CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Non-destructive estimation of anthocyanin content in yardlong bean based on tristimulus values and reflectance spectra

Crop Breeding and Applied Biotechnology
23(4): e46112341, 2023
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332023v23n4a36

Maliwan Bunluephan[1], Chutima Chuenduang[1], Sunisa Suamuang[2], Kitiya Amkul[2], Kularb Laosatit[2], Anupun Terdwongworakul[3] and Orn-u-ma Tanadul[2*]

Abstract: *Traditionally, the quantification of anthocyanin content requires destructive sampling followed by chemical analysis with elaborate process. The aim of this study was to use non-destructive methods for prediction of anthocyanin by using color values from handheld colorimeter. Five hundred and fifty-eight yardlong bean samples that have pale green to dark purple pod color were used for color measurement and anthocyanin analysis. The partial least square regression (PLSR) was used to correlate spectral data with anthocyanin content. The PLSR model could predict anthocyanin content with correlation coefficient of 0.900 and standard error of prediction of 1.994 A/gFw. The results indicated that colorimeter readings can be correlated with anthocyanin content in yardlong bean and could be used as an alternative to chemical analysis. This method is reliable, rapid and inexpensive and can be used to screen a large number of yardlong bean accessions or populations under high anthocyanin breeding program.*

Keywords: *Anthocyanins, colorimetry, vegetable, rapid method*

INTRODUCTION

Yardlong bean [*Vigna unguiculata* ssp. *sesquipedalis* (L.) Verdc] is a subspecies of cowpea, also known as vegetable cowpea, sitao, asparagus bean, string bean and snake bean (Purseglove 1977). It is an important vegetable crop in many warm countries, including China, Japan, Korea and Thailand (Kongjaimun et al. 2012). Yardlong bean is a climbing vine which produces long tender pod and is harvested before reaching maturity and consumed as green pods or stir-fry dish. The pod of yardlong bean can be smooth or rough with linear curved or coiled shape. Pod color varies from pale green, green, dark green, purple, purple red to dark purple. The difference in pod color is due to the contents of chlorophyll and anthocyanin in the pod. The higher the anthocyanin contents, the darker the pod color (Kuswanto et al. 2013).

Anthocyanins are the most important pigments, after chlorophyll, and both are visible to human eyes. Anthocyanins are water-soluble phenolic compounds belonging to flavonoid group which are responsible for red, purple and blue color in fruits, vegetables (Melo et al. 2009) and grains (Fioreze et al. 2023). They are natural pigments which are present in almost all higher plants and provide attractive colors. Anthocyanins play a role in pollinator attraction, seed

***Corresponding author:**
E-mail: agromt@ku.ac.th
ORCID: 0000-0002-6947-6846

Received: 21 July 2023
Accepted: 09 August 2023
Published: 01 October 2023

[1] Kasetsart University, Faculty of Agriculture at Kamphaeng Saen, Nakhon Pathom 73140, Thailand
[2] Kasetsart University, Faculty of Agriculture at Kamphaeng Saen, Department of Agronomy, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
[3] Kasetsart University, Faculty of Engineering at Kamphaeng Saen, Department of Agricultural Engineering, Kamphaeng Saen, Nakhon Pathom, 73140, Thailand

dispersal, and tolerance to pests, diseases and water stress conditions (Delgado-Vargas et al. 2000, Kuswanto et al. 2013). In addition to the importance of anthocyanin to plants, it is classified as an antioxidant which has anti-aging properties as vitamin C and prevents diseases in humans, such as heart disease and vascular disease, besides improving visual acuity (Khoo et al. 2017). Interest in the beneficial properties of anthocyanin on human health stimulate plant breeders to breed a new variety of yardlong bean with high amount of anthocyanin (Kuswanto et al. 2013). The quantification of anthocyanin components can be extremely complicated, including wet chemical extraction, separation and subsequent characterization of its components. Those procedures are very important to food technologists and horticulturists in order to assess the quality of products. For plant breeders, however, plant genetic germplasm screening is a prerequisite for selecting parental plants and later screening in population. Due to large number of samples, an easy, rapid, and accurate method is required by plant breeders.

Several methods for anthocyanin measurement have been reported, such as high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR), thin-layer chromatography, column chromatography and UV–visible absorption spectroscopy (Skrede and Wrolstad 2002, Takeoka and Dao 2002, Teng et al. 2020). The measurement of anthocyanin content can be influenced by the method used for analysis. Some reports showed that anthocyanin measurement by HPLC is better than by the pH differential method because HPLC method yields higher values, not because of its performance (Sachez-Moreno et al. 2003, Wu et al. 2004, Wu et al. 2006). Later Lee and Finn (2007) and Lee et al. (2008) confirmed that anthocyanin contents measured from the HPLC method were higher than those obtained by the pH differential method. Nevertheless, those methods rely on complicated and expensive equipment and time-consuming sample preparation. Moreover, researchers need to understand the operation of those machines.

The easiest method has been developed to measure anthocyanin content by using organic solvents under acidic condition to extract anthocyanin, and then optical characteristic across the visible spectrum by spectrophotometer was used to quantify the content of anthocyanin (Solovchenko et al. 2001). However, this method still produces hazardous waste, which has impacts on environment and human health. Compared with benchtop spectrophotometers, portable spectrophotometer is a handheld color analyzer used in scientific research to measure the intensity of a color by selective absorption of a specific light (Crocombe 2018). Portable spectrophotometer is a miniaturized benchtop spectrophotometer with the same technology. It was created for outside laboratory testing, so it can be used at the sampling site. It is affordable, easy to use, with rapid measurement, and can be employed to obtain reliable results without technicians or person with specific training. The only limitation of portable spectrophotometer is the narrow wavelength (Sanmartin et al. 2020). However, if the target wavelength falls within the machine's detection range, portable spectrophotometers are more suitable.

A tristimulus colorimeter was used to estimate anthocyanin concentration in intact raspberry fruit (Moore 1996). Hue angle, b* and L*, which were measured soon after thawing the intact raspberry fruit, showed good correlation with coefficient of determination, $R^2 = 0.76$ against the anthocyanin concentration. Vieira et al. (2018) used a portable colorimeter to predict anthocyanin content in phenolic extracts of fruits and vegetables. A simple mathematical model was developed and yielded good prediction performance with high correlation (r = -0.95) between the L* value and the extracts produced using 70% ethanol.

There have been previous reports on the use of a portable colorimeter for estimating the anthocyanin content. However, the use of visible reflectance spectra for developing a model to estimate the anthocyanin content in yardlong beans has not been documented. Hence, the main objective of this study was to concentrate on employing a portable colorimeter for estimating the anthocyanin content in intact yardlong beans. The predictors considered for this estimation include L*, a*, b*, gloss, and reflectance spectrum.

MATERIAL AND METHODS

Plant materials

Artificial pollination was made between yardlong bean purple pod variety and green pod variety to produce F_1 seeds at experimental field at Department of Agronomy, Faculty of Agriculture Kamphaeng Saen, Kasetsart University. The

F_1 seeds were grown and allowed to self-pollinate to produce F_2 seeds. Then, all the F_2 seeds were grown and allowed to self-pollinate. In total, 558 pod samples were collected at 9 days and 12 days after flowering and used for color measurement and anthocyanin analysis.

Color measurement

Fresh immature and mature yardlong pods were harvested and directly subjected to sample preparation. Each yardlong bean pod was cut into 15 cm long pieces by cutting the upper and lower parts off. The sample was cut again along the valves of the pod in order to make the sample surface fully cover the head of the device. The samples were measured for optical reflectance in the visible range of 400-700 nm three replicates using a handheld colorimeter (Spectro-guide, Sphere gloss BYK-Gardner, USA). The measurement was expressed in terms of CIE 'L*' (lightness), 'a*' (redness and greenness), 'b*' (yellowness and blueness), and reflectance spectrum in a wavelength range from 400 to 700 nm. The sensor was standardized using a white, black and green tile. After color measurement, the same sample was sliced into small pieces for anthocyanin determination.

Anthocyanin analysis

Anthocyanin content measurement was slightly modified from Taghavi et al. (2020). Briefly, yardlong bean pod samples from color measurement were cut into small pieces. Then, 0.5 grams of the sample were weighed and placed into 15 ml tube. Anthocyanins were extracted by adding 5 mL of methanol:concentrated HCl (0.1%) and incubated at 4 °C for 6 h. The extract was filtrated through qualitative cellulose filter paper. The filtrate was measured by UV-Vis spectrophotometer at 530 and 657 nm. Anthocyanin concentration was determined by the following formula and was given as A/g fresh tissue, where TA = total anthocyanin (A/gFw), A = absorbance at 530 and 657 nm, V = volume of extract (ml) and M = fresh weight of the sample (g) (Jayakumar et al. 1999).

$$TA = \frac{\mathrm{A530} - 0.3\mathrm{A657} * \mathrm{V}}{M}$$

Development of prediction model

After obtaining data from measuring all samples, including the anthocyanin content, visible light reflectance value, L*, a*, b* values, and gloss, equations were created to predict the anthocyanin content of yardlong bean. To prepare data for equation development, the sample data was divided into two groups: a calibration set of 66.7% and a prediction set of 33.3%. Both groups had a similar distribution of anthocyanin values, and the range of the anthocyanin values in the calibration set covered those in the prediction set. The samples were arranged in ascending order of anthocyanin value, and then the samples were alternately assigned to the two groups at a ratio of 2 to 1, starting with the samples with the lowest and highest anthocyanin values in the calibration set.

After dividing the data into the two groups, the visible light reflectance value, L*, a*, b* values, and gloss and the anthocyanin value of the calibration set were analyzed to create a prediction equation. Partial Least Squares Regression (PLSR) was used to develop a prediction model with The Unscrambler V.9.8 software (Camo, Oslo, Norway). The PLSR method was employed to address multicollinearity, which occurs when there are strong correlations between predictors, leading to instability in coefficient estimates. PLSR transforms the original predictors into a smaller set of uncorrelated latent variables, which are then substituted for the original predictors in the model. Moreover, the optimal number of latent variables was determined using the leave-one-out cross-validation technique. The reduction in the number of predictors achieved through PLSR proves valuable when working with high-dimensional data, where the number of predictors exceeds the number of observations.

After obtaining the appropriate equations, they were used to predict the anthocyanin content of the samples in the prediction set. Subsequently, the predicted anthocyanin values were compared to the measured anthocyanin values. The error was calculated as the standard error of prediction (SEP) using Equation (1), and bias was calculated using Equation (2).

$$SEP = \sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - y_i - Bias)^2}{n - 1}} \quad (1)$$

where:

$\hat{y}$ is predicted anthocyanin content;

y is measured anthocyanin content; and

n is the number of samples.

$$Bias = \frac{\sum_{i=1}^{n} (\hat{y}_i - y_i)}{n} \quad (2)$$

RESULTS AND DISCUSSION

Change in colorimetric components in relation to anthocyanin content

The pod color of F_2 yardlong bean population varied from pale green to dark purple (Figure 1A). The total anthocyanin content varied from 0.196 A/gFw in green pod color to 19.451 A/gFw in purple pod color (Figure 1B). The colorimetric parameters (L*, a* and b*) were used to characterize the color of yardlong bean. The L* represents lightness of the sample, with values ranging from 0 (black) to 100 (white). The a* and b* values indicate red-green and yellow-blue components of a color, respectively. The a* and b* have no specific numerical limits. Positive a* is red and negative a* is green, while positive b* is yellow and negative b* is blue. The results showed that the lowest anthocyanin content (0.196 A/gFw) had L*, a* and b* values of 67.72, -7.81 and 25.43, respectively. The highest anthocyanin (19.451 A/gFw) had L*, a* and b* values of 25.40, 15.64 and 2.55, respectively. The higher content of anthocyanins influenced the darker pod color, and lightness decreased. The a* value was negative for all green-colored pods and positive for all purple-colored pods, meaning that this value is highly related to color; however, there was no clear relationship between a* and anthocyanin content. The b* value of purple pods ranged from 0.63 to 11.82, while the value of green pods ranged from 13.32 to 30.33. The b* value was clearly separated between green and purple pod, except for only one purple pod sample, which had b* value of 13.57. Pods with high anthocyanin content tend to have low b* value. All the samples had a positive b* value, indicating that there was little or no blue color on the yardlong pod.

The difference in spectral reflectance feature of low, medium and high anthocyanin content in yardlong pod can be clearly seen in Figure 2. The low anthocyanin content sample (0.36 A/gFw) had higher reflectance than those with medium (8.41 A/gFw) and high (19.45 A/gFw) anthocyanin contents. An increase in anthocyanin content caused even more decrease in the green reflectance. A distinctive reflectance behavior of these yardlong bean pods was a peak in the red range around 700 nm. This agrees with the report of Merzlyak et al. (2003), who proposed to use reflectance at 700 nm to avoid chlorophyll interference for assaying anthocyanin content.

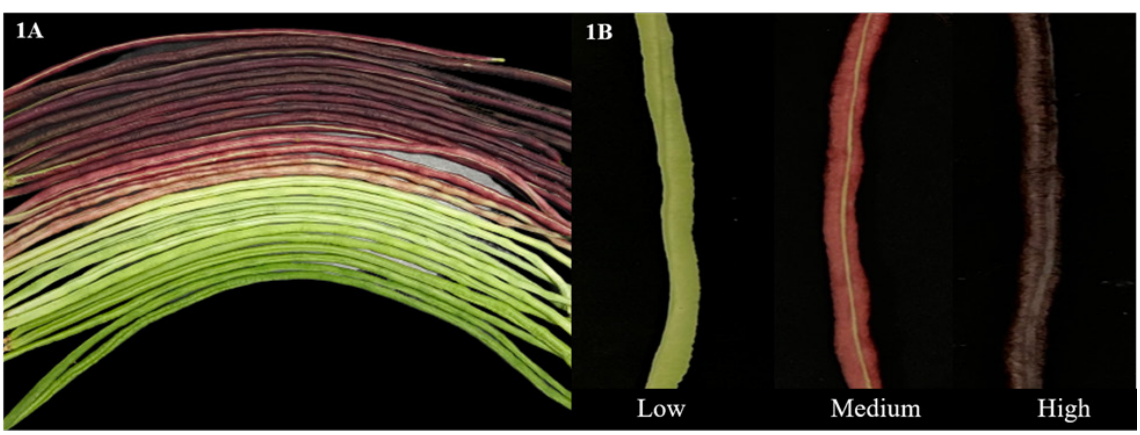

Figure 1. The variation of F_2 yardlong bean pod color (A) and yardlong bean pods that contain low, medium and high anthocyanin contents, respectively (B).

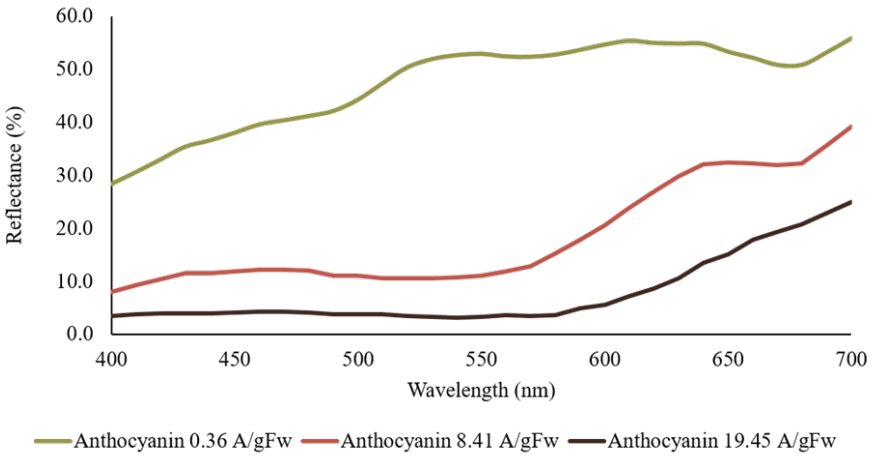

Figure 2. Effect of anthocyanin content on reflectance spectra of low, medium and high anthocyanin content in yardlong bean pod.

Correlations between anthocyanin content and colorimetric parameters

The anthocyanin content correlated with all colorimetric parameters, ranging from 0.243 (reflectance at 630 nm) to 0.862 (reflectance at 700 nm) (Table 1). Negative correlations were found between the anthocyanin content and L*, b*, gloss, and reflectance in the 500 to 620 nm range. This indicates that yardlong bean with a strong color ranging from green to orange tends to have a low anthocyanin content. However, a* and reflectance in the 400 to 490 nm range, as well as in the 630 to 700 nm range, positively correlated with the anthocyanin content.

Anthocyanin prediction

The anthocyanin content of yardlong bean samples ranged from 0.196 to 19.451 A/gFw (Table 2). Green-colored pods exhibited anthocyanin levels ranging from 0.196 to 1.535 A/gFw, while purple-colored pods had a higher levels and broader range of 1.591 to 19.451 A/gFw. These results indicate a correlation between pod color and anthocyanin content.

The prediction model was developed using PLSR based on the calibration set's sample data, and showed good performance in predicting the anthocyanin content of yardlong beans (Table 3). The correlation coefficient and the standard error of prediction obtained from the independent prediction set, distinct from the calibration set, were determined as 0.900 and 1.994 A/gFw, respectively (Figures 3 and 4). The accuracy of the prediction was comparable to the prediction of intact raspberry's anthocyanin concentration (r = 0.872), as estimated using the hue angle, b*, and

Table 1. Correlation coefficients between colorimetric parameters against anthocyanin content

Colorimetric parameters	r**	Colorimetric parameters	r	Colorimetric parameters	r	Colorimetric parameters	r
L*	-0.827	R450	0.835	R540	-0.795	R630	0.243
a*	0.686	R460	0.844	R550	-0.796	R640	0.592
b*	-0.851	R470	0.836	R560	-0.799	R650	0.703
Gloss	-0.491	R480	0.746	R570	-0.815	R660	0.781
R400*	0.755	R490	0.374	R580	-0.841	R670	0.799
R410	0.793	R500	-0.618	R590	-0.855	R680	0.811
R420	0.815	R510	-0.739	R600	-0.875	R690	0.847
R430	0.824	R520	-0.786	R610	-0.847	R700	0.862
R440	0.831	R530	-0.791	R620	-0.405		

* R400 = Reflectance at wavelength of 400 nm

** r = Correlation coefficient

Table 2. Descriptive statistics of anthocyanin content of samples in the calibration and prediction sets

	Min (A/gFw)	Mean (A/gFw)	Max (A/gFw)	Standard deviation (A/gFw)
Calibration set	0.196	5.477	19.451	4.538
Prediction set	0.268	5.448	17.766	4.471

Table 3. Anthocyanin prediction performance of the model developed using the partial least squares regression

Prediction variables	Number of factors	R	SEC[a] (A/gFw)	R	SEP[b] (A/gFw)	Bias (A/gFw)	Rc^{2c}	RMSE[d] (A/gFw)	Rp[e]	RMSE[f] (A/gFw)
L*, a*, b*, gloss, and reflectance in the range of 400 to 700 nm	11	0.925	1.736	0.900	1.994	0.087	0.855	1.734	0.804	1.990

[a] SEC = Standard error of calibration

[b] SEP = Standard error of prediction

[c] Rc^2 = coefficient determination of calibration

[d] RMSEC = Root mean square error of calibration

[e] Rp^2 = coefficient determination of prediction

[f] RMSEP = Root mean square error of prediction

L* values (Moore 1996). Lower R^2 value (0.59) was obtained in prediction of anthocyanin content in apple by the ratio of $(a^*/b^*)^2$ (Singha et al. 1991).

The variable that had the most significant impact on anthocyanin prediction was a* (green to red), as indicated by its highest absolute weighted regression coefficient. The second-best contributing variable was reflectance at 700 nm. The a* value represents the color component that spans from green (-a*) to red (+a*). This corresponded to the anthocyanin measurements taken using a spectrophotometer at 530 nm (green color) and 657 nm (red color).

This study demonstrated that the colorimetric method could effectively replace the spectrophotometric method for quantifying anthocyanins in intact yardlong beans. By using the colorimetric method, not only costs and time could be reduced, but the quantity of both toxic and non-toxic reagents used in traditional methods was also diminished.

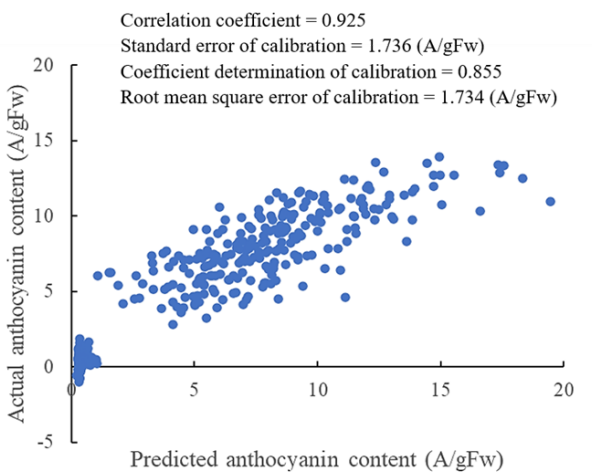

Figure 3. Scatter plot showing correlation between predicted anthocyanin content and measured anthocyanin content of the samples in the calibration set.

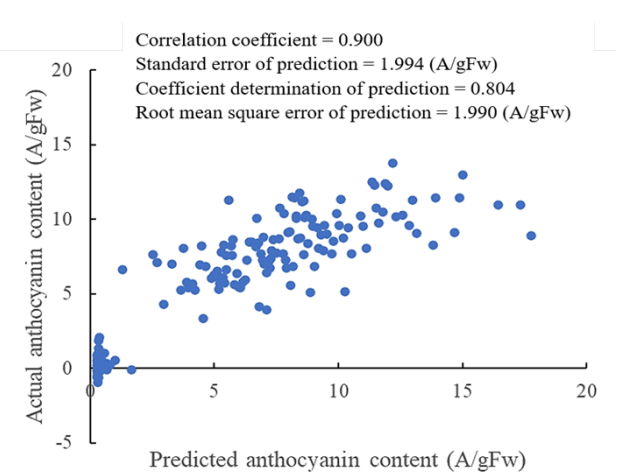

Figure 4. Scatter plot showing correlation between predicted anthocyanin content and measured anthocyanin content of the samples in the prediction set.

CONCLUSIONS

This is the first report of measuring anthocyanin content in yardlong bean by using colorimeter. The partial least squares regression model was developed, exhibiting a high correlation coefficient, which enables the prediction of anthocyanin content in yardlong beans. This model used the tristimulus values obtained from intact yardlong beans, measured using a colorimeter. The accuracy of the prediction was deemed satisfactory, allowing the model to serve as an alternative to the laboratory method. The conventional laboratory method relied on absorbance measurements at 530 nm and 657 nm, was destructive, and posed environmental hazards. The results conclusively demonstrated the feasibility of employing colorimetry as a means of screening anthocyanin content in yardlong beans.

ACKNOWLEDGMENTS

This research project was financially supported by Agricultural Research Development Agency (ARDA), Thailand no. PRP6305030170. We would like to thank Ms. Tarika Yimram for field work assistance. We also extend thanks to other graduate students who attended this project.

REFERENCES

Crocombe RA (2018) Portable spectroscopy. **Applied Spectroscopy 72**: 1701-1751.

Delgado-Vargas F, Jiménez AR and Paredes-López O (2000) Natural pigments: carotenoids, anthocyanins, and betalains characteristics, biosynthesis, processing, and stability. **Critical Reviews in Food Science and Nutrition 40**: 173-289.

Fioreze A, Konkol A, Spanholi D and & Fioreze S (2023). Evidence of maternal effect on the inheritance of flax (*Linum usitatissimum* L.) seed coat color. **Crop Breeding and Applied Biotechnology 23(2)**: e449423210

Jayakumar M, Eyini M, Selvinthangadurai P, Lingakumar K, Premkumar A and Kulandaivelu G (1999) Changes in pigment composition and photosynthetic activity of aquatic fern (*Azolla microphylla* Kaulf.) exposed to low doses of UV-C (254 nm) radiation. **Photosynthetica 37**: 33-38.

Khoo HE, Azlan A, Tang ST and Lim SM (2017) Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. **Food & Nutrition Research 61**: 1361779.

Kongjaimun A, Kaga A, Tomooka N, Somta P, Vaughan DA and Srinives P (2012) The genetics of domestication of yardlong bean, *Vigna unguiculata* (L.) Walp. Ssp. *unguiculata* cv.-gr. *Sesquipedalis*. **Annals of Botany 109**: 1185-1200.

Kuswanto K, Waluyo B and Hardinaningsih P (2013) Segregation and selection of observed yardlong bean (*Vigna sesquipedalis L. Fruwirth*) to get expected lines of purple pod. **Journal of Agricultural Science and Soil Science 3**: 88-92.

Lee J and Finn CE (2007) Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. **Journal of the Science of Food and Agriculture 87**: 2665-2675.

Lee J, Rennaker C and Wrolstad RE (2008) Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. **Food Chemistry 110**: 782-786.

Melo MJ, Pina F and Andary C (2009) Anthocyanins: nature's glamorous palette. In Stevens CV (ed) **Handbook of natural colorants.** John Wiley & Sons, New York, p. 135-150.

Merzlyak MN, Gitelson AA, Chivkunova OB, Solovchenko AE and Pogosyan SI (2003) Application of reflectance spectroscopy for analysis of higher plant pigments. **Russian Journal of Plant Physiology 50**: 704-710.

Moore PP (1996) Estimation of anthocyanin concentration from colorimeter measurements of raspberry fruit. **HortScience 31**: 640e-640.

Purseglove JW (1974) **Tropical crops: dicotyledons**. Longman Group Ltd., London, 719p.

Sachez-Moreno C, Cao G, Ou B and Prior RL (2003) Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. **Journal of Agricultural and Food Chemistry 51**: 4889-4896.

Sanmartin P, Gambino M, Fuentes E and Serrano M (2020) A simple, reliable, and inexpensive solution for contact color measurement in small plant samples. **Sensors 20**: 2348.

Singha S, Baugher TA, Townsend EC and D'Souza MC (1991) Anthocyanin distribution in 'Delicious' apples and the relationship between anthocyanin concentration and chromaticity values. **Journal of the American Society for Horticultural Science 116**: 497-499.

Skrede G and Wrolstad RE (2002) Flavonoids from berries and grapes. In Shi J, Mazza G and Maguer ML (eds) **Functional foods: Biochemical and processing aspects.** CRC Press LLC, New York, p. 71-133.

Solovchenko AE, Chivkunova OB, Merzlyak MN and Reshetnikova IV (2001) A spectrophotometric analysis of pigments in apples. **Russian Journal of Plant Physiology 48**: 693-700.

Taghavi T, Patel H and Rafie R (2020) Comparing pH differential and methanol-based methods for anthocyanin assessments of strawberries. **Food Science and Nutrition 10**: 2123-2131.

Takeoka G and Dao L (2002) Anthocyanins. In Hurst WJ (ed) **Methods of analysis for functional foods and nutraceuticals.** CRC Press LLC, Florida, p. 219-241.

Teng Z, Jian X, He F and Bail W (2020) Qualitative and quantitative methods to evaluate anthocyanins. **eFood 1**: 339-346.

Vieira LM, Marinho LMG, Rocha JDCG, Barros FAR and Stringheta PC (2018) Chromatic analysis for predicting anthocyanin content in fruits and vegetables. **Food Science and Technology 39**: 415-422.

Wu X, Beecher GR, Holden JH, Haytowitz DB, Gebhardt SE and Prior RL (2006) Concentration of anthocyanins in common foods in the United States and estimation of normal consumption. **Journal of Agricultural and Food Chemistry 54**: 4069-4075.

Wu X, Gu L, Prior RL and McKay S (2004) Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity. **Journal of Agricultural and Food Chemistry 52**: 7846-7856.