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Research article

Nondestructive evaluation of changes in total flavonoid, total phenol and DPPH scavenging activity during loquat (*Eriobotrya japonica* Lindl.) fruit development by chlorophyll fluorescence and RGB intensity values

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ABSTRACT: Loquat (*Eriobotrya japonica* Lindl.) is a kind of subtropical fruit cultivated in many countries. This study aimed to evaluate the changes in the content of total phenol, total flavonoid, and 1,1-Diphenyl-2-picrylhydrazyl (DPPH), scavenging activity during the seven developmental stages and to determine the optimum harvesting time of loquat fruits by using chlorophyll fluorescence and Red Green Blue (RGB) intensity detection. Our results revealed a high correlation between chlorophyll fluorescence parameters and the internal chemical parameters (total phenol, total flavonoid, and DPPH scavenging activity) in loquat. The best prediction model from ST1 to ST5 for total flavonoid with variable fluorescence (Fv) and Fv / maximum fluorescence (Fm), R^2 = 0.79, and for DPPH scavenging activity with Fm, Fv, and Fv/Fm, R^2 = 0.92 was achieved, respectively. Total phenol correlated with minimum fluorescence (Fo) and Fv from ST4 to ST7, with R^2 = 0.99. Red Green Blue (RGB) intensity is also highly correlated with the internal chemical parameters. Both total phenol and DPPH radical scavenging activities had the best correlations with the B intensity value (R^2 = 0.998, R^2 = 0.970) in the ST1-ST4 period, respectively. Total phenols and total flavonoids had the best correlation with the G intensity value (R^2 = 0.909, R^2 = 0.986) in the ST5-ST7 period, respectively. In conclusion, developmental stage ST5 is the optimum harvesting time for loquat fruit, and chlorophyll fluorescence and RGB intensity are both noninvasive measurements for quality assessment during the loquat development period and for the prediction of the optimal time for harvesting loquat fruit.

Keywords: quality assessment, seven developmental stages, the optimum harvesting time

Introduction

Loquat (*Eriobotrya japonica* Lindl.) was initially produced in temperate China (Hadjipieri et al., 2020). Its flesh is soft and juicy, sweet and sour, delicious and nutritious. The late autumn and early winter mark the flowering season of Loquat, and its fruit ripens during the spring (Pinillos et al., 2011). This means that loquat is a niche product that commands considerably high prices (Kahramanoğlu, 2020) as an early harvested fruit when other fruits are unavailable.

Harvesting loquat fruit during the optimal maturity stages is extremely important because its shelf life is limited to approximately five weeks (Shan et al., 2008). If the harvest is too early, loquat fruit with an insufficient accumulation of sugar content will affect the fruit quality and yield (Cañete et al., 2015), and late harvesting will affect the storage tolerance of the fruit making it vulnerable to mechanical damage. Loquat fruit classification is based mainly on observing fruit color and size or sampling destructive physiological index detection, which encompasses refractometric determination of sugar concentrations or determination of phenolic compounds by liquid mass spectrometry. The first method is easy, but the classification results are inaccurate, and loquat fruit cannot be classified on a large scale. Although the second method can yield more accurate results, the detection process complicated and time-consuming, requiring the destruction of fruit, and results for sampling fruit only.

Chlorophyll fluorescence measurement was used to detect fruit's ripening period and quality because it is simple, noninvasive, portable, and easy to operate (Lou et al., 2012). RGB intensity can be used to detect changes in nutritional quality because color development in the fruit ripening process is modified by pigment concentration in superficial tissues (Feng et al., 2020).

The objective of this study was to measure changes in the content of total phenols, total flavonoids, and DPPH scavenging activity and to determine the optimum harvesting time of loquat fruit during seven developmental stages by using measurements of chlorophyll fluorescence and RGB intensity as noninvasive techniques.

Materials and Methods

Materials

Different colors of loquat were hand-picked in Jinhua, a city in the province of Zhejiang (29°1′ N, 119°6′ E, altitude 600 m) and immediately transferred to the laboratory in Apr. Fruits with no damage were divided into seven developmental stages according to the fruit ripening stage (ST1-ST7), as shown in Figure 1. ST1-ST3 belonged to the green fruit stage (ST1 = dark green; ST2

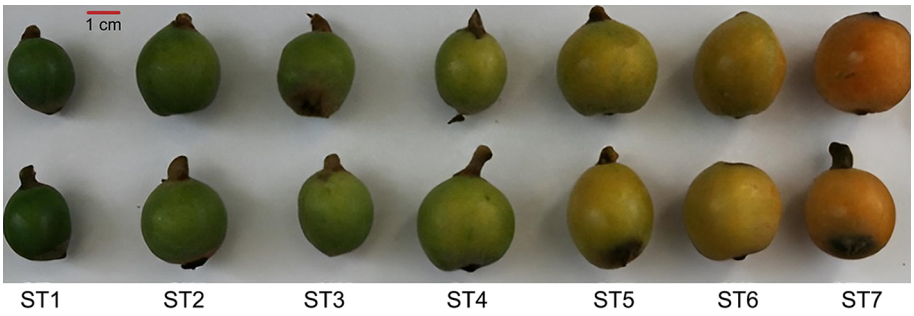

Figure 1 – Loquat at different developmental stages (ST1-ST7).

= a lighter green that does not contain any yellow; ST3 = a lighter green that contains a little yellow), ST4-ST6 belonged to the color transition stage (ST4 = more green and less yellow; ST5 = more yellow and less green; ST6 = a lighter yellow that contains a little green), and ST7 belonged to the mature stage, completely yellow fruit. Aluminum trichloride (analytical pure, N° 100617) was purchased from Tianjin Guangfu Fine Chemical Research Institute; potassium acetate (analytical pure, N° F20040821) was obtained from Sinopharm Group Chemical Reagent Co., Ltd; anhydrous sodium carbonate (analytical pure, N° 110501) and methanol (analytical pure, N° 120902) was provided by Jiangsu Qiangsheng Functional Chemical Co., LTD; folin phenol reagent (N° 20111216) was purchased from the Shanghai Lida Biotechnology Co., LTD; DPPH Free Radical (N° D9132) and Trolox (N° 238813) were purchased from the SIGMA High-tech Co., Ltd; rutin standard product (N° 100080-200707) and gallic acid (N° 110831-200803) was provided by the China Food and Drug Testing Institute.

Content analysis of total phenol

The phenolic compounds were extracted with 80 % methanol solution until the extracted pulp turned white. This was followed by filtration reservation. The content of total phenol (TP) in the extracts was assessed by the Emmons' method with slight modifications (Emmons et al., 1999). For the analysis, 0.5 mL of each sample or gallic acid standard solution or blank was added to a separate test tube and appropriately diluted with 8.25 mL of distilled water, respectively, followed by 0.5 mL of Folin-Ciocalteau solution. After mixing for exactly 1 min, 0.75 mL of 20 % sodium carbonate was added. After further mixing, the solution was incubated at 40 °C for 40 min in darkness. The absorbance was read at 755 nm, which was repeated three times. The concentration of total phenol was calculated from a calibration curve using gallic acid standard solution (0.01, 0.02, 0.03, 0.04, and 0.05 mg mL^{-1}).

Content analysis of total flavonoid

Total flavonoid (TF) content was analyzed by a colorimetric method with a slight modification (Bonvehí et al., 2001). It was extracted using 80 % methanol solution until the extracted pulp turned white, followed by filtration reservation. Next, the tube containing 0.5 mL of each extract or standard solution was added to 1.5 mL of methanol solution, 0.1 mL of 2 % aluminum chloride solution, 0.1 mL of 1 M potassium acetate solution, and 2.8 mL of distilled water. After mixing, the tube was left to react at room temperature for 30 min. Finally, the absorbance value was read at 415 nm by a spectrophotometer. This was repeated three times. The contents of total flavonoids were calculated from a calibration curve using rutin (0.04, 0.08, 0.12, 0.16, and 0.2 mg mL^{-1}) as a standard.

DPPH scavenging effect

The DPPH free radical scavenging activity was analyzed by the method with a slight modification (Lu et al., 2012). The reactions were triggered in 10 mL of methanol solution containing 0.03 g L^{-1} DPPH (2,2-diphenyl-1-trinitrophenylhydrazine) and 0.1 mL of different loquat sample extracts. The 0.1 mL of different loquat sample extracts was reacted with distilled water as a negative control. After incubating at 37 °C for 30 min in the dark, the absorbance was read at 517 nm. This was repeated three times. The concentration of Trolox as a standard ranged from 0 to 16.5 μmol, and the results were expressed as Trolox equivalent antioxidant activity. The DPPH free radical scavenging capacity was calculated by the formula:

$$\text{Scavenging activity}(\%) = \left[\frac{A_0 - (A_1 - A_s)}{A_0}\right] \times 100$$

A_0 = the absorbance value of 10 mL DPPH solution; A_1 = the absorbance value of sample extract mixed with distilled water; As = the absorbance value of sample extract mixed with DPPH solution.

Determination of chlorophyll fluorescence

After dark-adapted treatment for 30 min, chlorophyll fluorescence parameters included the minimum fluorescence (Fo), and the maximum fluorescence (Fm) was measured by the MiniPAM (Pulse-Amplitude

Modulation) fluorescence meter (Dai et al., 2009). The variable fluorescence (Fv = Fm – Fo) of Photosystem II (PSII) and photochemical efficiency (Fv/Fm) could be calculated. Each loquat was measured evenly at all three locations.

Determination of RGB intensity values

Loquat fruits were photographed by a Canon EOS 600D camera. The lighting was from natural light on a sunny morning in late spring, and all photographs were taken three times in the same place with a 70 μmol s^{-1} per m^2 light intensity. The mean values of RGB intensity from photos (JPEG image format) were extracted by the Image J program (version 1.8.0).

Statistical analysis

Multiple linear regression models were run on an SAS software program (SAS, version 9.0). The models included a stepwise multiple linear regression analysis of an REG program with SAS. In a step-by-step model, the independent variables are repeatedly added to or removed from each step of the program according to the significance of the independent variables. The program ends with a significance condition that all the independent variables meet and produce the model and determine which independent variables contribute to changes in dependent variables. Each analysis was repeated thrice, and each extraction had at least three parallel experiments. Correlations within the data were shown by Pearson correlation coefficient (R).

Results and Discussion

Changes in total flavonoid, total phenol, and DPPH radical scavenging activities

Flavonoids and phenolic compounds are the main components that engage in antioxidant activity (Tosun et al., 2009) which is an essential index for evaluating fruit quality (Zhang et al., 2021). Therefore, loquat fruit with a higher flavonoid and phenol content are more beneficial to human health (Xu et al., 2014).

It was observed that the content of TF was high in young fruit at ST1 (152 mg per 100 g). This decreased as the fruit developed, and then increased at ST7. In the last growth stage of ST7, the content of TF was 100.92 mg per 100 g of fresh weight (Figure 2).

The content of phenolic compounds in fruit is related to fruit maturity (Zadernowski et al., 2005). In our study, total phenol content decreased from ST1 to ST2 and then maintained a steadily decreasing trend before ST6. In the last stage of development, ST7, the TP content increased again. A number of researchers have found that phenols increase during the last developmental stage in red-colored fruit because of the maximal flavonol and anthocyanin accumulation (Gerasopoulos and Stavroulakis, 1997). These studies are consistent with our results, in which both total flavonoid and total phenol increased in the last stage, ST7, of development. In our study, the total phenol amount of loquat ranged from 82.09 to 186.2 mg per 100 g of fresh weight. The research of loquat cv. Mogi revealed that the amount of TP was high in young loquat fruit, which then decreased from four to two weeks prior to maturity but increased in the last two weeks before maturity (Ding et al., 2001), a pattern that was very similar to our experience. We found that the content of TP was 186.2 mg per 100 g in loquat fruit in ST1, which decreased steeply from ST1 to ST2, declining slowly from ST2 to ST6 (the lowest content of 82.09 mg per 100 g was reached in ST6), then rising robustly in ST7 to a higher content of 130.2 mg per 100 g in loquat fruit.

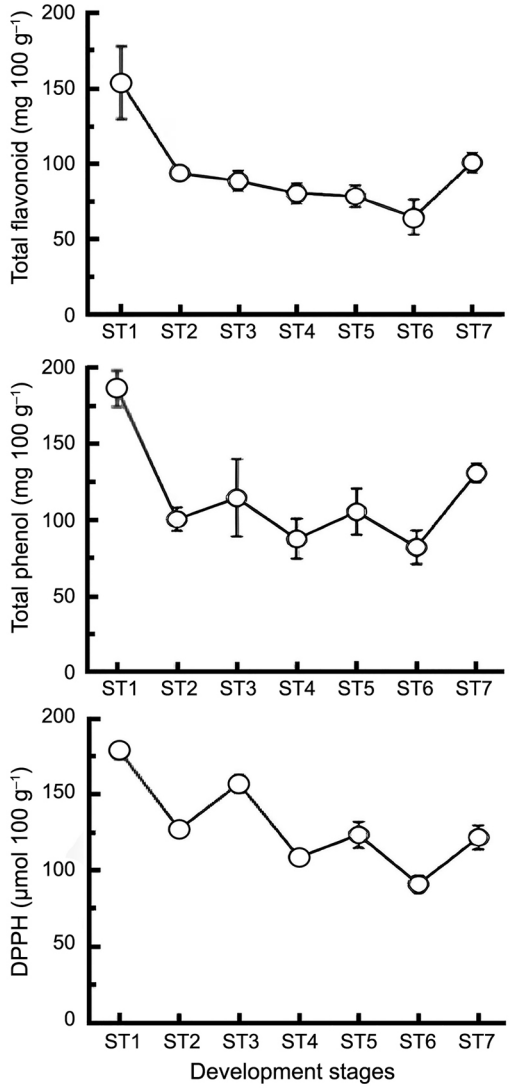

Figure 2 – Variation trend of in total flavonoid, total phenol, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity during development of loquat fruit.

The radical scavenging capacity of loquat decreased gradually from ST1 to ST6 and increased in ST7. The variation trend of radical scavenging capacity is similar to the variation trend of TP in development stages ST1 to ST7 of loquat fruit. Our results show that the content of TP may be a closely linked contributor to the radical scavenging capacity in loquat.

Correlations of radical scavenging capacity with total phenol and total flavonoid

DPPH scavenging activity had a positive and significant correlation with TF and TP, and the correlation coefficients (r) were 0.84 ($p < 0.05$) and 0.85 ($p < 0.05$), respectively. The results confirmed that TP mainly contributes to DPPH scavenging activity in loquat fruits. A significant correlation ($r \geq 0.892$) was observed between TP and antioxidant capacity under storage regimes in two loquat (*Eriobotrya japonica*) cultivars (Goulas et al., 2014). In contrast, vegetables such as onion (Khiari et al., 2009) and *Brassica oleracea* L. (Bidchol et al., 2011) had little correlation between phenolic content and antioxidant potential. In addition, a high correlation was found between DPPH scavenging activity and TF, with an r value of 0.84 ($p < 0.05$) in our study. The results show that TF is also an essential to radical scavenging capacity in loquat fruits.

Correlations between chlorophyll fluorescence, RGB intensity and total flavonoid, total phenol, DPPH scavenging activities

Our study used chlorophyll fluorescence measurements to detect the best ripening period and nutritional quality of loquat fruit. During the induction of the Kautsky effect, if the excitation light at the beginning was insufficient to cause the excitation of the PSII center, then the stable fluorescence signal Fo generated first is known as minimum fluorescence. The fluorescence reached the maximum value in this experiment, which was called Fm. The fluorescence that rises from Fo to Fm is Fv (the variable fluorescence), which is obtained by Fm – Fo. Fv/Fm can reflect the efficiency of light energy conversion of the PSII center. This technique has been successfully applied to the evaluation of fruit ripeness of mango, grape, papaya (Kolb et al., 2006), mulberry (Lou et al., 2012) and jujube (Lu et al., 2012). Furthermore, the content analysis of anthocyanins (Bahar et al., 2012; Ferrandino et al., 2017), phenols, and flavonoids in grape berries (Ferrandino et al., 2017), mulberry and jujube were evaluated without injury caused by chlorophyll fluorescence (Lou et al., 2012; Lu et al., 2012).

Fo, Fm, Fv and Fv/Fm decreased steadily from ST1 to ST7 (Figure 3). This is because the chlorophyll content decreases during the maturation of the loquat, and the chlorophyll fluorescence intensity emitted from the loquat is almost certainly affected by the chlorophyll content. An appropriate fit was established for the relationship between chlorophyll fluorescence parameters and TF, TP, and DPPH scavenging activities during loquat development (ST1-ST7) by using a polynomial model including step-by-step linear regression analysis and the regression (REG) procedure (Table 1). The optimal model for TF prediction was obtained using Fv and Fv/Fm, with an R^2 of 0.68. The optimal prediction model of TP was obtained by combining Fm, Fv, and Fv/Fm with an R^2 of 0.64. The optimal prediction model for DPPH radical scavenging activity from ST1 to ST7 was obtained by combining Fm and Fv, with an R^2 of 0.81 (Table 1). The prediction models for TF, TP, and DPPH scavenging activities were obtained respectively using all the R, G, and B intensity values, with R^2 values of 0.751, 0.426, and 0.392 during the developmental period of ST1-ST7 (Table 2).

Internal nutritional ingredients (TF, TP), DPPH scavenging activity, and chlorophyll fluorescence parameters during the ST1-ST5 developmental stages were also fitted by a polynomial model including stepwise linear regression analysis and the REG procedure (Table 1). Interestingly, we found that from ST1 to ST5, the optimally fitted equation for TF prediction was obtained using Fv and Fv/Fm, with R^2 of 0.79. The optimal fitted equation for TP prediction

Table 1 – Variables in model, correlation coefficients (R^2) and fitted equations in the prediction of total flavonoid (TF), total phenol (TP), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, during loquat fruit development. Model based on minimum fluorescence (Fo) = X1; maximum fluorescence (Fm) = X2; variable fluorescence (Fv) / maximum fluorescence (Fm) = X3; and Fv = X4, respectively; y represents the corresponding substance content; NS stands for no suitable fitting formula at the significance level (no correlation).

Stages (ST)	Chemical parameters	R^2	Variables in model	Fitted equations
ST1-ST7	TF	0.68	Fv, Fv/Fm	y = 204.93 – 349.31 X3 + 0.11 X4
	TP	0.64	Fm, Fv, Fv/Fm	y = 327.22 – 0.39 X2 – 374.29 X3 + 0.61 X4
	DPPH	0.81	Fm, Fv	y = 796.69 – 1.79 X2 + 2.42 X4
ST1-ST5	TF	0.79	Fv, Fv/Fm	y = 796.69 – 1.79 X2 + 2.42 X4
	TP	0.38	Fv	y = 54.32 + 0.06 X4
	DPPH	0.92	Fm, Fv, Fv/Fm	y = 3004.12 – 3.66 X2 – 3451.92 X3 + 5.25 X4
ST4-ST7	TF	NS		
	TP	0.99	Fo, Fv	y = 217.56 – 0.48 X1 + 0.12 X4
	DPPH	NS		

Table 2 – Variables in model, correlation coefficients (R^2) and fitted equations in the prediction of total flavonoid (TF), total phenol (TP), DPPH radical scavenging activity (DPPH), during loquat fruit development by the stepwise multiple linear regressions within the regression (REG) procedure model based on Red = R (X1), Green = G (X2), and Blue = B (X3) intensity values; y represents the corresponding substance content.

Stages (ST)	Chemical parameters	R^2	Variables in model	Fitted equations
ST1-ST7	TF	0.751	R, G, B	y = 147.39 – 0.014 X1 – 0.426 X2 – 0.613 X3
	TP	0.426	R, G, B	y = 135.27 + 0.263 X1 – 0.80 X2 + 0.51 X3
	DPPH	0.392	R, G, B	y = 468.60 + 0.322 X1 – 2.25 X2 + 6.85 X3
ST1-ST4	TF	0.722	R	y = 101.82 – 0.19 X1
	TP	0.998	B	y = –19.67 + 3.50 X3
	DPPH	0.970	B	y = –296.67 + 24 X3
ST5-ST7	TF	0.986	G	y = 174.24 – 0.830 X2
	TP	0.909	G	y = 223.35 – 1.067 X2
	DPPH	0.419	G	y = 663.28 – 1.98 X2

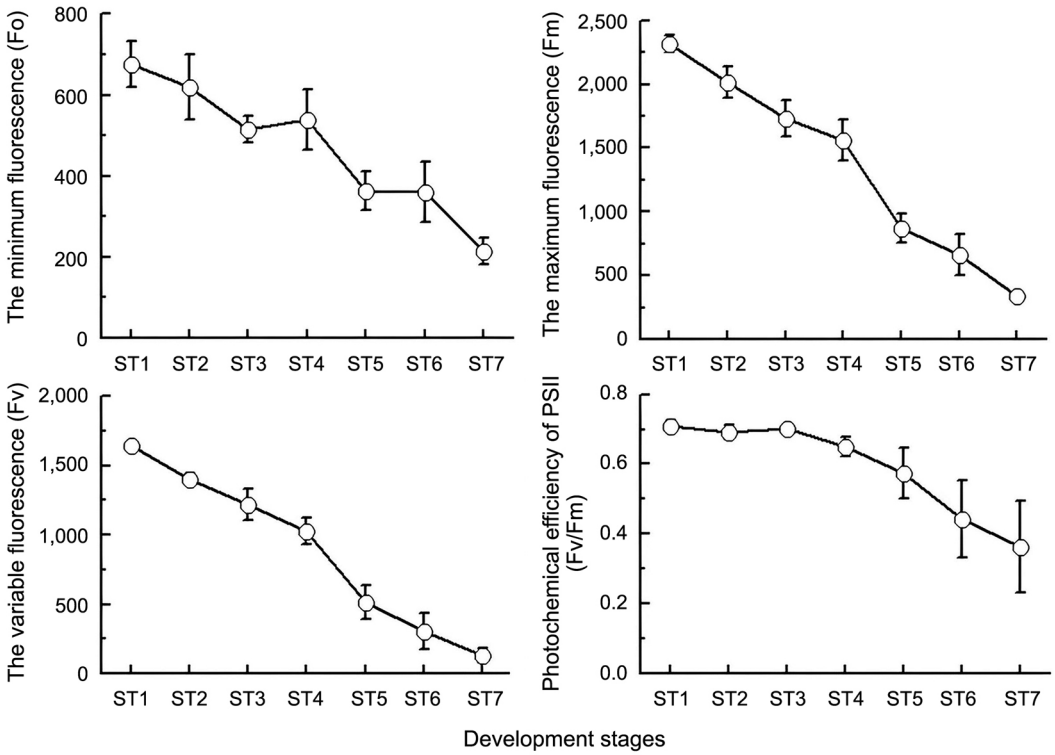

Figure 3 – Changes in minimum fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv) and Fv/Fm of chlorophyll fluorescence parameters during development of loquat fruit.

was obtained by combining Fv, with R^2 of 0.38, and the optimal model for predicting DPPH scavenging activity was obtained by using Fm, Fv, and Fv/Fm, with R^2 of 0.92. We also found that TP had a higher correlation with Fo and Fv in the last four stages (ST4-ST7), with an R^2 of 0.99. In contrast, TF and DPPH free radical scavenging activity had no significant correlation with chlorophyll fluorescence parameters. Fo, Fm, and Fv were positively correlated with DPPH scavenging activity followed by TP and TF ($0.82 \leq R^2 \leq 0.920$, $p < 0.05$) in the last four developmental stages (ST4-ST7) of mulberry fruit (Lou et al., 2012). A similar result was obtained where Fo, Fm, and Fv had prominent positive correlation with DPPH scavenging activity, followed by TP, and TF ($0.729 \leq R^2 \leq 0.920$, $p < 0.05$) in the later ripening developmental stages of jujube fruit (Lu et al., 2012). These results differ from those in our study of loquat fruit, which show that the correlations between chlorophyll fluorescence parameters and DPPH scavenging activity, TP, and TF in different fruit exhibit multiple patterns.

Compared to the four chlorophyll fluorescence parameters, correlations of RGB intensity with TF and TP were higher, and RGB intensity values were less

correlated with DPPH scavenging activities during the developmental period ST1-ST7. However, if we used different single color intensity values in the periods ST1-ST4 and ST5-ST7, the color values showed a better correlation with DPPH scavenging activity, TP, and TF. Total phenol showed the best correlation with B intensity value (R^2 = 0.998) in the period ST1-ST4 and with G intensity value (R^2 = 0.909) in the period ST5-ST7; DPPH scavenging activities were best correlated with B intensity value (R^2 = 0.970) in the period ST1-ST4 and with G intensity value (R^2 = 0.419) in the period ST5-ST7; total flavonoid had the best correlation with G intensity value (R^2 = 0.986) in the period ST5-ST7. In the last three stages, chlorophyll fluorescence and RGB parameters were suitable for estimating the content of TP due to the acceptable correlation (R^2 = 0.99, 0.909), and chlorophyll fluorescence performed better than RGB intensity values. RGB parameters were more suitable for analyzing the changes in TF, with an R^2 of 0.986 compared to chlorophyll fluorescence (no correlation). In the first few stages, chlorophyll fluorescence and RGB parameters were suited to estimating DPPH scavenging activities, with an R^2 of 0.92 during ST1-ST5 and an R^2 of 0.97 during ST1-ST4.

In conclusion, developmental stage ST5 is the optimum harvesting time for loquat fruit because it has sufficient storage tolerance and relatively high content of total phenol and flavonoid, and DPPH scavenging activity. Our study proved strong correlations between chlorophyll fluorescence, RGB intensity, and TF, TP, and DPPH scavenging activity in loquat fruit. Therefore, chlorophyll fluorescence parameters and RGB intensity values may be tools for quality assessment during loquat development, especially for the assessment of DPPH scavenging activity in the early stage (ST1-ST5) and total phenols in the last several developmental stages (ST4-ST7) by chlorophyll fluorescence and for the assessment of total flavonoid in the last three developmental stages (ST5-ST7) by RGB intensity. The high correlation between chlorophyll fluorescence and total phenol in loquat fruit during the ST4-ST7 developmental stages and between RGB intensity and total flavonoid in loquat fruit during the ST5-ST7 developmental stages indicated the feasibility of using chlorophyll fluorescence parameters and RGB intensity values to estimate the optimal picking time of loquat fruit noninvasively. Therefore, chlorophyll fluorescence and RGB intensity may serve as noninvasive methods for nutrient quality assessment during loquat development and for predicting the optimal time for harvesting loquat fruits.

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Authors' Contributions

Conceptualization: Lu H. **Project administration:** Lu H, Li S. **Supervision:** Lu H. **Funding acquisition:** Li S. **Writing-original draft:** Li S. **Visualization:** Li S. **Validation:** Li S. **Investigation:** Sun Y. **Formal analysis:** Sun Y. **Resources:** Lu H. **Methodology:** Lu H. **Data curation:** Li S. **Writing-review &editing:** Sun Y.

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