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RAPID ASSESSMENT OF LYCOPENE AND β-CAROTENE IN SPINY BITTER GOURD (*MOMORDICA COCHINCHINENSIS* (LOUR.) SPRENG)

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Abstract

A simple spectrophotometric method was developed for the analysis of lycopene, β-carotene and total carotenoids in the spiny bitter gourd. Lycopene, β-carotene and total carotenoids were extracted from spiny bitter gourd aril samples using three accelerated solvent extraction methods. The supernatants of the extracted samples were then analyzed for carotenoids using spectrophotometry at the wavelengths of 450, 470 and 502 nm. The proposed method was validated for its analytical performance parameters including simplicity, accuracy and effectiveness. The method was applied to the determination of lycopene, β-carotene and total carotenoids in 43 spiny bitter gourd genotypes. Over all genotypes, the lycopene, β-carotene and total carotenoidscontents obtained using the proposed spectrophotometric method were not significantly different from those obtained from HPLC methods. A spiny bitter gourd genotype with high lycopene, β-carotene and total carotenoids was identified by the HPLC method, and this result was similar to the results of the spectrophotometric method. The highest positive correlation was found between HPLC and spectrophotometric method III for lycopene ($r = 0.94$; $p \leq 0.01$), β-carotene ($r = 0.92$; $p \leq 0.01$) and total carotenoids ($r = 0.93$; $p \leq 0.01$). The results indicated that the present spectrophotometric method could be used as an alternative to chromatographic analysis for the determination of the lycopene, β-carotene and total carotenoids contents in spiny bitter gourd. This method is reliable, rapid and inexpensive and can be usedto screen a large number of accessions in spiny bitter gourd breeding programs.

Key words: Gac fruit, Spectrophotometer, Indirect selection, Carotenoids, Phytochemical.

Introduction

The modern lifestyle and the consumption of low quality food can cause several health problems such as diabetes, hypertension, heart disease and cancer (Voung & King, 2003; Voung *et al*., 2006; Ishida & Chapman, 2009). Functional food products, including many vegetables and fruits that contain useful phytochemicals, have health benefits beyond ordinary food, and appropriate consumption of these functional food products reduces the risk of many chronic diseases.

Spiny bitter gourd or gac fruit (*Momordica cochinchinensis* (Lour.) Spreng) is an underutilized climbing plant in the Cucurbitaceae family. Its aril is rich in lycopene and β-carotene, which can reduce the risk of several diseases such as cancer of the prostate, colon and stomach as well as coronary heart disease (Vuong *et al*., 2006; Ishida & Chapman, 2009). The species is distributed widely in many Asian countries, including Vietnam, India, Bangladesh, China, Laos, Myanmar, Malaysia and Thailand (Bootprom *et al*., 2012; Bootprom *et al*., 2015). The spiny bitter gourd aril contains higher levels of β-carotene and lycopene than any known fruit crop, and it also contains high quality fatty acids (Ishida *et al*., 2004). The fatty acids in spiny bitter gourd play an important role in the absorption of lycopene and β-carotene (Ishida *et al*., 2004). Thus, arils of the spiny bitter gourd are currently used for the production of many functional food products, such as encapsulated arils, frozen arils, dry arils, beverages, snacks, health foods and cosmetics.

For the quality control of raw materials and breeding of spiny bitter gourd for a high lycopene content, reliable and effective methods for the determination of lycopene are required. The methods available for lycopene analysis include spectrophotometry-based methods and high performance liquid chromatographic (HPLC) methods used in tomato products (Luterotti *et al*., 2013). The spectrophotometric method is simple, rapid and cost-effective and can be used as a tool for the analysis lycopene in other plants. The HPLC method, in contrast, is more effective and accurate, but it is also more complex and time-consuming. The spectrophotometric method is an alternative method for large numbers of samples. A positive and significant correlation between the values obtained with the spectrophotometric method and the HPLC method has been reported in tomato (Davis *et al*., 2003a), vegetables (Barba *et al*., 2006), orange juice (Meléndez-Martínez *et al*., 2011) and yellow maize flour (Luterotti *et al*., 2013). However, the HPLC method is more sensitive for the quantification of lycopene in fruit and vegetable samples, and is more specific than the spectrophotometric method (Càmara *et al*., 2010)

The HPLC method is not appropriate for the evaluation of lycopene in large numbers of accessions or segregating materials in breeding programs because it is difficult, costly and time-consuming. For the spiny bitter gourd, the spectrophotometric method may be useful as an alternative method for screening spiny bitter gourd accessions for high lycopene contents if the results are closely related to those of the HPLC method. Although the spectrophotometric method was developed for the analysis of lycopene and total carotenoid contents in many plant species, the development of this method in

spiny bitter gourd plant is still lacking. Moreover, the relationship between these methods in spiny bitter gourd is not known. The objectives of this study were to evaluate carotenoids, lycopene and β-carotene in spiny bitter gourd accessions using the spectrophotometric and HPLC methods and to determine the relationship between these methods. This information will be useful for the quality control and breeding of spiny bitter gourd with a high lycopene and β-carotene content.

Materials and methods

Plant materials: Forty-three genotypes of spiny bitter gourd were used in this study. Forty-one F_1 plants were obtained from the hybridization of parental plants from various sources of origin and two accessions (KKU ac.11-134 and KKU ac.12-161) were introduced from Vietnam for comparison. These genotypes were planted from seeds as individual plants in May 2013-May 2014 at the Fruit Crop Research Station, Faculty of Agriculture, Khon Kaen University. Agronomic practices, including irrigation, fertilizer application, insecticides and fungicides were applied appropriately and mini-sprinkler irrigation was available to avoid drought. Six months after transplanting, the female flowers were artificially pollinated and tagged to identify the parents and days to maturity. At maturity, or about 60-90 days after pollination, the ripe fruits (identified by the red skin of the fruits) were harvested. Four fruits from each genotype were harvested, and the arils were separated from the fruits and stored separately as four replicates at -20ºC until phytochemical analysis.

Reagents and standards: Butylated hydroxyl toluene (BHT), carotenoid standards for the HPLC method, lycopene and β-carotene were purchased from Sigma Chemical Co. (Louis, MO, USA). Methanol, acetonitrile and dichloromethane used in the extraction of carotenoids and used in the HPLC and spectrophotometer analysis were purchased from Labscan (RCI, Labscan, Thailand). All chemicals and reagents used in the experiments were of analytical grade.

Extraction of carotenoids: Three extraction methods were used for carotenoid analysis for 43 genotypes of spiny bitter gourd. Aril samples (0.1 g) were used for the analysis. These samples were placed in plastic cups and covered with lids. The cups were wrapped in aluminum foil to protect the aril samples from direct sunlight. These samples were used for all extraction methods.

The extraction methods used in this study are described briefly. In method I, the samples were extracted with ethanol in a mortar and ground until the aril samples were pale. The ground samples were then put in a Falcon tube with 3 mL of tetrahydrofuran (Vuong *et al*., 2006). In method II, the samples were mixed with 100 mL of extraction solvent (n-hexane/DW/ethanol, 56:10:34 v/v/v) until the complete exhaustion of color. In method III, the samples were mixed with 100 mL of extraction solvent (n-hexane/acetone/ethanol, 50:25:25 v/v/v) (Bohm *et al*., 2002; Kubola & Siriamornpun, 2011).

The extracted samples were loaded in Falcon tubes and then vortexed (speed 6) for 1 minute. The samples were the supplemented with 5 mL of 95% n-hexane and 5 mL of distilled water and centrifuged at 5000 rpm at 25ºC for 10 minutes. The supernatants of the extracted samples were used for the analysis of carotenoids.

The samples obtained from methods I, II and III were analyzed by a spectrophotometer at the wavelengths of 450, 470 and 502 nm. The samples obtained from method III were also filtered through 0.45 µm membrane filters and sub-samples of 20 µl were injected for HPLC analysis at a wavelength of 450 nm.

Carotenoid analysis by spectrophotometry: A spectrophotometer (10S UV/visible spectrophotometer, Thermo Scientific Genesys, Australia) was used for carotenoid analysis in spiny bitter gourd aril samples. Thespectrophotometer analyzed the samples in the visible spectrum ranging from 190 to 1100 nm. The samples were diluted with hexane, and 3 mL of the diluted samples was used for analysis at the wavelengths of 450, 470 and 502 nm. Hexane was used as the blank. A quartz cuvette was used in the spectrophotometer. The coefficient of $A^{1\%}$ was used for the calculation of lycopene and β-carotene, and the extinction coefficient of 1% was used as the absorbance coefficient for hexane.

Carotenoid analysis by high performance liquid chromatography (HPLC): An HPLC method (Kubola & Siriamornpun, 2011) with minor modifications was used for the analysis of the carotenoid, β-carotene and lycopene contents in the arils of ripe fruits. The composition of the solvents and the isocratic conditions used were described previously (Kubola & Siriamornpun, 2011). Analysis was performed using a Shimadzu (Japan) LC-20AC pump, an SPD-M20A diode array detector and a LUNA C-18 column (4.6 × 250 mm i.d., 5 µM). The mobile phase consisted of acetonitrile l (solvent A)/dichloromethane (solvent B) and methanol (solvent C) at a flow rate of 1 mL/min. At this flow rate, one sample took 30 minutes to pass through the column, and the absorbance was measured at the wavelength of 450 nm.

Statistical analysis: The data were analyzed for the individual methods according to a completely randomized design, and the combined analysis of variance for all methods was performed for all parameters that showed homogeneity of variance. The least significant difference (LSD) test at $p>0.05$ was used to compare means. All calculations were carried out in the STATIX8 software package.

Results

Variations in lycopene, β-carotene and total carotenoid levels: The spectrophotometric and HPLC methods were used for the evaluation of lycopene, β-carotene and total carotenoid levels in 43 spiny bitter gourd genotypes. Significant differences were observed among the 43 genotypes of gac fruit regarding lycopene, β-carotene and carotenoid levels determined using all methods (Table 1). Lycopene levels determined by three different spectrophotometric methods ranged from 94.5 to 3798.0 µg/g fresh weight, whereas it ranged from 300.1 to 4014.7 µg/g fresh weight by the HPLC method (Table 1). Method II had the lowest range (94.5 to 2323.1 µg/g fresh weight), whereas method III had the highest range (172.0 to 3798.0 µg/g fresh weight).

Table 1. Maximum (max), minimum (min) and means for lycopene, Beta-carotene and total carotenoids in 43 spiny bitter gourd genotypes determined by three spectrophotometric methods (method I, II and III) and HPLC method.

	Lycopene (µg/g fresh weight)				Beta-carotene (µg/g fresh weight)				Total carotenoids (µg/g fresh weight)			
	Method I	Method II	Method III	HPLC	Method I	Method II	Method III	HPLC	Method I	Method II	Method III	HPLC
Max.	2543.8	2323.1	3798.0	4014.7	827.5	755.1	884.2	454.4	2705.2	2351.8	3855.3	4179.8
Min.	123.4	94.5	172.0	300.1	8.8	28.7	19.6	121.6	179.7	169.5	271.4	427.8
Mean	890.4	783.9	996.3	951.1	222.1	208.3	214.2	211.0	1112.6	992.1	1210.5	1162.1
SD.	556.0	497.4	697.7	701.1	180.2	189.9	206.5	83.6	577.8	530.4	720.5	715.9
SE.	42.4	37.9	53.2	53.5	13.7	14.5	15.7	6.4	44.1	40.4	54.9	54.6
C.V.(%)	27.4	27.7	34.3	34.2	41.8	46.6	46.4	16.4	23.9	25.7	30.2	29.3
F-test	**	**	**	**	**	**	**	**	**	**	**	**

** = Significant at p<0.01
SD = Standard deviation, SE = Standard errors, C.V. = Calculation from of variance data

Table 2. The simple linear regression, coefficient of determination (R^2) and correlation coefficients between three spectrophotometric methods and HPLC method in analysis of lycopene, β-carotene and total carotenoid content in 43 spiny bitter gourd genotypes.

Method	Equation	R^2	r
Lycopene			
HPLC vs method I	y = 0.95x – 101.84	0.59**	0.77**
HPLC vs method II	y = 1.17x + 32.72	0.71**	0.85**
HPLC vs method II	y = 0.96x – 7.48	0.89**	0.94**
***B*-carotene**			
HPLC vs method I	y = 0.40x + 123.36	0.67**	0.82**
HPLC vs method II	y = 0.40x + 128.32	0.74**	0.86**
HPLC vs method II	y = 0.38x + 129.54	0.85**	0.92**
Total carotenoids			
HPLC vs method I	y = 0.90x + 157.66	0.55**	0.74**
HPLC vs method II	y = 1.07x + 101.91	0.64**	0.80**
HPLC vs method II	y = 0.93x + 26.83	0.87**	0.93**

** Significant at p<0.01

The β-carotene levels determined by the three spectrophotometric methods ranged from 8.8 to 882.5 µg/g fresh weight, while it ranged from 121.6 to 454.4 µg/g fresh weight by the HPLC method. The ranges of β-carotene were rather similar for method I (8.8 to 827.5 µg/g fresh weight) and method III (19.6 to 884.2 µg/g fresh weight); however, it was rather narrow for method II (28.7 to 755.1 µg/g fresh weight).

Total carotenoid levels determined by the three spectrophotometric methods ranged from 169.5 to 3855.3 µg/g fresh weight, while the total carotenoid levels determined by the HPLC method ranged from 427.8 to 4179.8 µg/g fresh weight. Method II had the lowest range (169.5 to 2351.8 µg/g fresh weight), while method III had the highest range (271.4 to 3855.3 µg/g fresh weight). Of note, the range of values provided by spectrophotometric method III were rather similar to those determined by the HPLC method.

Relationship between the HPLC and spectrophotometric methods: The correlation coefficients between the HPLC and spectrophotometric methods for lycopene, β-carotene and carotenoid contents were calculated for 43 spiny bitter gourd genotypes (Table 2).Strong positive correlations were found between the HPLC method and spectrophotometric method I for lycopene (r = 0.77), β-carotene (r = 0.82) and total carotenoids (r = 0.74). There were also positive correlations between the HPLC method and spectrophotometric method II regarding the lycopene (r = 0.85), β-carotene (r = 0.86) and total carotenoid (r = 0.80) contents. Similarly, there was a consistently strong and positive correlation between HPLC and spectrophotometric method III for thelycopene (r = 0.94), β-carotene (r = 0.92) and total carotenoid (r = 0.93)contents. The results show that spectrophotometric method III was more strongly and positively correlated with the HPLC method than spectrophotometric methods I and II.

Thus, we focused on the relationship between HPLC method and spectrophotometric method III for the determination of phytochemicals (Fig. 1).

Discussion

Spectrophotometry-based methods have been used for the analysis of lycopene, β-carotene and total carotenoids in various crops such as tomato (Davis *et al*., 2003a), watermelon (Davis *et al*., 2003b), vegetables (Barba *et al*., 2006) and orange juice (Meléndez-Martínez *et al*., 2011). However, studies have been limited to a small number of genotypes, and information on the development of this method for the analysis of gac fruit is still lacking.

In this study, lycopene, β-carotene and total carotenoids were determined by three spectrophotometry-based methods and an HPLC method in 43 spiny bitter gourd genotypes. The results indicate that the spectrophotometric method can be used to identify different spiny bitter gourd genotypes according to their lycopene, β-carotene and total carotenoid contents, similar to the HPLC method. The carotenoid levels were the highest, the lycopene levels were intermediate, and the β-carotene levels were the lowest. High CV values and F-ratios indicate considerable variations in these phytochemicals.

The average lycopene, β-carotene and total carotenoids contents were 300.1, 121.6 and 427.8 µg/g fresh weight, respectively, determined by HPLC. These levels were similar to the findings of Vuong *et al*. (2006), who reported average lycopene, β-carotene and total carotenoids content of 408, 83 and 497 µg/g fresh weight, respectively. In addition, the lycopene and β-carotene contents were estimated to be 380 and 101 µg/gfresh fruit, respectively, by Aoki *et al*. (2002). In contrast, Ishida*et al*. (2004) reported average lycopene and β-carotene contents of 2300 and 750 µg/g fresh weight, respectively. The differences in these observations could be due to differences in plant materials. In this study, the phytochemical content determined by HPLC was rather similar to the levels provided by the spectrophotometric methods, especially spectrophotometric method III. This result is similar to that of Luterotti *et al*. (2013), reported in tomato products and yellow maize flour.

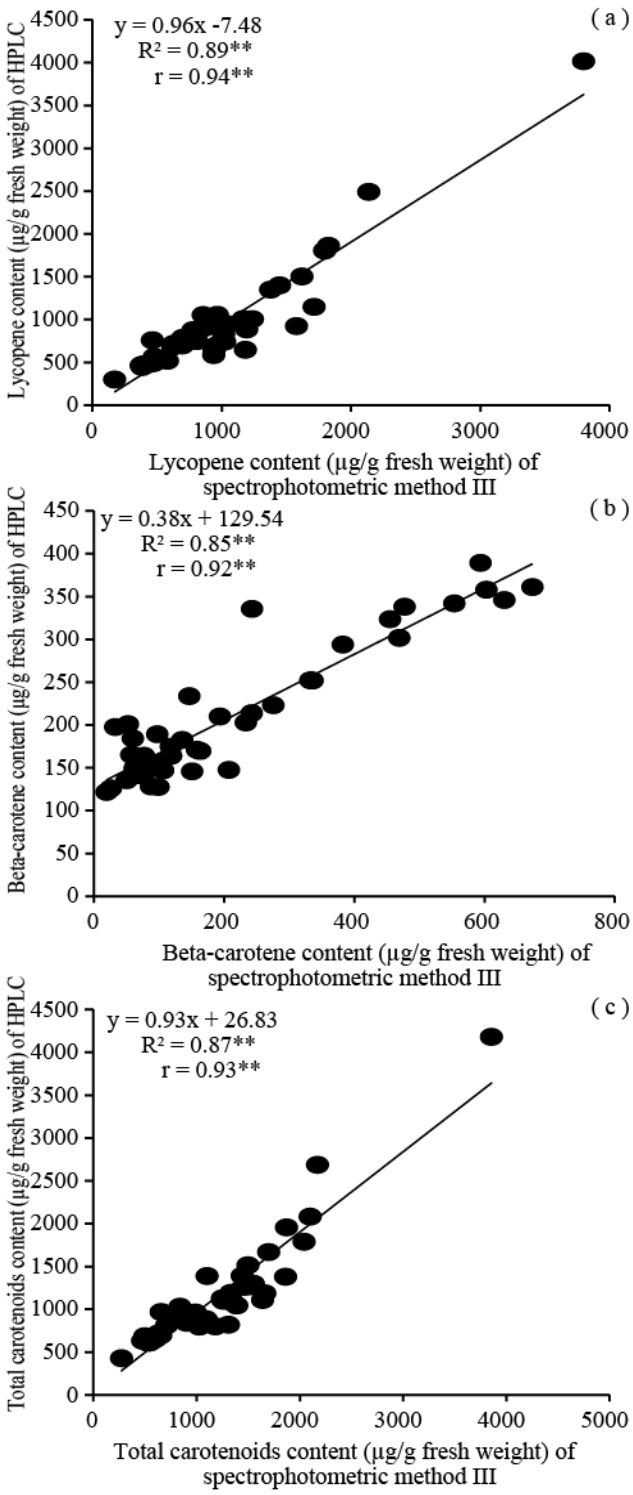

Fig. 1. Relationships between HPLC and spectrophotometric method III for determination of lycopene content (a), β-carotene content (b), and total carotenoid content (c) in 43 spiny bitter gourd genotypes.

Although the application of spectrophotometric methods for the analysis of lycopene, β-carotene and total carotenoids has been investigated in many plant species (Davis *et al*., 2003a; Barba *et al*., 2006; Meléndez-Martínez *et al*., 2011; Luterotti *et al*., 2013), there is limited information on the application of this method to gac fruit. An understanding of the relationship between the HPLC method and spectrophotometric methods may be helpful in choosing a simple method for the determination of phytochemicals in spiny bitter gourd accessions. In the present study, three spectrophotometric methods with different extraction methods were compared with the HPLC method for the determination of lycopene, β-carotene and total carotenoids in the arils of the spiny bitter gourd. All correlation coefficients between the spectrophotometric methods and the HPLC method were positive and significant for the lycopene, β-carotene and total carotenoidcontents. The current study supports previous studies in which that high correlations are found between the HPLC method and the spectrophotometric methods for the analysis of lycopene, β-carotene and total carotenoids in tomato and orange (Melendez-Martinez *et al*., 2011, Luterotti *et al*., 2013).

Interestingly, the correlation coefficients between spectrophotometric method III and the HPLC method had the highest coefficients of determination for lycopene, β-carotene and total carotenoids. Thus, the HPLC method could be largely explained by spectrophotometric method III for the analysis of lycopene (R^2 = 0.89), β-carotene (R^2 = 0.85) and total carotenoids (R^2 = 0.87). This result is in agreement with those observed in which a high positive relationship was found between spectrophotometric method III and the HPLC method, likely because the same extraction method was used for phytochemical analysis.

To the best of our knowledge, this study is be the first report on the relationship between spectrophotometric methods and HPLC methods for the analysis of lycopene, β-carotene and total carotenoids in spiny bitter gourd. The present study demonstrates that spectrophotometry can be used as a tool for the analysis carotenoids, including lycopene and β-carotene in spiny bitter gourd arils. The spectrophotometric method is rapid and accurate for measuring phytochemicals and can be used for screening the spiny bitter gourd germplasm or for large breeding populations.

Conclusions

This study demonstrated that spectrophotometric methods can be developed for the analysis of lycopene, β-carotene and total carotenoids in spiny bitter gourd arils. The results show high correlation coefficients between the spectrophotometric methods and the HPLC method for the analysis of lycopene, β-carotene and total carotenoids. Based on its rapid, precise and inexpensive assessment of phytochemicals, the spectrophotometric method can be used as an alternative to the HPLC method for the determination of lycopene, β-carotene and total carotenoids in spiny bitter gourd. This method can be used in spiny bitter gourd selection programs to increase lycopene, β-carotene and carotenoid levels and to evaluate fruit quality in the functional food industry.

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