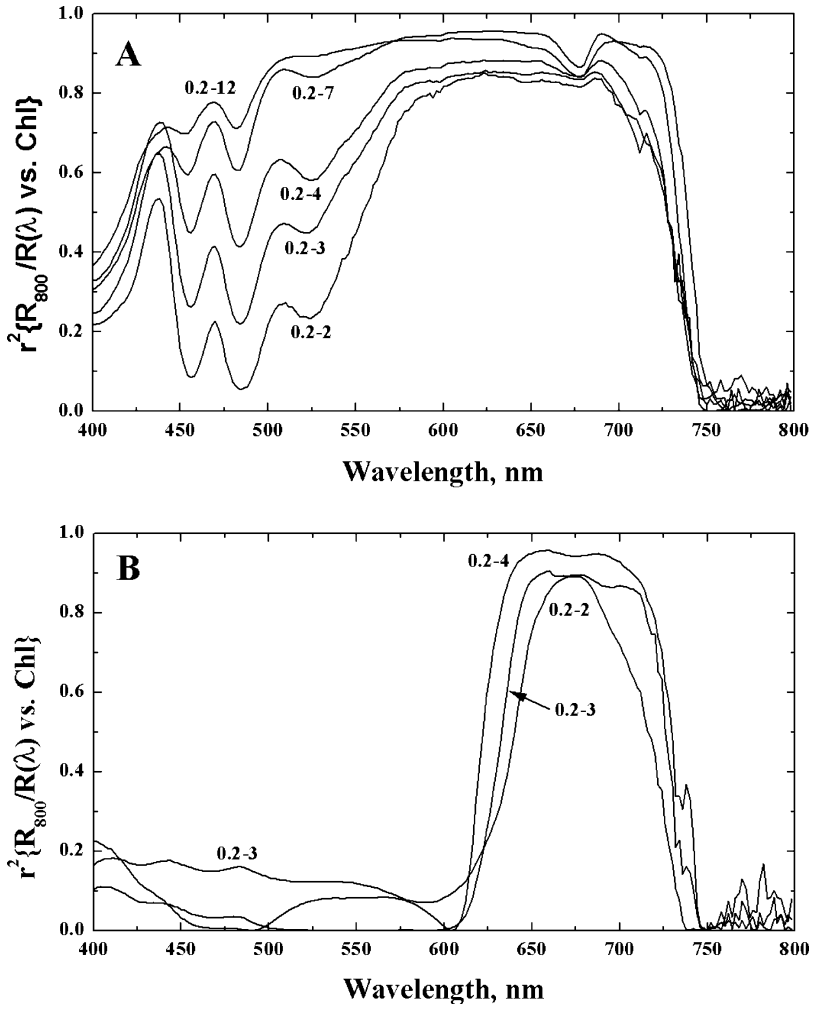

Fig. 4. The spectra of the determination coefficient of the relationship between $R_{800}/R(\lambda)$ and chlorophyll content for green-to-yellow (A) and red (B) apple fruit. Chlorophylls content ranges (nmol/cm^2) are indicated on each curve.

nmol/cm^2, a broad flat maximum of the correlation ($r^2 = 0.8–0.9$) was observed in the orange–red region of the spectrum between 600 and 710 nm in anthocyanin-free fruit, and between 650 and 710 nm for anthocyanin-containing fruit. In anthocyanin-containing fruit, a sharp decrease of r^2 occurred at wavelengths shorter than 640 nm. For anthocyanin-free fruit, correlation with chlorophyll content in the blue region was lower than in the 600–700 nm range; however, two prominent maxima of the determination coefficient at 430–440 and 460–470 nm attributable to chlorophyll *a* and *b*, respectively, were found. At wavelengths below 430 nm, r^2 underwent a sharp decrease. In anthocyanin-containing fruit, reciprocal reflectance in the 400–600 nm range showed a weak correlation with peel chlorophyll content (Fig. 4B), and determination coefficient spectra in this band contained no spectral details.

Reciprocal reflectances in the red, corrected to that in the NIR, might be used for chlorophyll assessment in apple fruit. The index, R_{800}/R_{678}, where a reflectance minimum at 678 nm was used as a term sensitive to chlorophyll, was found to be

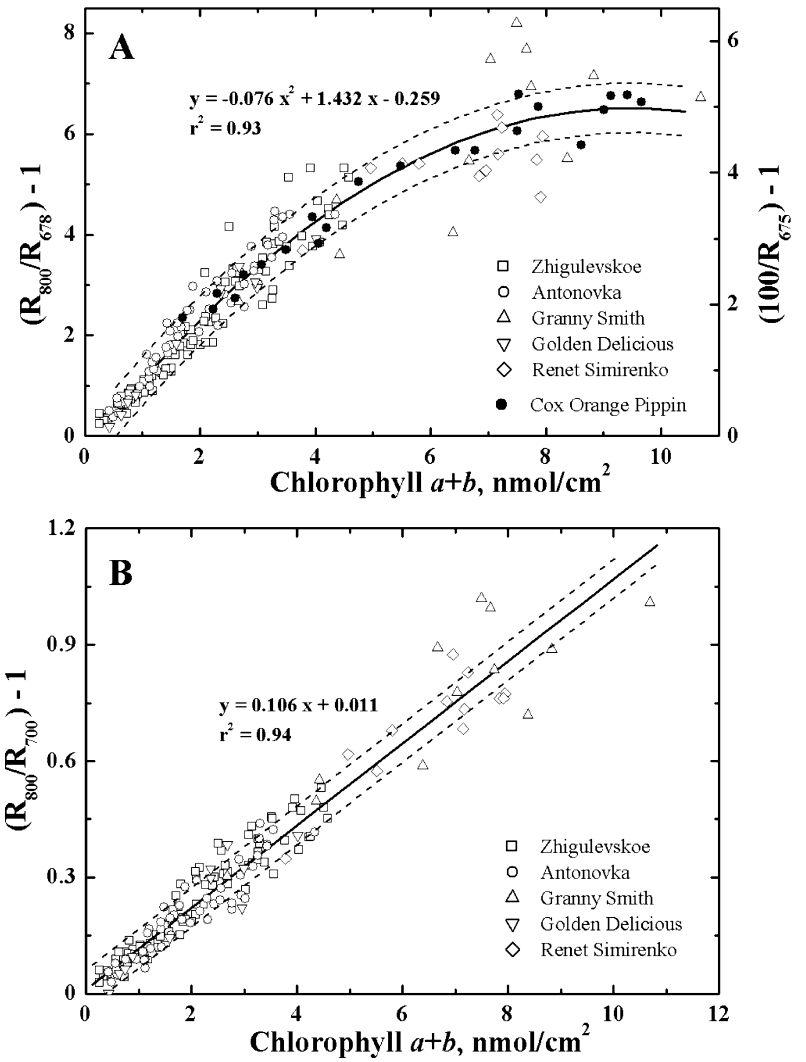

Fig. 5. Reflectance indices (R_{800}/R_{678})−1 and ($100/R_{675}$)−1 (A), and (R_{800}/R_{700})−1 (B) plotted vs. peel chlorophyll content in apple fruit studied. Solid lines represent the best-fit functions; dashed lines represent the standard deviation (STD). (A) The index, (R_{800}/R_{678})−1, is plotted on the left scale for the apple cultivars studied. The index, ($100/R_{675}$)−1, was calculated for Cox Orange Pippin apples from the data by Knee (1980) and is shown on the right scale.

linearly related to chlorophyll content in the range 0.4–5 nmol/cm^2 (Fig. 5A, Table 1). When chlorophyll exceeded 6 nmol/cm^2, the index was almost insensitive to chlorophyll content because of saturation of the relationship 'R_{678} versus chlorophyll' (Fig. 5A). This is consistent with findings by Knee (Fig. 2 in Knee (1980)) for Cox Orange Pippin apples.

In accordance with our previous studies (Gitelson and Merzlyak, 1994, 1996, 1997) and signature analysis (Fig. 4), we suggest using for chlorophyll estimation reciprocal reflectances at 640 and 700 nm, which are quite far from the region of strong pigment absorption. Two indices, R_{800}/R_{700} and R_{800}/R_{640}, showed high correlation with chlorophyll content ($r^2 = 0.93$) in a wide range of the

Table 1
The reflectance indices for pigment content estimation in apple fruit in form, [Index] = $a+b$* [Pigment content, nmol/cm^2]

Index	Pigment content range (nmol/cm^2)	Parameters				
		n	r^2	a	b	Estimation error of pigment determination (nmol/cm^2)
Chlorophylls						
$R_{800}/R_{678}-1$	0.3–5	148	0.92	0.56	0.86	0.36
	0.3–11*	166	0.93	–	–	0.49
$R_{800}/R_{700}-1$	0.2–11	166	0.94	0.011	0.106	0.054
$R_{800}/R_{640}-1$	0.3–11	132	0.93	0.11	0.31	0.16
Carotenoids						
$R_{800}/R_{520}-R_{800}/R_{550}$	0.6–4.5	79	0.83	0.06	0.20	0.08
$R_{800}/R_{520}-R_{800}/R_{700}$	0.6–4.5	79	0.80	0.13	0.23	0.10
Anthocyanins						
$R_{800}/R_{550}-R_{800}/R_{700}$	5.0–48.1	23	0.93	−0.51	0.41	1.59
Carotenoids-to-Chlorophylls ratio						
$(R_{500}-R_{678})/R_{800}$	0.34–2.65	78	0.88	−0.14	0.16	0.03
$(R_{480}-R_{678})/R_{800}$	0.34–2.65	78	0.94	−0.09	0.20	0.02

Non-linear regression in form, [Index] = $a+b$ [Pigment content, nmol/cm^2] + c*(Index)^2 ($a=-0.26$, $b=1.43$, $c=-0.08$). r^2 is determination coefficient, all correlations are significant at $P<0.001$, n is the number of fruit studied.

pigment variation for the fruit of all cultivars studied including those containing anthocyanins (Fig. 5B, Table 1).

3.3.2. Carotenoids to chlorophylls ratio and carotenoids content

To estimate the contribution of carotenoids to reflectance spectra, one needs to remove a significant effect of chlorophyll absorption. Normalization of reciprocal reflectance to reflectance at 678 nm (red chlorophyll absorption band) removes the chlorophyll effect to a certain degree; thus, the function $(R_{800}/R_\lambda)/R_{678}$ depends on other factors, but chlorophyll, and it was almost invariant relative to chlorophyll content (see references in Gitelson et al., 2002). Spectra $(R_{800}/R_\lambda)/R_{678}$ of the apple fruit for carotenoid ranging from 0.6 to 2.0, 0.6 to 3.0, and 0.6 to 4.0 nmol/cm^2 are shown in Fig. 6. The spectral curves were close in the green to the red region, suggesting nearly the same contribution of chlorophyll *a* and chlorophyll *b* to the function $(R_{800}/R_\lambda)/R_{678}$ for fruit with different peel carotenoid content. In the region of combined chlorophyll and carotenoid absorption (400–550 nm), the spectra were slightly different in magnitude (increasing with an increase in carotenoid content) and possessed similar spectral features: a band near 460 nm, a shoulder at 450–460 nm and an increase at shorter wavelengths (Fig. 6, curves 1–3). Thus, the spectra of $(R_{800}/R_\lambda)/R_{678}$ as well as the standard deviation (STD) of these spectra (Fig. 6A) manifest spectral features of carotenoids. The shape of the bands and the positions of maxima near 425 (as a shoulder), 460 and 485 nm in the STD spectrum (Fig. 6A) are consistent with carotenoid absorption. An increase of the STD at wavelengths shorter than 420 nm, however, suggests the contribution of a compound(s) different from both chlorophyll and carotenoids.

Previously, we found that the rates and onset of both fruit ripening and leaf senescence could be estimated using reflectances in the bands of strong absorption by chlorophylls and carotenoids with the Plant Senescence Reflectance Index (PSRI), $(R_{678}-R_{500})/R_{NIR}$. In leaves, this index was correlated with changes in the carotenoid to chlorophyll ratio (Merzlyak et al., 1999). Fig. 6B and Table 1 show the relationship of the index, $(R_{678}-R_{500})/R_{800}$, versus the carotenoid to chlorophyll molar ratio in apple fruit peel. The index allowed reliable assessment of the carotenoid to chloro-

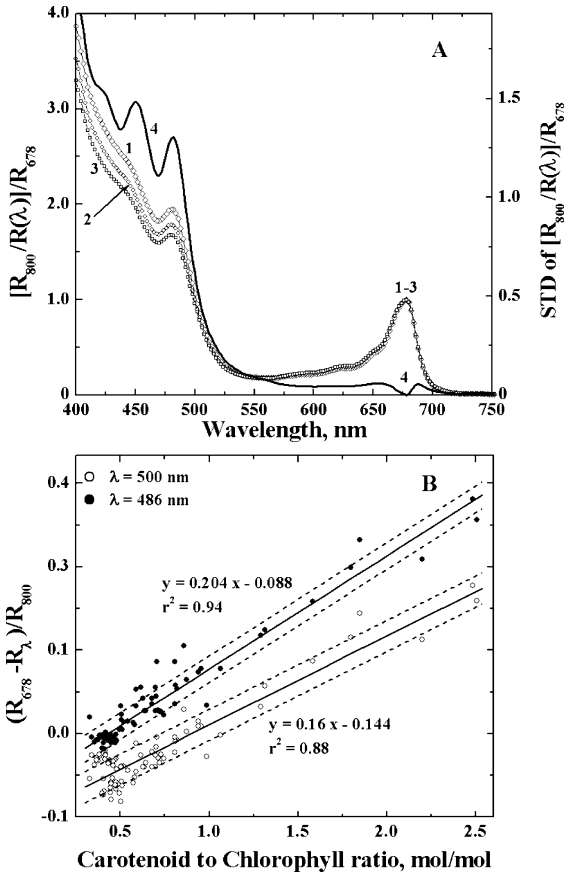

Fig. 6. The average spectra $[R_{800}/R(\lambda)]/R_{678}$ (left scale) with different ranges of carotenoids, their standard deviation (STD; right scale) spectrum (A) and the relationships of Plant Senescence Reflectance Indices (PSRI), $(R_{678}-R_{500})/R_{800}$ and $(R_{678}-R_{486})/R_{800}$, vs. the carotenoids-to-chlorophylls molar ratio. Anthocyanin-free apple fruit. (A) Spectra 1, 2 and 3 are presented for fruit with carotenoid content ranging from 0 to 1, 0 to 3 and 0 to 4 nmol/cm^2, respectively. (B) Solid lines in B represent the best-fit function; dashed lines represent the STD.

phyll ratio ($r^2 = 0.88$) in a wide range of its changes in anthocyanin-free fruit. The reflectance in the long-wave carotenoid maximum at 486 nm (Fig. 6A) was also used as a term in PSRI. The index, $(R_{678}-R_{486})/R_{800}$, was found to be linearly correlated with the carotenoid to chlorophyll ratio ($r^2 = 0.94$) and more sensitive to the carotenoid to chlorophyll ratio than that with R_{500} (Fig. 6B, Table 1).

To reveal bands sensitive to carotenoid content on the variable chlorophyll absorption background, determination coefficient spectra of the relationship 'R_{800}/R_λ versus carotenoid content' were calculated for fruit with different chlorophyll content (Fig. 7A). A very low correlation with carotenoid content was found in the NIR region of the spectrum. In the blue region, prominent maxima attributable to carotenoid absorption were situated near 520, 480 and 455 nm and at a shoulder near 430 nm. The maxima were mostly evident in fruit with low chlorophyll content. With an increase in chlorophyll content, these maxima underwent progressive flattening, and a weak

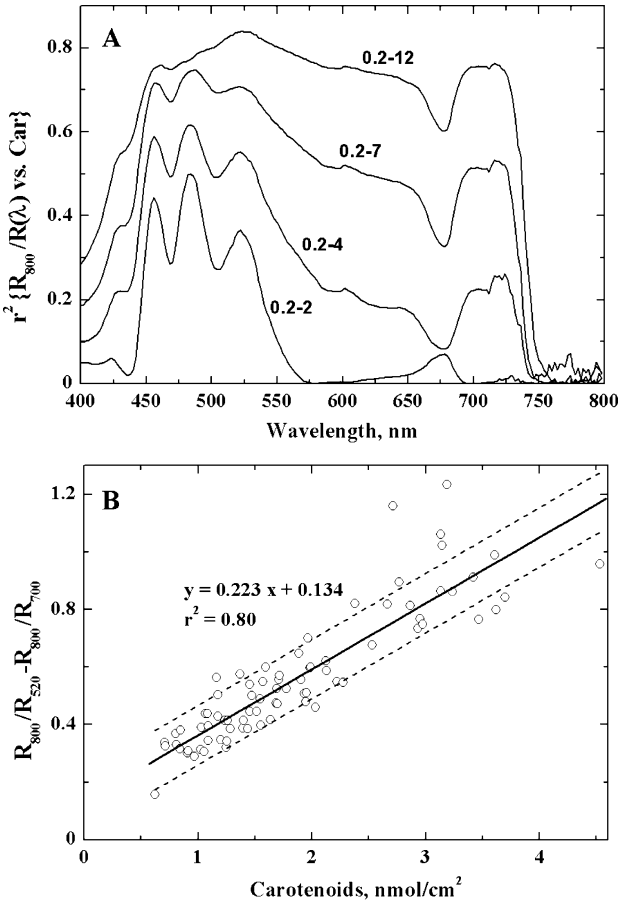

Fig. 7. (A) The determination coefficient spectra of the relationship 'reflectance ratio $R_{800}/R(\lambda)$ vs. carotenoids content' for fruit with different chlorophyll content. Numbers indicate the chlorophyll content range (nmol/cm^2). (B) The reflectance index $R_{800}(1/R_{520}-1/R_{700})$ versus peel carotenoid content for anthocyanin-free fruit. The solid line represents the best-fit function; the dashed lines represent the STD.

correlation with carotenoid content was found between 430 and 400 nm. For the entire data set including fruit with high chlorophyll content, the r^2 maximum was located near 520 nm.

Since reflectance at 520 nm is dependent on both carotenoid and chlorophyll absorption, compensation for the contribution of chlorophyll absorption to the reflectance spectrum is necessary for carotenoid estimation. Reciprocal reflectance at 700 nm as well as that at 550 nm closely correlated with chlorophyll content (Fig. 3B, Fig. 5B) and could serve as measures of chlorophyll contribution at 520 nm. Thus, the difference between reciprocal reflectances at 520 and 700 nm (or 550 nm) can be used as a measure of carotenoid content (Gitelson et al., 2002). The index in the form $R_{800}(1/R_{520}-1/R_{700})$ exhibited a linear correlation ($r^2=0.80$) with carotenoid content, providing reliable assessment of carotenoids ranging from 0.6 to 4.5 nmol/cm^2 (Fig. 7B, Table 1).

3.3.3. Anthocyanins

As can be seen from Fig. 2B, Fig. 8A, anthocyanin absorption induced a dramatic decrease of the reflectance in the green region of the spectrum (Fig. 2B). The presence of anthocyanins (even in low amounts) disturbs a close correlation 'R_{550} versus R_{700}' characteristic of anthocyanin-free fruit (Fig. 3B). Employing the signature analysis of reflectance spectra presented in Fig. 2B as was carried out previously (Fig. 6), spectral features of anthocyanins and carotenoids appeared in the STD of function $(R_{800}/R_\lambda)/R_{678}$ (Fig. 8A). Attributable to carotenoids, absorption maxima at 455 and 480 nm could be seen in fruit with low-to-moderate anthocyanin content (Fig. 8A). In the spectra of fruit with high anthocyanin content, the maximum of reflectance variation attributable to anthocyanins (cf. panels A and B in Fig. 2; see also Merzlyak and Chivkunova, 2000) was observed in the green region of the spectrum around 530–550 nm (Fig. 8A). The position and magnitude of the STD maximum depended on the anthocyanin content range. The variation of function $(R_{800}/R_\lambda)/R_{678}$ in the green increased with an increase of anthocyanin content up to 20 nmol/cm^2. Furthermore, an increase in the pigment content was accompanied by a decrease of the STD peak, its

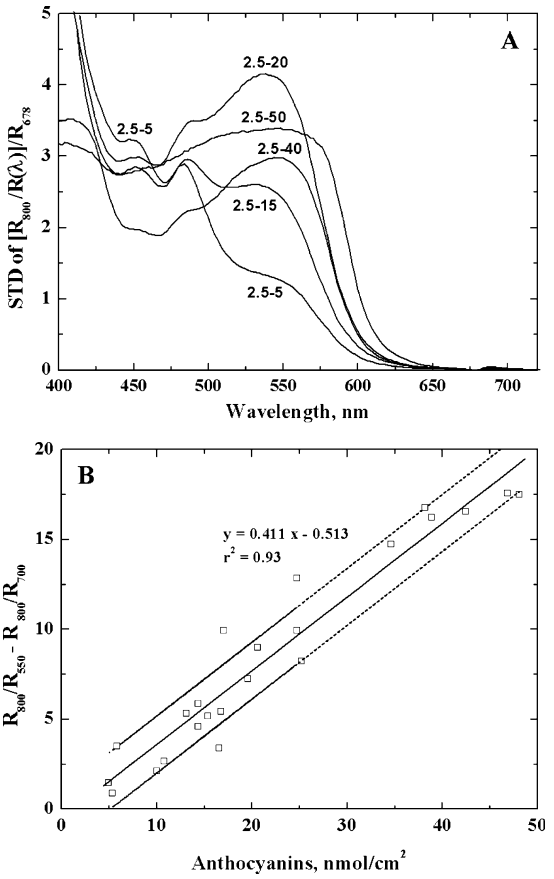

Fig. 8. The STD spectra of the function $[R_{800}/R(\lambda)]/R_{678}$ for fruit with various anthocyanin content (A) and the relationship of the reflectance ratio $R_{800}(1/R_{550}-1/R_{700})$ versus the anthocyanin content (B). (A) Anthocyanin content indicated in nmol/cm^2 near the curves. (B) The solid line represents the best-fit function; dashed lines represent the STD.

flattening and a shift of the maximum toward longer wavelengths. For anthocyanin content as high as ca 50 nmol/cm^2, anthocyanins possessed a weak (if any) absorption between 650 and 750 nm.

To devise an index for anthocyanin assessment, it is essential to find a spectral band where reflectance depends primarily upon anthocyanin content and minimally on other pigment. According to the data presented in Figs. 6 and 8A for anthocyanin-free fruit, the strong influence of carotenoid absorption between 420 and 500 nm decreased significantly at longer wavelengths. At 550 nm, near the anthocyanin absorption maximum in vivo (Lancaster et al., 1994; Merzlyak and

Chivkunova, 2000), the contribution of carotenoid to reflectance was low (Fig. 6). Chlorophyll did contribute to reflectance at 550 nm; this contribution was significant especially in the case of fruit with low anthocyanin content against the high background of chlorophyll (lower curves in Fig. 8A). In the green fruit, both $(R_{550})^{-1}$ and $(R_{700})^{-1}$ depended mostly on chlorophyll ($r^2 > 0.90$), whereas, in the red fruit, $(R_{550})^{-1}$ was governed by the combined anthocyanin and chlorophyll absorption (Fig. 3B). To remove the contribution of chlorophyll absorption at 550 nm, reciprocal reflectance at 700 nm, $(R_{700})^{-1}$, was subtracted from $(R_{550})^{-1}$; the difference $[(R_{550})^{-1} - (R_{700})^{-1}]$ was dependent mostly upon the anthocyanin content (Gitelson et al., 2001). To compensate for the NIR reflectance, R_{800} was introduced in the index in the form $R^*_{800}(1/R_{550} - 1/R_{700})$, which we suggest for anthocyanin estimation. The index correlated closely ($r^2 = 0.93$) with anthocyanins ranging from 2.5 to 50 nmol/cm^2 (Fig. 8B, Table 1) regardless of the fruit chlorophyll and carotenoid content.

4. Discussion

In spite of complicated morphological, anatomical and optical properties and the fact that our understanding of the contribution of various pigments into light absorption is still insufficient, considerable progress was achieved during the last 10 years in the development of non-destructive techniques for the assessment of pigment content in plant leaves (Gitelson and Merzlyak, 1994, 1996, 1998; Penuelas and Filella, 1998; Gamon and Surfus, 1999; Merzlyak et al., 1999; Gitelson et al., 2001, 2002). An attempt has been undertaken in this study to develop techniques for the assessment of major pigments in apple fruit.

Non-destructive assessment of pigments in apple fruit peel is complicated by obstacles including overlapping light absorption by individual pigments and the non-linear relationship of reflectance versus pigment content in the bands of strong absorption (Figs. 2 and 3). A close correlation between carotenoids and chlorophyll $a+b$ and between chlorophyll a and chlorophyll b is inherent in apple fruit as well as high carotenoid to chlorophyll and chlorophyll b to chlorophyll a ratios (Fig. 1; see also Blanke and Lenz, 1989). Although reflectance between 640 and 680 nm possesses spectral features attributable to chlorophyll a and b, an intercorrelation in their content (Fig. 1A) makes finding the band(s) specifically sensitive to individual chlorophyll very difficult. The development of a non-destructive technique for carotenoid estimation is complicated by the chlorophyll interference absorption in anthocyanin-free fruit and by the interference absorption of chlorophyll and anthocyanins in anthocyanin-containing fruit (Fig. 2B, Fig. 8A). In turn, the analysis of anthocyanin content requires elimination of the effects by carotenoids and chlorophylls (Fig. 8). The development of a non-destructive technique suitable for application in a wide range of pigment content presumes that spectral features of pigment remain the same in different cultivars and through different stages of fruit development. However, significant changes in carotenoid composition are known to occur during fruit ripening (Knee, 1988). In vivo spectral properties of anthocyanins in fruit are strongly dependent on local pH, interaction with metal ions, aggregation, co-pigmentation effects of flavonoids, etc. (Mosko-witz and Hrazdina, 1981; Lancaster et al., 1994). The precision of peel pigment determination by reflectance measurement of whole fruit is limited by the contribution of pulp pigments to the overall light absorption, which is difficult to estimate; it is responsible for uncertainties in the relationship of reflectance with analytically measured pigment content. Nevertheless, the data presented in this paper suggest the applicability of reflectance spectroscopy for analysis of total chlorophyll, carotenoid and anthocyanins in apple fruit.

Independent of anthocyanin presence, reciprocal reflectance at the red chlorophyll absorption maximum was highly sensitive to small-to-moderate chlorophyll content and showed a linear response to chlorophyll content (up to 5 nmol/cm^2), indicating that the index, R_{800}/R_{678}, could be applied for Antonovka, Zhigulevskoe and Golden Delicious fruit. With a further increase in chlorophyll content, the index possessed a curvilinear relationships with chlorophyll content (Fig. 5A,

Table 1). These results were consistent with data obtained by Knee (1980) with Cox Orange Pippin (Fig. 5A). The signature analysis showed that more precise assessment of a wide range of chlorophyll content could be done at wavelengths located quite far from reflectance minima (around 640 and 700 nm) to avoid saturation of the 'absorption versus pigment' relationship (Figs. 2–5). The indices R_{800}/R_{700} (Fig. 5B, Table 1) and R_{800}/R_{640} (Table 1) exhibited a close linear correlation with apple peel chlorophyll through the entire range of chlorophyll content studied; they allow for a more reliable chlorophyll estimation than using the spectral band near 680 nm.

Fig. 3B indicates a strong correlation of R_{550} versus R_{700} in anthocyanin-free fruit, the spectral feature previously discovered in the leaves of diverse plant species (Gitelson and Merzlyak, 1998). In red fruit, this close correlation failed even at a small anthocyanin content because of increased absorption by anthocyanins at R_{550}. It should also be noted that when chlorophyll content is small and carotenoid content is high, the contribution of carotenoid to absorption at 550 nm might be significant (Fig. 6A); it makes the relationship 'R_{550} versus R_{700}' weaker (lower curve in Fig. 4A). Nevertheless, R_{550} could serve as a precise measure of chlorophyll content in anthocyanin-free apple fruit (Fig. 3).

Signature analysis of reciprocal reflectance of anthocyanin-free fruit (Fig. 6A) revealed several carotenoid maxima in the blue region near 485, 460 and 425 nm (as a shoulder). The same bands were presented as minima on the determination coefficient spectra of the relationship '$(R_{800}/R_{\lambda})/R_{678}$ versus chlorophyll' (Fig. 4A). In addition, the spectra in Fig. 6A showed the presence of compound(s) with absorption increasing from 430 to the 400 nm. This spectral feature was especially expressed in sun-exposed fruit sides and was putatively attributed to flavonoids (Merzlyak and Chivkunova, 2000; Merzlyak et al., 2002).

The proportion of carotenoids and chlorophylls reflects the physiological changes occurring in fruit during its development. In our previous studies, the difference of reflectance in the blue and the red regions of the spectrum was found to be an indicator of the pigment transformation occurring during leaf senescence and fruit ripening (Merzlyak et al., 1999). For apples, we suggested to use reflectance at either 480 or 500 nm in PSRI. The indices, $(R_{678}-R_{500})/R_{800}$ and $(R_{678}-R_{480})/R_{800}$, were linearly correlated with the carotenoid to chlorophyll molar ratio in a wide range of its changes in the peel of anthocyanin-free apples. The reflectance at the long-wave carotenoid absorption maximum (486 nm) as a term in PSRI improved the sensitivity and accuracy of the carotenoid to chlorophyll estimation (Fig. 6B, Table 1). In the green fruit with a low carotenoid to chlorophyll ratio (Granny Smith and Renet Simirenko), chlorophyll absorption was a main factor governing reflectance R_{480} and, thus, caused severe interference impairing sensitivity of the index (Fig. 6B). Therefore, the index $(R_{678}-R_{480})/R_{800}$ is preferable for carotenoid to chlorophyll estimation in cultivars with low or medium chlorophyll content (such as Antonovka or Golden Delicious) or in ripening fruit with yellowish coloration. A stage when R_{678} matches R_{500} (i.e. PSRI turns to zero) was suggested as an indicator of the onset of fruit ripening and leaf senescence (Merzlyak et al., 1999). For apple fruit studied, $PSRI = 0$ corresponded to carotenoid to chlorophyll values of 0.9 ± 0.03 for R_{500} and 0.43 ± 0.02 for R_{480}. Thus, the index $(R_{678}-R_{480})/R_{800}$ allowed earlier detection of ripening onset and a more precise estimation of the rate of the ripening process.

In the development of an index for carotenoid assessment in plants, one should pay particular attention to finding the spectral band(s) depending mostly on carotenoid content on a high chlorophyll background (Gitelson et al., 2002). Fig. 7A indicates that in anthocyanin-free fruit, carotenoid spectral features are most pronounced at low chlorophyll content and tend to disappear with an increase in chlorophyll content. The use of the region between 425 and 480 nm for carotenoid estimation is rather questionable, especially in fruit with high chlorophyll content. In this region of strong pigment absorption, the relationship 'absorption versus carotenoids' saturated, thus, sensitivity of the index to pigment content dropped significantly. Therefore, the spectral range 520–530 nm appeared as the one most suitable for

carotenoid assessment against a variable chlorophyll background. Because chlorophyll also exhibit absorption in this range, compensation for their contribution to reflectance R_{520} was necessary. To remove the chlorophyll contribution, reciprocal reflectances R_{550} and R_{700}, which are proportional to chlorophyll content, were employed. The difference of reciprocal reflectances $[(R_{520})^{-1}-(R_{700})^{-1}]$ was proportional to the carotenoid content, and indices for carotenoid assessment in the forms $R_{800}(1/R_{520}-1/R_{500})$ and $R_{800}(1/R_{520}-1/R_{700})$ were linearly correlated with peel carotenoid content (Fig. 7B; Table 1). In fruit with high chlorophyll content (Granny Smith), efficiency of the index decreased slightly because chlorophyll absorption became a main factor governing R_{520}; anthocyanin absorption strongly overlaps those of carotenoids and chlorophylls (Fig. 2B, Fig. 3B, Fig. 8A), making non-destructive carotenoid determination in anthocyanin-containing fruit hardly feasible.

A reflectance band maximally sensitive to variation in anthocyanin content was found in the green region of the spectrum (Fig. 8A), near its in vivo absorption maximum around 550 nm (Merzlyak and Chivkunova, 2000). Chlorophyll also absorbs in the green range; to remove the chlorophyll absorption contribution at 550 nm, a reciprocal reflectance at 700 nm has been used. The index $R^{*}_{800}(1/R_{550}-1/R_{700})$ almost did not depend on chlorophyll content and was linearly related to anthocyanins (Fig. 8B, Table 1). It should be noted that in fruit with anthocyanin content higher than that recorded in Zhigulevskoe apple, reflectances with high sensitivity to anthocyanins are situated at wavelengths longer than 550 nm.

As a result of the study, we can conclude that reflectance spectroscopy is a suitable and sensitive tool for the non-destructive determination of peel content of chlorophyll, carotenoids and anthocyanins, providing reliable information about pigment dynamics in whole fruit during development. Further investigations are required to broaden and improve the developed indices for other apple cultivars. In this respect, particular attention should be paid to the pigment spectral signature search to achieve a more selective and precise assessment. The indices developed are of potential use for the non-destructive estimation of pigment content in fruit of other plant species.

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