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Nondestructive evaluation of the changes of total flavonoid, total phenols, ABTS and DPPH radical scavenging activities, and sugars during mulberry (*Morus alba* L.) fruits development by chlorophyll fluorescence and RGB intensity values

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ABSTRACT

The use of chlorophyll fluorescence measurements and RGB intensity values to noninvasively evaluate changes of the internal chemical parameters (total flavonoid, total phenols, ABTS cation and DPPH radical scavenging activities and sugars) was investigated during all the development stages (ST) and the last four development stages of mulberry fruits in this study. The appropriately fitted relationships in this study revealed that chlorophyll fluorescence and RGB intensity have high correlations with the internal chemical parameters of mulberry fruits. Especially, each of the internal chemical parameters has higher correlations with chlorophyll fluorescence and RGB intensity during the period of ST4–ST7 than during the period of ST1–ST7 respectively. High correlations between chlorophyll fluorescence and the internal chemical parameters were found during the period of ST4–ST7, with R^2 ranged from 0.82 to 0.94. RGB intensity values showed fine correlations with the internal chemical parameters with R^2 ranged from 0.93 to 0.97 during the period of ST4–ST7. Therefore, chlorophyll fluorescence and RGB intensity values may be potential methods for quality evaluation of mulberry fruits during development, however, it is of great importance to select the most adequate parameter for the evaluation of different chemical components.

1. Introduction

Mulberry is a perennial woody plant native to warm, temperate, and subtropical regions of Asia, Africa, North America, and southern Europe (Watson & Dallwitz, 2007). There are 24 species of *Morus* and one subspecies, with at least 100 known varieties (Ercisli & Orhan, 2007). Mulberry can grow in a wide range of topographical, climatic and soil conditions. Mulberry fruit are a traditional Chinese medicine, which can be used for the treatment of sore throat, fever, hypertension, and anemia (Li et al., 2009). In China, mulberry fruits are eaten fresh and are also used in marmalades, juices, liquors, natural dyes and in the cosmetics industry. Previous studies on the fruits *Morus* species showed the presence of fats and fatty acids, vitamin C, minerals, phenols, and flavonoids (Ercisli & Orhan, 2007) and, in the case of black mulberry fruits, some organic acids (Koyuncu, 2004) and anthocyanins (Dugo, Mondello, Errante, Zappia, & Dugo, 2001). Recent research in mulberry has revealed that the extract of mulberry may have antidiabetic (Asano et al., 2001), antihyperglycemic (Andallu & Varadacharyulu, 2003), antiviral (Du et al., 2003), antioxidative (Kim, Park, & Lee, 1998), hypolipidemic (El-Beshbishy, Singab, Sinkkonen, & Pihlaja, 2006), and neuroprotective (Kang, Hur, Kim, Ryu, & Kim, 2006) effects. Cui et al. (2006) found that biomedical functions for mulberry have been partly attributed to the properties of suppressing inflammatory mediators and protein tyrosine phosphatase of the flavonoids and soluble sugar contained in mulberry leaf, fruit, and root bark. However, the most important ability of mulberry for its pharmaceutical value is its antioxidant capacity (Pan & Lou, 2008).

The ideal method to evaluate fruit maturity has to be simple, precise, quick, reliable, and nondestructive (Lechaudel, Urban, & Joas, 2010). Recently, the nutritional quality of fruit during ripening has become an increasingly important problem. Therefore, predicting the behavior of nutritional quality nondestructively during the development of fruits is important. The nondestructive techniques for quality evaluation have gained in popularity. Some such efforts using near-infrared (NIR) spectroscopy (Guthrie & Walsh, 1997; Walsh, Golic, & Greensill, 2004), visual spectral analysis (Jha, Chopra, & Kingsly, 2005), acoustic and ultrasound techniques (Mizrach, 2000; Shmulevich, Galili, & Howarth, 2003) have been reported for mango, but these instruments are costly and

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difficult to carry to the fruit orchards. Chlorophyll fluorescence measurements are not only noninvasive but are simple and rapidly taken, and portable chlorophyll fluorometers for field measurements are now readily available (DeEll, Van Kooten, Prange, & Murr, 1999; Smillie, Hetherington, Nott, Chaplin, & Wade, 1987). It has been used to evaluate the degree of ripeness in mango fruits (Lechaudel et al., 2010), grape berries and 'Golden' papaya fruit successfully (Bron, Ribeiro, Azzolini, Jacomino, & Machado, 2004; Kolb et al., 2006). In addition, anthocyanins in olive (*Olea europaea* L.) fruits at different degrees of pigmentation were also assessed nondestructively by measuring chlorophyll fluorescence (Agati et al., 2005).

Fruit ripening is usually accompanied by a change in the skin color due to a modification of pigment concentration in the superficial tissues (Agati et al., 2005). Usually, color development from green to purple is modulated by an accumulation of anthocyanins (Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002) together with the degradation of chlorophylls and carotenoids (Minguez-Mosquera & Gallardo-Guerrero, 1995). The change in the skin color of mulberry fruits at different development stages is obvious and this feature suggests the use of color analysis to detect the change of nutritional quality in mulberry fruits is possible.

The objective of this study is to make a nondestructive assessment of the nutritional quality including total phenols, total flavonoid, sugars and antioxidant activity in mulberry fruits with different skin colors, which correspond to different development levels by using chlorophyll fluorescence parameters and red, green, and blue (RGB) intensity values respectively. To our knowledge, this investigation has not been carried out before.

2. Materials and methods

2.1. Collection and preparation of mulberry fruit samples

Mulberry fruits (*Morus alba* L.) with the widest range of skin colors were manually picked from Zhejiang Normal University (Jinhua, Zhejiang, People's Republic of China) on May 13, 2011, and then immediately transported to our laboratory. Seven developmental stages (ST1–ST7) were selected according to fruit shape, size and color as shown in Fig. 1. The fruits were then stored in polyethylene bags at −18 °C until analysis.

2.2. Measurement of chlorophyll fluorescence

Before chlorophyll fluorescence measurements, fruits were dark-adapted for 30 min, and then chlorophyll fluorescence parameters, including the minimum fluorescence (*Fo*) and the maximum fluorescence (*Fm*), were measured using a MINIPAM (pulse-amplitude modulation) fluorometer (WALZ, Effeltrich, Germany) as reported by Dai et al. (2009). Photochemical efficiency of PSII (*Fv*/*Fm*) and the variable fluorescence ($Fv = Fm - Fo$) were also calculated. The chlorophyll fluorescence of each fruit was randomly and evenly measured at three locations.

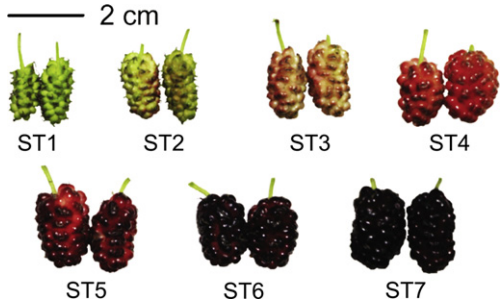

Fig. 1. Mulberry fruits at different pigmentation stages: stage 1 (ST1)–stage 7 (ST7).

2.3. Image acquisition and RGB intensity values

Mulberry fruit images were acquired using the method as our previous study reported (Zheng et al., 2011). Mulberry fruits were photographed using a Canon EOS 50D camera with a Canon EF-S 18–55 mm f/3.5–5.6 IS lens at 50 mm. The lighting for images is entirely from natural light in the room (avoid direct sunshine) on a sunny morning in the early summer, and all image acquisitions were carried out at least in triplicate at about the same place with light intensity of about 70 μmol/m^2/s. The average RGB intensity values from color images (TIFF image format) were obtained using the color histogram tool of Image J (version 1.4.3.67, freeware at http://rsb.info.nih.gov/ij/).

2.4. Total flavonoid (TF) and total phenols (TP) determination

The TF and TP were determined according to the method described in our earlier paper (Lu, Lou, Zheng, Hu, & Li, 2011). The TP content of mulberry fruit was determined using Folin–Ciocalteu reagent, the TF content was evaluated by a colorimetric assay method. The TP and TF contents were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh mulberry fruits and milligrams of rutin per 100 g of fresh mulberry fruits, respectively.

2.5. DPPH radical scavenging activity determination

The DPPH free radical scavenging activity was carried out according to the method of our previous study (Lu et al., 2011) with some modifications. The extracts (0.1 mL) of sample in ethanol were reacted with 10 mL of 0.03 g/L DPPH (2,2-diphenyl-1-picrylhydrazyl) ethanol solution at room temperature. The extract (0.1 mL) with 10 mL distilled water was used as control. The absorbance was measured at 517 nm after 30 min of reaction in the dark. DPPH radical scavenging capacity was expressed as Trolox equivalent (μmol of Trolox/100 g of fresh mulberry fruits).

2.6. ABTS assay

The ABTS assay was based on the method of Re et al. (1999) with slight modification. $ABTS^{\bullet+}$ reagent was produced by reacting 10 mL of 7 mM ABTS solution with 178 μL of 140 mM potassium persulfate aqueous in the dark at room temperature for 13 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to appropriate absorbance. One-tenth of a milliliter of diluted sample was added to 3.9 mL of diluted $ABTS^{\bullet+}$ solution to react in the dark at room temperature for 6 min, and the absorbance at 732 nm was recorded. Trolox was used as standard with the final concentration ranging from 0 to 16.5 μM. Results were expressed as Trolox equivalent antioxidant capacity (μmol of Trolox/100 g of fresh mulberry fruits).

2.7. Sugars content

A DuoFlow HPLC system (Bio-RAD, USA) equipped with a pump system and a refractive index detector (RID-10A) was used for sugar analysis. Mulberry slurries (10 g) were diluted with 50 mL of purified water, and then mixed with 5 mL of zinc acetate solution (3 mL of glacial acetic acid was added to 21.9 g of zinc acetate, and water were added to a final volume of 100 mL) and 5 mL potassium ferrocyanide solution (10.6 g of potassium ferrocyanide was dissolved in purified water, and the final volume was brought up to 100 mL with purified water). The final weight of the extract solution was added to 100 g by purified water and then stirred about

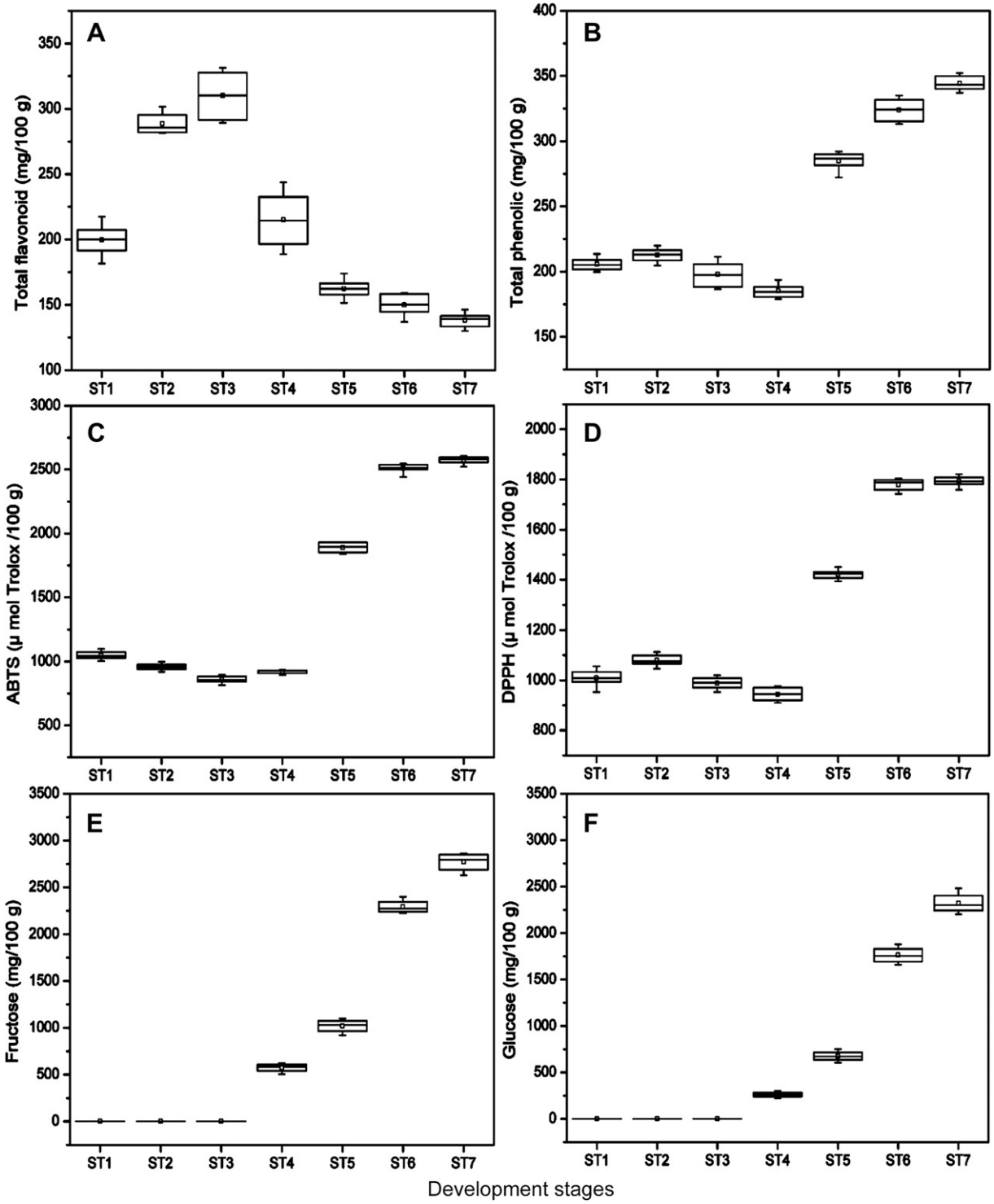

Fig. 2. Changes in total flavonoid (A), total phenolic (B), ABTS radical scavenging activity (C), DPPH radical scavenging activity (D), fructose (E), and glucose (F) during development of mulberry fruit. Box plots show the median (line in the box) and the average values (square in the box). The whiskers extend to the min and max range, whereas the box extends from the 25th to the 75th percentile.

30 min. The homogenate was filtered by filter paper at room temperature. Filtrate was filtered through a 0.45 μm membrane filter before HPLC analysis. The sample was then analyzed onto an Amethyst-Amino column (Sepax, 4.6 × 250 mm). The elution solvent used contained 80% acetonitrile and 20% deionized water. The column was operated at 30 °C with 0.8 mL/min flow rate and the sample injection volume was 50 μL.

2.8. Statistical analysis

A multiple linear regression model was performed using SAS software (SAS, version 9.0). This model consists of a stepwise multiple linear regressions within the REG procedure of SAS. In a stepwise model, independent variables are removed or added iteratively from the model in each step of the procedure according

to their significance ending with a model that includes all independent variables that meet the significance criteria. The purpose of the model is to determine which independent variables from the proteomic analysis were contributing to the variation of the dependent variable.

3. Results and discussion

3.1. Total flavonoid, total phenols, sugars, ABTS and DPPH radical scavenging activities changes

Flavonoids, including flavanols, flavones and condensed tannins, are plant secondary metabolites. Consumption of the flavonoid-containing fruits and vegetables has been linked to protection against cancer and heart disease (Hertog, Hollman, & Venema, 1992; Juan & Chou, 2010). In our study, the content of TF in mulberry fruit increased with fruit development, peaked at ST3, and then decreased as fruit developed (Fig. 2A). The TF content at ST3 and ST7 of mulberry fruit were 310 and 138 mg/100 g fresh weight respectively.

The content of TP in mulberry fruit increased from ST1 to ST2, and then decreased until ST4. At the later stages of fruit development (ST4–ST7), TP content rose steadily (Fig. 2B). Some researchers showed that in red-colored fruits, phenols increase during the last ripening stage, due to the maximal accumulation of anthocyanins and flavonols (Bridle & Timberlake, 1978; Gerasopoulos & Stavroulakis, 1997), which belong to flavonoids. However, in our study, total flavonoids at the last ripening stage of mulberry fruit decreased steadily. Therefore, it means other components, not anthocyanins and flavonols, contributed to the total flavonoid content at the last ripening stage of mulberry fruit. The total phenols content of mulberry fruit ranged from 185 to 344 mg GAE/100 mg fresh weight during fruit development in this study (Fig. 2B). Earlier, total phenols content in three different species of mulberry fruit was reported which ranged from 181 to 1422 mg GAE/100 g fresh weight (Ercisli & Orhan, 2007). In addition, Lin and Tang (Lin & Tang, 2007) found that the total phenols content in mulberry fruit (*M. alba*) was 1515 mg GAE/100 g fresh matter. Bae and Suh (2007) reported that the mulberry fruit contained 959.9–2570.4 μg/g fresh samples of phenols substances. The variation of phenols compounds in the fruits depends on many factors, such as degree of maturity at harvest, genetic differences, and environmental conditions during fruit development (Zadernowski, Naczk, & Nesterowicz, 2005).

Different assays have been introduced to measure antioxidant capacity of foods and biological samples. In recent years, a wide range of spectrophotometric assays has been adopted to measure antioxidant capacity of foods, the most popular being ABTS and DPPH assay (Floegel, Kim, Chung, Koo, & Chun, 2011). Antioxidant capacity describes the ability of redox molecules in foods and biological systems to scavenge free radicals. It was observed in this study that during mulberry fruit development, the antioxidant capacity measured by ABTS assay decreased from ST1 to ST3, than increased steadily at later stages of fruit development (Fig. 2C). The antioxidant capacity measured by DPPH assay showed a similar change pattern to TP (Fig. 2D) and the results indicated that TP may be the major contributor to the DPPH radical scavenging activity in this study.

The individual sugars were characterized and assigned according to their chromatographic and spectral behaviors and they are glucose and fructose. The change of glucose and fructose during mulberry fruit development was similar in this study (Fig. 2E, F). There were no glucose and fructose in mulberry fruit until ST4, and they both increased steadily from ST4 to ST7. Özgen, Serçe, and Kaya (2009) reported that the main sugars in mulberry species of *Morus nigra* L. and *Morus rubra* L. fruits were glucose (about 52%) and fructose (about 48%), however, sucrose was detected only in some accessions and it only reached 1% of total sugars in a few accessions. In our experiment, the result showed that there was no sucrose in *M. alba* L.

3.2. Correlations of internal chemical parameters with RGB intensity values and chlorophyll fluorescence

The relationship between the chlorophyll fluorescence parameters and total flavonoid, total phenols, and ABTS and DPPH radical scavenging activities was appropriately fitted by using a multivariate model consisting of a stepwise multiple linear regressions within the REG procedure during mulberry fruits development (ST1–ST7), as shown in Table 1. The best prediction model for total flavonoid was obtained by combining *F*m, *F*v and *F*v/*F*m, with $R^2 = 0.70$. While the best prediction models for total phenols and ABTS cation and DPPH radical scavenging activities were all obtained by combining *F*o, *F*m, *F*v and *F*v/*F*m, with R^2 of 0.63, 0.69 and 0.64 respectively. Compared to chlorophyll fluorescence, correlations of RGB intensity values with the internal chemical parameters (total flavonoid, total phenols and ABTS and DPPH radical scavenging activities) were much higher during the period of ST1–ST7 (Table 2). *B* intensity value had a best correlation with the total flavonoid ($R^2 = 0.76$) in the multiple linear regressions model. The best prediction models for total phenols and ABTS cation and DPPH radical scavenging activities were all obtained by combining *R*, *G*, and *B* intensity values, with R^2 of 0.87, 0.90 and 0.90 respectively during the period of ST1–ST7. Therefore, RGB intensity values are more suited to estimate the changes of total flavonoid, total phenols and ABTS and DPPH radical scavenging activities noninvasively respectively during mulberry fruits development (ST1–ST7).

Interestingly, by comparing these changes in chlorophyll fluorescence parameters and RGB intensity values and in internal chemical parameters determined in this study from ST4 to ST7, we

Table 1
Variables in model, correlation coefficients (R^2) and fitted equations in the prediction of total flavonoid (TF), total phenols (TP), ABTS cation radical scavenging activity (ABTS), DPPH radical scavenging activity (DPPH), fructose and glucose during mulberry fruits development by the stepwise multiple linear regressions within the REG procedure model based on *F*o (x_1), *F*m (x_2), *F*v (x_3) and *F*v/*F*m (x_4).

Stages (ST)	Chemical parameters	R^2	Variables in model	Fitted equations
ST1–ST7	TF	0.70	*F*m, *F*v, *F*v/*F*m	$y = -311.95 + 1.61x_2 - 2.02x_3 + 444.02x_4$
	TP	0.63	*F*o, *F*m, *F*v, *F*v/*F*m	$y = 630.63 + 0.98x_1 - 1.73x_2 + 1.90x_3 - 377.33x_4$
	ABTS	0.69	*F*o, *F*m, *F*v, *F*v/*F*m	$y = 6208.82 + 10.62x_1 - 21.44x_2 + 23.79x_3 - 4280.39x_4$
	DPPH	0.64	*F*o, *F*m, *F*v, *F*v/*F*m	$y = 3399.36 + 5.19x_1 - 9.78x_2 + 10.73x_3 - 1967.05x_4$
ST4–ST7	TF	0.82	*F*o, *F*m, *F*v	$y = -7.67 - 0.78x_1 + 1.49x_2 - 1.49x_3$
	TP	0.88	*F*o, *F*m, *F*v, *F*v/*F*m	$y = 1067.16 + 4.85x_1 - 7.46x_2 + 8.01x_3 - 623.91x_4$
	ABTS	0.87	*F*o, *F*m, *F*v	$y = 6089.85 + 37.27x_1 - 54.65x_2 + 54.88x_3$
	DPPH	0.92	*F*o, *F*m, *F*v, *F*v/*F*m	$y = 5293.78 + 25.42x_1 - 39.10x_2 + 41.43x_3 - 2396.10x_4$
	Fructose	0.93	*F*o, *F*m, *F*v	$y = 5932.58 + 49.95x_1 - 62.74x_2 + 59.92x_3$
	Glucose	0.94	*F*o, *F*m, *F*v	$y = 4992.41 + 47.32x_1 - 57.62x_2 + 54.63x_3$

Table 2
Variables in model, correlation coefficients (R^2) and fitted equations in the prediction of total flavonoid (TF), total phenols (TP), ABTS cation radical scavenging activity (ABTS), DPPH radical scavenging activity (DPPH), fructose and glucose during mulberry fruits development by the stepwise multiple linear regressions within the REG procedure model based on R (x_1), G (x_2), and B (x_3) intensity values.

Stages (ST)	Chemical parameters	R^2	Variables in model	Fitted equations
ST1–ST7	TF	0.76	B	$y = 41.88 + 4.96x_3$
	TP	0.87	R, G, B	$y = 365.94 - 1.67x_1 - 0.71x_2 + 2.30x_3$
	ABTS	0.90	R, G, B	$y = 2972.87 - 17.80x_1 - 8.35x_2 + 19.66x_3$
	DPPH	0.90	R, G, B	$y = 1947.47 - 9.63x_1 - 4.27x_2 + 13.66x_3$
ST4–ST7	TF	0.97	G	$y = 84.67 + 2.80x_2$
	TP	0.93	R, G, B	$y = 234.91 - 1.16x_1 - 9.47x_2 + 15.89x_3$
	ABTS	0.93	R, G, B	$y = 1856.00 - 13.21x_1 - 81.88x_2 + 134.34x_3$
	DPPH	0.95	R, G, B	$y = 1204.77 - 7.90x_1 - 47.27x_2 + 86.55x_3$
	Fructose	0.96	R, B	$y = 1927.12 - 35.93x_1 + 91.04x_3$
	Glucose	0.96	R, B	$y = 1415.68 - 33.67x_1 + 88.71x_3$

found that at the same time that *F*o, *F*m, *F*v and *R*, *G*, and *B* values decrease, the total phenols, ABTS and DPPH radical scavenging activities, glucose content, and fructose content increase. However, total flavonoid decrease steadily from ST4 to ST7. The relationship between the internal chemical parameters (total flavonoid, total phenols, ABTS cation and DPPH radical scavenging activities and sugars) and the chlorophyll fluorescence parameters was also fitted by using a multivariate model consisting of a stepwise multiple linear regressions within the REG procedure during the last four stages of mulberry fruits development, as shown in Table 1. We found that the model based on combining *F*o, *F*m and *F*v showed a best correlation with the total flavonoid, ABTS cation radical scavenging activity, fructose and glucose, with R^2 of 0.82, 0.87, 0.93 and 0.94 respectively among the chlorophyll fluorescence parameters (*F*o, *F*m, *F*v, and *F*v/*F*m) at the last four selected stages (ST4–ST7). The best prediction models for total phenols and DPPH radical scavenging activity were both obtained by combining *F*o, *F*m, *F*v and *F*v/*F*m, with R^2 of 0.88 and 0.92 respectively at the last four selected stages (ST4–ST7) (Table 1). The relationships between the internal chemical parameters (total flavonoid, total phenols, ABTS cation and DPPH radical scavenging activities and sugars) and the *R*, *G*, and *B* values were shown in Table 2. It is worth noting that color parameters showed fine correlations with the internal chemical parameters with R^2 ranged from 0.93 to 0.97 at the last four selected stages. *G* value had a best correlation with the total flavonoid ($R^2 = 0.97$). The model based on combining *R*, *G*, and *B* intensity values showed a best correlation with the total phenols, ABTS cation and DPPH radical scavenging activities, with R^2 of 0.93, 0.93 and 0.95 respectively. The best prediction models for fructose and glucose were both obtained by combining *R* and *B* intensity values, with R^2 of 0.96 and 0.96 respectively.

Therefore, chlorophyll fluorescence and color intensity may be potential methods for quality evaluation of mulberry fruits during development noninvasively, especially during the later ripping stages. Lu et al. (2011) found that *F*o, *F*m, and *F*v had high positive correlations with DPPH radical scavenging activity, followed by total phenols, reducing sugar, ascorbic acid, and total flavonoid ($0.729 \leq r \leq 0.920$, $P < 0.05$), and showed negative correlations with soluble sugar, pH, and carotenoids ($-0.885 \leq r \leq -0.826$) during later ripping stages of jujube fruit. Kolb et al. (2006) reported that *F*o fluorescence was always better correlated to sugar concentrations than *F*m and *F*v/*F*m. In addition, there were good relationships between *F*o values and weight loss values for both grape cultivars "Thompson and Flame seedless" (Ramin, Prange, DeLong, & Harrison, 2008). However, they did not use multivariate models but only considered one variable at a time. Our previous study (Zheng et al., 2011) has reported that red, green, and blue (RGB) intensity values show well prediction in anthocyanins, ascorbic acid and DPPH radical scavenging activity of bayberry juice during storage. *F*o and *F*m fluorescence correlated with skin color was found in papaya fruits (Bron et al., 2004). However, in this study, it seems that color intensity values are more suitable to noninvasively estimate the internal chemical parameters of mulberry fruits.

In conclusion, our observations demonstrate that chlorophyll fluorescence and RGB intensity have high correlations with the internal chemical parameters of mulberry fruits. The high correlations between RGB intensity values and internal chemical parameters and between chlorophyll fluorescence parameters and internal chemical parameters found at the last four development stages of mulberry fruits in this study support possible nondestructive methods to evaluate the chemical changes and ripening degrees of mulberry fruits using chlorophyll fluorescence and color parameters. Therefore, chlorophyll fluorescence and RGB intensity values may be potential methods for quality evaluation of mulberry fruits during development noninvasively, but the selection of the most adequate parameter is of great importance to predict different chemical components. However, it seems that color intensity values are more suitable to estimate the quality of mulberry fruits. Most likely, the method can be extended to other fruits, which accumulate anthocyanins in their skin during development of their fruits.

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