Original Article

Thai Journal of Pharmaceutical Sciences

# Simple and rapid spectrophotometric method for quality determination of roselle (*Hibiscus sabdariffa*)

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**Received:** June 9, 2016
**Accepted:** Nov 15, 2016
**Published:** Dec 01, 2016

**Keywords:**
Anthocyanins, chemometrics, *Hibiscus sabdariffa*, phenolic compounds, quality, spectrophotometry

## ABSTRACT

A simple and rapid spectrophotometric method has been developed to determine the quality of roselle (*Hibiscus sabdariffa*). The absorbance at 530 nm (coloring intensity) and the ratio between the absorbances at 380 and 530 nm (degradation index) of its 50% methanol extract were suggested as the criterion parameters to discriminate between the qualified and unqualified samples. The contents of total anthocyanins and total phenolic compounds were also estimated by the partial least square and multiple linear regression models established from the absorbances at 330, 380, and 530 nm. Their reliability was indicated by the low root-mean-square error of prediction = 0.11-0.13 and high correlation between the actual and prediction values of the tested samples ($R$ = 0.99).

## INTRODUCTION

Roselle (*Hibiscus sabdariffa* L. family Malvaceae) is a worldwide popular herb. Its pharmacological activities and clinical studies have been investigated, and many extensive reviews have been published [1-4]. The main activities were, for examples, antioxidation, reducing blood pressure in pre- and mild-hypertensive patients, improving lipid profile, and diuretic. Its main phytochemical constituents were organic acids, anthocyanins, and other phenolic compounds [1,2]. The official quality specification of roselle is published in European Pharmacopoeia; acid content is assayed and coloring intensity is determined [5]. Thailand and China were the world's largest suppliers [6]. However, there was only one comprehensive study on the quality of Thai roselle [7], and none of the publication has been found for the Chinese roselle. Most of the quality study was on the African roselle [8-13]. The quality of Sudanese roselle was said to be the best but its production quantity was low and all was exported to Germany. The other important producers were Mexico, Egypt, Senegal, Tanzania, Mali, and Jamaica but the production is mostly used domestically [6]. The other two quality studies of roselle were that from Malaysia [14] and Turkey [15]. Beside the general quality control parameters, the total titratable acid and the contents of its active constituents, i.e., anthocyanins and phenolic compounds have been reported [5,7,8,10,12,14-16]. The total contents of anthocyanins and phenolic compounds were determined by spectrophotometric and Folin-Ciocalteu methods, respectively, whereas the contents of two main anthocyanins (delphinidin 3-sambubioside and cyanidin 3-sambubioside) were analyzed by high performance liquid chromatography [12].

One obstacle for the quantitation of total anthocyanin content in routine work is the high expenditure of standard anthocyanins. The reddish color of roselle, which referred

to the color of anthocyanins, was the alternative quality parameter [5,12,15,16]. Visual inspection is the easiest technique to classify between the qualified and the unqualified samples [7,11] and it was one of the commercial specification [6,13]. However, some borderline samples are not easy to be discriminated due to human visual limitation. Color of a sample could be more accurately determined by measuring for its spectrophotometric absorbance at 520 nm [5] or CIELAB color [12]. Furthermore, the contents of total anthocyanins and each of its main anthocyanins could be estimated from CIELAB color [17]. However, different cultivar of roselle possesses different color. It is orange-red, dark purple, and purplish-red for the samples from Sudan, China, and Thailand, respectively [6]. The quality of roselle produced in Thailand has been investigated [7] but its color measurement has never been studied. Therefore, this study suggested a simple and rapid method for the quality determination of Thai roselle based on its color using spectrophotometry. Moreover, the models for prediction of the contents of total anthocyanin and total phenolic compounds were also constructed from ultraviolet/visible (UV/Vis) spectral data using chemometric methods, i.e., partial least square (PLS) and multiple linear regression (MLR).

## MATERIALS AND METHODS

### Plant Materials

32 roselle samples throughout Thailand were purchased from local market during 2003 to 2004. They were identified by Associate Professor Uthai Sotanaphun. The voucher specimens (US03001-US03020 and US04001-US04012) are deposited at the Herbarium of the Department of Pharmacognosy, Silpakorn University, Thailand. Their quality grading was assigned based on their color as reddish group ($n$ = 16) and brownish group ($n$ = 16). 60% of samples (10 reddish samples and 9 brownish samples) were randomly selected as training set, and the rest samples were used as test set. All samples were dried, ground to fine powder, passed through sieve mesh 0.250 mm, stored at 4°C and warmed to room temperature before use.

### UV/Vis Spectroscopic Data

Exactly 1.0000 g of the fine sample was extracted by sonication with 5 ml of 50% methanol for 15 min. The extract (0.2 ml) was diluted with 0.8 ml of 50% methanol and mixed well. The solution (0.3 ml) was further diluted and mixed well with 4.9 ml of 50% methanol. UV/Vis spectra in the range of 190-1100 nm of the final solution were collected by a UV/Vis spectrophotometer (Agilent 8453 Model G 11C3A, USA).

### Determination of Total Anthocyanin and Total Phenolic Contents

All data of the contents of total anthocyanins (% calculated as delphinidin 3-sambubioside) and total phenolics (% calculated as gallic acid) of all samples were from our previous study (Table 1) [7].

### Data Analysis

Principal component analysis (PCA), PLS, and MLR were carried out using the Unscramble 9.8® (Camo Process AS, Norway). Data of all UV/Vis spectra (250-650 nm) were pretreated by subtraction with the absorbance at 700 nm before analyses (Figure 1).

## RESULTS AND DISCUSSION

This was the continuous study on the quality control of roselle of Thailand. 32 samples and the analysis result of their total anthocyanin and total phenolic contents were obtained from our previous investigation (Table 1) [7]. Based on the color visualized by naked eye, all samples ($n$ = 32) were divided into reddish (qualified) and brownish (unqualified) groups. Total anthocyanin contents of all reddish samples were more than 1.0% calculated as delphinidin 3-sambubioside, suggesting this value as a critical value for quality grading of roselle samples. However, standard anthocyanin was need for the analysis. It is expensive and not easy to purify in laboratory. To

**Table 1:** Summarized total anthocyanins content (% calculated as delphinidin 3-sambubioside) and total phenolic content (% calculated as gallic acid) of all sample set of reddish and brownish roselle (mean±SD [minimum-maximum])

| Sample set ($n$) | Total anthocyanins | Total phenolics |
|---|---|---|
| Reddish | | |
| Total (16) | 1.80±0.56 | 3.01±0.40 |
| | (1.04-2.95) | (2.52-3.58) |
| Training (10) | 1.72±0.50 | 3.03±0.40 |
| | (1.04-2.64) | (2.52-3.58) |
| Test (6) | 1.92±0.68 | 3.00±0.45 |
| | (1.24-2.95) | (2.52-3.57) |
| Brownish | | |
| Total (16) | 0.50±0.20 | 2.27±0.34 |
| | (0.05-0.68) | (1.51-2.64) |
| Training (9) | 0.53±0.20 | 2.26±0.34 |
| | (0.05-0.68) | (1.61-2.64) |
| Test (7) | 0.47±0.20 | 2.28±0.36 |
| | (0.06-0.66) | (1.51-2.55) |

SD: Standard deviation

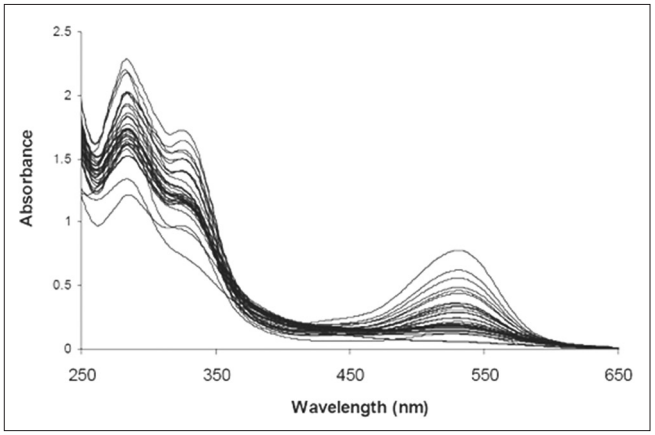


**Figure 1:** Ultraviolet/visible spectra of 50% methanol extracts of all samples in the range of 250-650 nm

develop a fast, simple, and reliable method for quality grading in routine work, 60% of samples (training set, $n$ = 19) were randomly selected, and the rest samples (test set, $n$ =13) were used to challenge the method. All samples were extracted with 50% methanol to ensure that anthocyanins and other phenolic compounds could be dissolved in the solvent. UV/Vis spectra of the extracts of all samples were measured, and the results are shown in Figure 1.

The difference between the spectra of the reddish and brownish samples in the training set was studied by PCA. As shown in Figure 2, the score and loading plots of PC1 versus PC2 explained for 97% of the variables. The reddish and brownish groups were clearly distinguished at the opposite side of the diagonal between the axes of PC1 and PC2 (Figure 2a). The reddish samples were mainly located in the quarter between the positive axis of PC1 and the negative axis of PC2 which corresponded to the higher absorbances at 330 and 530 nm, and the lower absorbance at 380 nm (Figure 2b) than the brownish samples. $A_{380}$ was the absorbance of browning degradation products of anthocyanins. It was possibly interfered by other constituents and shifted from generally at 420 nm [18]. However, $A_{380}$ of the reddish group (0.260 ±0.040) was not significantly lower than that of the brownish group (0.301 ± 0.051). $A_{330}$ and $A_{530}$ were the maximum absorption wavelengths of hydroxycinnamates and anthocyanins, respectively, with little shifts from the references [19]. Even though $A_{330}$ of the reddish group (1.388 ± 0.214) was higher than that of the brownish group (1.129 ± 0.123), their ranges (1.126-1.696 and 0.917-1.288, respectively) were overlapped. $A_{530}$ or coloring intensity [5] was the only wavelength that the ranges between the two groups were separately. They were 0.215-0.620 (0.372 ± 0.129) and 0.058-0.200 (0.159 ± 0.045), respectively. $A_{530}$ of all reddish samples were more than 0.200. Therefore, this value might be assigned as a cut-off criterion for the grading distinguishing between the qualified (reddish) and the unqualified (brownish) roselle. However, their different was very small and easy to error. Degradation index was the other suggested parameter. It has been generally used to explain the quality of the natural products containing anthocyanins. It was calculated from the ratio between $A_{420}$ and $A_{520}$ [18]. In this study, $A_{380}$ and $A_{530}$ were used for instead. Degradation indexes of the reddish and brownish samples were 0.433-1.133 (0.761 ± 0.221) and 1.447-5.614 (2.173 ± 1.309), respectively. Their difference was obviously enlarged. The lower degradation index indicated the better quality of the sample. The value of not more than 1.2 might be suggested as the cut-off criterion between the qualified and the unqualified samples. Using of both coloring intensity and degradation index for quality grading was validated by the samples in the test set. The results are shown in Figure 3. Coloring intensity and degradation indexes of all reddish and brownish samples in the test set were in accordance with above quality criteria.

For more detail on the contents of total anthocyanins and total phenolic compounds, PLS models based on UV/Vis spectral data in the range of 250-650 nm were developed, and the results are shown in Figure 4. The optimal number of PLS factors was determined by the lowest root-mean-square error of cross-validation (RMSECV) obtained from the cross-validation process. The models were tested by the tested sample set and the error of prediction was expressed as root-mean-square error of prediction (RMSEP). The validation statistics of the models of total anthocyanins and total phenolics are shown in Tables 2 and 3, respectively. Both models used only 2 PLS factors and had satisfied validation results (RMSEP = 0.1502 and 0.1093, respectively). However, PLS model constructed from variable-rich spectroscopic data set compare to the number of samples possibly caused an overfitting problem and misleading prediction results of unknown samples [20]. Therefore, only data really involving the models should be included. As indicated in the previous section, $A_{330}$, $A_{380}$, and $A_{530}$ were the important data discriminating between the qualities of roselle samples. High values of regression coefficients of these wavelengths in PLS models (Figure 4) also suggested them as the high influencing variables which referred to the quality of the samples. Regression coefficients of $A_{330}$ and $A_{530}$ were the positive values, whereas that of $A_{380}$ was negative. These data confirmed the results of PCA that the qualified (reddish) samples had higher $A_{330}$ and $A_{530}$, and lower $A_{380}$ than the unqualified (brownish) samples. Then, PLS and MLR models using only these three variables were constructed. A better prediction was obtained for the models of total anthocyanins (RMSEP = 0.1091 and 0.1151 for PLS and MLR models, respectively) (Table 2). Only $A_{380}$ and $A_{530}$ were significant in MLR model. However, when only these two wavelengths were included in the model,

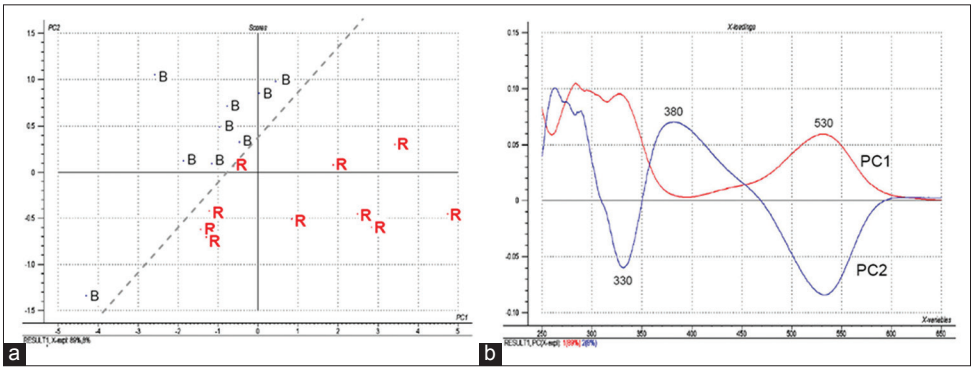


**Figure 2:** Principal component analysis of training set, (a) score plot and (b) loading plot, using ultraviolet/visible spectra in the range of 250-650 nm; R = Reddish samples, B = Brownish samples

the validation result was not satisfied (RMSEP = 0.2083). Prediction results of the tested samples using these models are shown in Table 4. The effective and simple models for the prediction of total anthocyanin content are as follows:

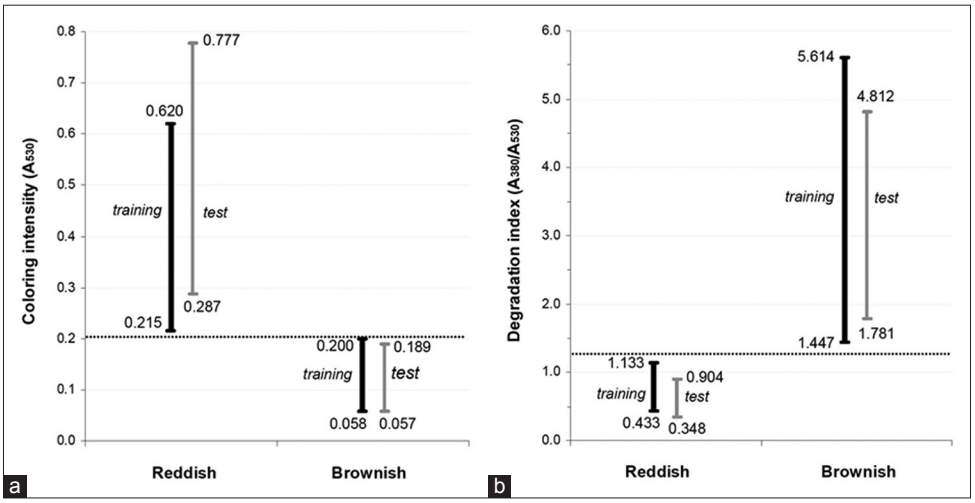


**Figure 3:** (a) Coloring intensity and (b) degradation indexes of reddish and brownish samples of training and test set

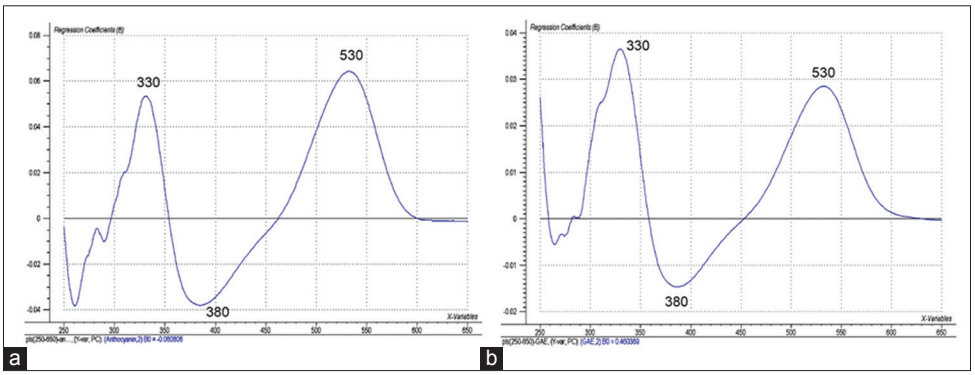


**Figure 4:** Partial least square prediction models of (a) total anthocyanin content (% calculated as delphinidin 3-sambubioside) and (b) total phenolic content (% calculated as gallic acid) using ultraviolet/visible spectra in the range of 250-650 nm

**Table 2:** Validation parameters of the prediction models of total anthocyanin content

| Method | Wavelength (nm) | Number of PLS factor | RMSECV | *R* (LOOCV)[a] | RMSEP | *R* (test)[b] |
|---|---|---|---|---|---|---|
| PLS | 250-650 | 2 | 0.1503 | 0.9768 | 0.1502 | 0.9314 |
| PLS | 330, 380, 530 | 2 | 0.1353 | 0.9812 | 0.1091 | 0.9935 |
| MLR | 330, 380, 530 | - | 0.1443 | 0.9788 | 0.1151 | 0.9921 |
| MLR | 380, 530 | - | 0.1506 | 0.9769 | 0.2083 | 0.9810 |

[a]Correlation between measured and predicted values during leave one out cross-validation, [b]Correlation between measured and predicted values of test set. RMSECV: Root-mean-square error of leave one out cross-validation, RMSEP: Root-mean-square error of prediction, PLS: Partial least square, MLR: Multiple linear regression

**Table 3:** Validation parameters of the prediction models of total phenolic content

| Method | Wavelength (nm) | Number of PLS factor | RMSECV | *R* (LOOCV)[a] | RMSEP | *R* (test)[b] |
|---|---|---|---|---|---|---|
| PLS | 250-650 | 2 | 0.1762 | 0.9419 | 0.1093 | 0.9830 |
| PLS | 330, 380, 530 | 1 | 0.1512 | 0.9573 | 0.1273 | 0.9881 |
| PLS | 330, 530 | 1 | 0.1459 | 0.9602 | 0.1273 | 0.9880 |
| MLR | 330, 380, 530 | - | 0.1778 | 0.9409 | 0.1461 | 0.9878 |

[a]Correlation between measured and predicted values during leave one out cross-validation, [b]Correlation between measured and predicted values of test set. RMSECV: Root-mean-square error of leave one out cross-validation, RMSEP: Root-mean-square error of prediction, PLS: Partial least square, MLR: Multiple linear regression

PLS: Total anthocyanins = − 0.064 + 0.857$A_{330}$* − 2.884$A_{380}$* + 3.487$A_{530}$*

MLR: Total anthocyanins = − 0.005 + 0.725$A_{330}$ − 2.688$A_{380}$* + 3.690$A_{530}$*

*$P < 0.05$.

For total phenolic content, validation result of PLS model established from $A_{330}$, $A_{380}$, and $A_{530}$ (RMSEP = 0.1273) was not much different from that of $A_{250-650}$, and the result of MLR model using $A_{330}$, $A_{380}$, and $A_{530}$ was worsened (RMSEP = 0.1461). $A_{330}$ and $A_{530}$ were significant in PLS model and when another model established from these two variables, the same result was obtained (RMSEP = 0.1273). Prediction results of the tested samples using these models are shown in Table 5. The effective and simplest model for the prediction of total phenolic content is as follow:

PLS: Total phenolics = 0.234 + 1.684$A_{330}$* + 1.094$A_{530}$*

*$P < 0.05$.

## CONCLUSION

This study found that the UV/Vis absorbance at 330, 380, and 530 nm of 50% methanol extract were the important parameters referring to the quality of roselle. The specification based on the absorbance at 530 nm (coloring intensity) and the ratio between the absorbances at 380 and 530 nm (degradation index) was set up to specify the quality of the samples. PLS and MLR models established from the absorbances of these three wavelengths were also suggested to estimate the contents of total anthocyanins and total phenolic compounds. All suggested methods were reliable, simple, and rapid; used less chemical and could be applied in routine work.

**Table 4:** Total anthocyanin content (% calculated as delphinidin 3-sambubioside) of tested samples using the prediction models

| Sample | Measured value | PLS | | MLR | |
|---|---|---|---|---|---|
| | | 250-650 nm | 330,380,530 nm | 330,380,530 nm | 380,530 nm |
| 1 | 1.38 | 1.35 | 1.40 | 1.42 | 1.53 |
| 2 | 2.55 | 2.42 | 2.51 | 2.53 | 2.64 |
| 3 | 2.95 | 2.81 | 3.05 | 3.13 | 3.58 |
| 4 | 1.59 | 1.28 | 1.35 | 1.36 | 1.41 |
| 5 | 1.83 | 1.52 | 1.57 | 1.56 | 1.54 |
| 6 | 1.24 | 1.13 | 1.18 | 1.20 | 1.34 |
| 7 | 0.51 | 0.50 | 0.49 | 0.49 | 0.47 |
| 8 | 0.66 | 0.65 | 0.64 | 0.64 | 0.61 |
| 9 | 0.58 | 0.61 | 0.58 | 0.59 | 0.61 |
| 10 | 0.47 | 0.46 | 0.45 | 0.45 | 0.45 |
| 11 | 0.37 | 0.37 | 0.35 | 0.36 | 0.37 |
| 12 | 0.06 | −0.17 | −0.05 | −0.02 | 0.16 |
| 13 | 0.62 | 0.65 | 0.65 | 0.65 | 0.64 |

PLS: Partial least square, MLR: Multiple linear regression

**Table 5:** Total phenolic content (% calculated as gallic acid) of tested samples using the prediction models

| Sample | Measured value | PLS | | | MLR |
|---|---|---|---|---|---|
| | | 250-650 nm | 330, 380, 530 nm | 330, 380 nm | 330, 380, 530 nm |
| 1 | 2.64 | 2.61 | 2.55 | 2.55 | 2.53 |
| 2 | 3.46 | 3.40 | 3.34 | 3.34 | 3.32 |
| 3 | 3.57 | 3.62 | 3.40 | 3.40 | 3.28 |
| 4 | 2.67 | 2.52 | 2.46 | 2.46 | 2.47 |
| 5 | 3.11 | 2.93 | 2.92 | 2.92 | 2.93 |
| 6 | 2.52 | 2.55 | 2.48 | 2.48 | 2.44 |
| 7 | 2.53 | 2.29 | 2.30 | 2.30 | 2.30 |
| 8 | 2.55 | 2.47 | 2.49 | 2.49 | 2.48 |
| 9 | 2.46 | 2.44 | 2.46 | 2.46 | 2.44 |
| 10 | 2.39 | 2.29 | 2.30 | 2.30 | 2.29 |
| 11 | 2.19 | 2.12 | 2.13 | 2.13 | 2.12 |
| 12 | 1.51 | 1.58 | 1.48 | 1.48 | 1.44 |
| 13 | 2.32 | 2.40 | 2.40 | 2.40 | 2.39 |

PLS: Partial least square, MLR: Multiple linear regression

## ACKNOWLEDGMENTS

The authors would like to thank Faculty of Pharmacy, Silpakorn University, for provision of laboratory facilities.

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