การศึกษาวิธีที่เหมาะสมและตรวจสอบความถูกต้องสำหรับวิเคราะห์ปริมาณไลโคปีนและเบตาแคโรทีนในผลฟักข้าวด้วยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูง
HPLC Method Optimization and Validation for Determination of Lycopene and Beta-carotene in Gac Fruit

นิพนธ์ต้นฉบับ

Original Article

ชยุต ฟองสุข[1], ลลนา คงคาเนรมิตร[2], ฐาปนีย์ หงส์รัตนาวรกิจ[3] และ วรพรรณ สิทธิถาวร[4]*

[1] นิสิตบัณฑิตศึกษา
[2] สาขาวิชาวิทยาศาสตร์เภสัชกรรม คณะเภสัชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ อ.คลองหลวง จ.ปทุมธานี 12120
[3] สาขาวิชาเภสัชเคมี
[4] สาขาวิชาเภสัชเวท
[1,3,4] คณะเภสัชศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ อ.องครักษ์ จ.นครนายก 26120

*** Corresponding author:** chayutmd@gmail.com

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Chayut Fongsuk[1], Lalana Kongkaneramit[2], Tapanee Hongratanaworakit[3] and Worapan Sitthithaworn[4]*

[1] Graduate student
[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Thammasat University, Khlong Luang, Pathumthani 12120, Thailand
[3] Department of Pharmaceutical Chemistry
[4] Department of Pharmacognosy
[1,3,4] Faculty of Pharmacy, Srinakharinwirot University, Ongkharak, Nakhonnayok, 26120, Thailand

*** Corresponding author:** chayutmd@gmail.com

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บทคัดย่อ

วัตถุประสงค์: เพื่อหาสภาวะที่เหมาะสมในการวิเคราะห์และตรวจสอบความถูกต้องของวิธีวิเคราะห์ที่ได้พัฒนาในการตรวจสอบปริมาณไลโคปีนและเบตาแคโรทีนในเปลือก เนื้อ และรกหุ้มเมล็ดของผลฟักข้าว (*Momordica cochinchinenssis* Spreng., Family Cucurbitaceae) **วิธีการศึกษา:** การศึกษานี้ใช้เทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูงแบบผันกลับ คอลัมน์ C18 อัตราการไหล 1.5 มิลลิลิตรต่อนาที ที่ 25 องศาเซลเซียส ปริมาตรที่ฉีด 20 ไมโครลิตร ตรวจวัดที่ความยาวคลื่น 475 นาโนเมตร และทดลองใช้เฟสเคลื่อนที่หลายชนิด **ผลการศึกษา**: พบว่าการใช้ อะซิโตไนไตรล์ต่อไดคลอโรมีเทน อัตราส่วน 75:25 โดยปริมาตร เป็นระบบที่เหมาะสมในการวิเคราะห์ โดยระบบการวิเคราะห์นี้มีความสามารถในการทำซ้ำได้ เห็นได้จากค่า %RSD ของไลโคปีนและเบตาแคโรทีนเท่ากับ 2.60 และ 3.87 ตามลำดับ มีค่าความถูกต้องแสดงในรูปของร้อยละการคืนกลับของไลโคปีนและเบตาแคโรทีนอยู่ในช่วง 99.59 – 103.20 และ 97.02 – 100.14 ตามลำดับ โดยใช้เวลาในการวิเคราะห์ 9 นาทีต่อตัวอย่าง และเวลาที่ใช้ในการชะไลโคปีนและเบตาแคโรทีนเท่ากับ 4.1 และ 6.5 นาที ตามลำดับ เมื่อนำวิธีการดังกล่าวไปใช้ในการวิเคราะห์ปริมาณไลโคปีนและเบตาแคโรทีนในผลฟักข้าว พบว่าพบปริมาณไลโคปีนมากที่สุดในรกหุ้มเมล็ด (9.45 ± 0.27 มิลลิกรัมต่อกรัมน้ำหนักแห้ง) ตามด้วยเนื้อ (1.15 ± 0.06 มิลลิกรัมต่อกรัมน้ำหนักแห้ง) และเปลือก (0.74 ± 0.07 มิลลิกรัมต่อกรัมน้ำหนักแห้ง) **สรุป:** วิธีวิเคราะห์มีความจำเพาะ ความแม่นและความเที่ยง ตามหลักเกณฑ์การตรวจสอบความถูกต้อง AOAC guideline 2012 สามารถวิเคราะห์ปริมาณได้ในระยะเวลาสั้นซึ่งเป็นข้อดีในการใช้ต่อไปในอนาคต เช่น การวิเคราะห์ความคงตัว หรือควบคุมคุณภาพในการผลิตต่อไป

คำสำคัญ: ฟักข้าว, ไลโคปีน, เบตาแคโรทีน, โครมาโตกราฟีของเหลวสมรรถนะสูง

Abstract

Objective: To optimize and validate an HPLC method for determination of lycopene and beta-carotene in peel, pulp and aril of gac fruit (*Momordica cochinchinensis* Spreng., Family Cucurbitaceae). **Method:** Reverse phase HPLC with a C18 column was used in this study. A flow rate of 1.5 mL/min, column temperature of 25°C, 20 µL of injection volume and detection wavelength (λ) of 475 nm was performed with various isocratic mobile phase system. **Results**: The most suitable mobile phase was acetonitrile: dichloromethane (75:25 v/v). The HPLC method showed sufficient reproducibility, i.e., %RSD for lycopene and beta-carotene was 2.60 and 3.87, respectively. The accuracy, i.e., %recovery, for lycopene and beta-carotene was in the range of 99.59 – 103.20% and 97.02 – 100.14%, respectively. The analysis took 9 minutes for a sample and retention time of peaks of lycopene and beta-carotene were 4.1 and 6.5 minutes, respectively. For the content of lycopene and beta-carotene in fully ripening gac fruit, the highest lycopene content was found in aril (9.45 ± 0.27 mg/g dry weight), followed by pulp (1.15 ± 0.06 mg/g dry weight) and peel (0.74 ± 0.07 mg/g dry weight). **Conclusion:** The method was proved to be specific, accurate and precise as indicated by AOAC guideline 2012. This validated method has provided a short run time per sample and it is advantageous for further studies such as stability of the extract or quality control in manufacturing industry.

Keywords: *Momordica cochinchinensis*, Gac, lycopene, beta-carotene, HPLC

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Introduction

Momordica cochinchinensis Spreng (Family Cucurbitaceae), commonly called Gac, is a tropical plant that is indigenous to countries in South and Southeast Asia including Vietnam and Thailand. It is reported that gac fruit is the richest source of carotenoids, especially lycopene and beta-carotene of all known fruits.[1] The membrane around the seeds or aril has been used not only as colorant in food but also as carotenoid source in food supplement.[2] Carotenoid analysis may be carried out by different methods, such as spectrophotometry, colorimetry and HPLC.[3] Spectro-

photometry or colorimetry can be used for rapid analysis but high sensitivity and high selectivity method such as HPLC is preferable.[4] Reverse-phase HPLC especially with C_{18} or C_{30} column is commonly used to determine carotenoids content in gac fruits.[5] Some studies of composition of carotenoids in gac were reported by Aoki et al[6], followed by Ishida et al[9] and Vuong et al.[1]

In 2002, Aoki et al used C_{18} column for HPLC and performed with a gradient elution program consisting of the mobile phase containing a mixed solution of (A) acetonitrile: dichloromethane: methanol (70:20:10 v/v) and (B) dichloromethane.[6] The run time took 40 minutes. Ishida et al used C_{30} column with an isocratic mobile phase of methyl tert-butyl ether (MTBE): methanol and: ethyl acetate (40:50:10 v/v) for separating gac carotenoid's isomer.[7] The run time took 14 minutes. The method developed by Vuong et al[1] was similar to Aoki's method. In that the samples were applied on a C_{18} column and mobile phase was a mixture of acetonitrile, dichloromethane and methanol (65:25:10, v/v), so the obtained results were similar.

None of these authors have mentioned a validation data for their methods. Although these methods allow accurate quantification of individual carotenoids and separation of isomers; many of them require long retention times and mobile phase system with gradient solvents, or containing aqueous solvent which could induce on-column precipitation of carotenoids during elution. Therefore, a suitable HPLC method must be developed for gac fruit. The purpose of this work was to develop an isocratic non-aqueous reverse phase HPLC procedure for the rapid separation, suitable, reliable and quantification of lycopene and beta-carotene analysis in gac fruit and validate the developed method.

Methods

Chemicals and reagents

Lycopene (secondary standard, 11.4% in inert material) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Beta-carotene standard (98.5%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The methanol, dichloromethane and acetonitrile used in the HPLC analysis were purchased from Merck (Darmstadt, Germany). Double distilled water was obtained from a Milli-Q System (Millipore, Bedford, MA, USA).

Instruments

HPLC was carried out on a Shimadzu system, equipped with two high pressure pumps (LC-20AD), vacuum degasser (DGU-20A5R), autosampler (SIL 20ACHT), column oven (CTO 20AC), a diode array detector (SPD-M20A) and the CBM-20A System Controller. All data acquired were proceeded by LabSolutions software (Shimadzu Japan).

Preparation of standard and working solutions

Lycopene standard solution was prepared by weighing 10 mg of the lycopene standard into 10-mL volumetric flask and dissolving in 8 mL dichloromethane. The solution was sonicated for 15 minutes and then filtered with 0.45 µm membrane filters. The lycopene standard stock solution was adjusted to 10 mL to a final concentration of 114 µg/mL.

The stock solution of beta-carotene standard was prepared by weighing 1 mg of beta-carotene standard into 10-mL volumetric flask and dissolving in 10 mL dichloromethane. The final concentration was 100 µg/mL. (Beta-carotene concentration was 98.5 µg/mL). The stock solution of lycopene 300 µL was diluted to 1,000 µL by dichloromethane to obtain a working standard solution (34.2 µg/mL). The stock solution of beta-carotene 60 µL was diluted to 1,000 µL by dichloromethane to obtain a working standard solution (5.91 µg/mL).

Chromatographic condition

Analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A diode array detector, and an Inertsil® C-18 column (150 x 4.6 mm i.d., 5 µm). Several isocratic mobile phase were assayed; (1) acetonitrile: methanol (90:10 v/v), (2) acetonitrile: methanol (60:40, v/v), (3) acetonitrile: methanol: dichloromethane (71: 22: 7, v/v), and (4) acetonitrile: dichloromethane (75:25, v/v). The mobile phase was filtered through a 0.45 µm membrane, and degassed ultrasonically before used. The mobile phase flow rate was 1.5 mL/min. The column temperature was 25 ± 0.5 °C and the absorbance was detected at 475 nm.

System suitability

The test was carried out to establish the parameter such as percentage relative standard deviations (% RSD) for RT, peak area response and tailing factor resolution factor. This test was performed by analyzing six replicates (n = 6) of a

working standard (34.2 µg/mL) and the % RSD of the parameters was calculated.

Method validation

The method validation was investigated based on the Association of Official Analytical Chemists, AOAC 2012.[8] The validation parameters included specificity, linearity, range, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Specificity

The method specificity was assessed by injecting lycopene and beta-carotene standard, sample, blank and mixture of standard and sample separately.

Linearity and range

The stock standard solution of lycopene and beta-carotene were diluted to five concentration levels from 10 – 60 and 2 – 10 µg/mL, respectively, with dichloromethane and injected into HPLC instrument (n = 3). The calibration curve was constructed. Linear equation, coefficient of determination (R^2) and test range were reported.

Accuracy

A spike technique was used and the analysis was performed in triplicate. Percent recovery was determined by recovery studies at three concentration levels of mixed standard (15, 25 and 40 µg/mL for lycopene and 3, 5 and 7 µg/mL for beta-carotene) and three samples from each concentration were injected.

Precision

Precisions of the method were evaluated by analyzing the three concentration levels of mixed standard (15, 25 and 40 µg/mL for lycopene and 3, 5 and 7 µg/mL for beta-carotene) in triplicate on the same day for intra-day precision and on three consecutive days (n=3) for inter-day precision. The mean and % RSD was calculated for intra-day and inter-day precision.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by analyzing different concentrations of the standards and measuring the signal-to-noise ratio. LOD is the concentration that gives a signal-to-noise ratio approximately 3:1, while LOQ is the concentration that gives a signal-to-noise ratio approximately 10:1 with % RSD (n = 3) of less than 10%.

Plant materials and sample preparations

Gac fruits were randomly selected from Talat Thai Market, Pathum Thani, Thailand. All of these fruits were fully ripe as observed by the fruit was red color, the pulp was orange and the seed aril was red. They were cleaned and separated to pulp, peel and aril (seed membrane). Each part was dried at 45 °C in hot air oven for 3 days, and then blending in a blender to obtain a representative sample for carotenoid analysis.

Determination of lycopene and beta-carotene content of extract

The extraction method was described previously by Barba et al.[4] Each part of the fruits powder (2 g) was placed in a vessel which was protected from light and mixed with 100 mL of extraction solvent (hexane, acetone and ethanol; 50:25:25 v/v/v). The mixture was magnetically stirred for 1 hour; then water (15 mL) was added. The upper layer was collected in a round-bottomed flask, then evaporated to dryness. 1 mg of residue was dissolved in mobile phase to the final volume of 10 mL. The final solution was filtered through 0.45µm membrane filters and the solution (10 µL) was injected for HPLC analysis.

Results and Discussion

The optimized HPLC conditions

It was found that the mobile phase consisting of acetonitrile and dichloromethane in the ratio of 75:25 v/v with a flow rate of 1.5 mL/min, injection volume of 10 µL, column temperature at 25 ± 0.5 °C and detection wavelength (λ) of 475 nm resulted in the shortest retention time (4 min for lycopene and 6 min for beta-carotene) with satisfactory resolution and tailing factor (Figure 1 and Table 1). In addition, symmetrical peak shape was observed for both lycopene and beta-carotene. Thus, the optimized condition was applied for the entire study.

It was found that the mobile phase consisting of acetonitrile and dichloromethane in the ratio of 75:25 v/v with a flow rate of 1.5 mL/min, injection volume of 10 µL, column temperature at 25 ± 0.5 °C and detection wavelength (λ) of 475 nm resulted in the shortest retention time (4 min for lycopene and 6 min for beta-carotene) with satisfactory resolution and tailing factor (Figure 1 and Table 1). In addition, symmetrical peak shape was observed for both lycopene and

beta-carotene. Thus, the optimized condition was applied for the entire study.

Method validations

The optimized HPLC condition was validated. The results were within the accepted criteria with related to the Association of Official Analytical Chemists, AOAC, 2012.[8] Thus the condition was reliable for sample analysis. The results of validation parameters were shown below:

Specificity

The specificity of the analytical of lycopene and beta-carotene was confirmed by comparing the retention time and peak spectra obtained in the standard and sample analyses as shown in Figure 2. The retention time of lycopene and beta-carotene observed in the chromatogram from the sample resembled to that observed in the standard carotenoid chromatogram. Representative UV spectra of standard and sample were matching.

Linearity and range

The mean peak areas obtained from HPLC method were plotted against the corresponding concentrations to obtain the calibration curve. The calibration curve for lycopene at the concentration range of 17.31 – 46.15 μg/mL and beta-carotene at the concentration range of 3.70 – 7.93 μg/mL was linear with the correlation coefficient (R^2) that was greater than 0.99 (Table 2).

Accuracy

This experiment expressed the closeness of results obtained by that method to the true value. Acceptable percent recovery is 80 – 115 %.[8] The result of accuracy showed percent recovery at all three levels of concentration in range 99.59 – 103.20% for lycopene and 97.02 – 100.14% for beta-carotene as shown in Table 3.

Precision

The precision of the method was evaluated as intraday and inter-day precision. They were examined by analyzing three known concentrations for three consecutive days. % RSD values of both intraday and inter-day precision was not more than 3.87% (Table 3). Acceptable values for repeatability and reproducibility were not more than 6% and 11%, respectively.[8]

LOD and LOQ

The LOQ values for lycopene and beta-carotene were 316.35 ng/mL and 650.10 ng/mL, respectively, whereas the LOD values for lycopene and beta-carotene were 158.18 ng/mL and 325.05 ng/m, respectively.

The content of lycopene and beta-carotene

The chromatograms of lycopene and beta-carotene in each part of gac fruits were shown the same retention time (Figure 3). The content of lycopene and beta-carotene in each part of gac fruits was shown in Table 4. The highest content of both lycopene and beta-carotene was found in the gac aril while the lowest content was found in the gac peel.

The previous report of lycopene and beta-carotene content in each part of gac fruits were summarized and shown in Table 5. The discrepancies in lycopene and beta-carotene content might be due to different sample preparation and extraction. It was noticed that the results of this study were close to the study reported by Kubola and Siriamornpun.[9] The sample preparation and the extraction solvent used in this study were the same as mentioned in Kubola and Siriamornpun.[9] On the other hand, the yielded of lycopene and beta-carotene in this study was greater than extraction from fresh fruits as reported by Vuong et al[1], Aoki et al[6] and Ishida et al.[7] who extracted lycopene and beta-carotene from fresh fruits.

The extraction solvent used in this study and that reported by Kubola and Siriamornpun[9] was a mixture of hexane, acetone and ethanol (50:25:25 v/v/v). The solvent mixture has been reported to exhibited the best result for carotenoid extraction in several carotenoid containing plants.[4] In that study, the solvent was tested for extraction from fresh materials which polarity of acetone and ethanol could enhance the water miscibility of hexane in the cells and facilitate the extraction of non-polar compound such as carotenoids. It was found that this solvent mixture was also effective for extraction of carotenoids from dry samples because it took one extraction step and required low solvent volume. In addition, the carotenoid yield was greater than yield mentioned by other reports.[1,6,7] However, higher polarity solvent such as acetone and ethanol may hamper the extraction of carotenoids due to the poor solubility of this compound in these solvent. Thus, further investigation for more appropriate solvent mixture for complete carotenoid extraction from dried materials is need to be performed.

In addition to sample preparation and extraction solvent, the discrepancies in carotenoid contents might be due to degradation of carotenoids during extraction, analysis, transport, and storage. Another reason might be in the ripening of the fruits because carotenoid contents can change dramatically during fruit ripening.[9]

Conclusion

This simple isocratic HPLC method provided reliable results and a short runtime (9 minutes) for the quantification of lycopene and beta-carotene. The optimized and validated method and the sample extraction process in this study should be beneficial for determination of lycopene and beta-carotene in dried gac fruits.

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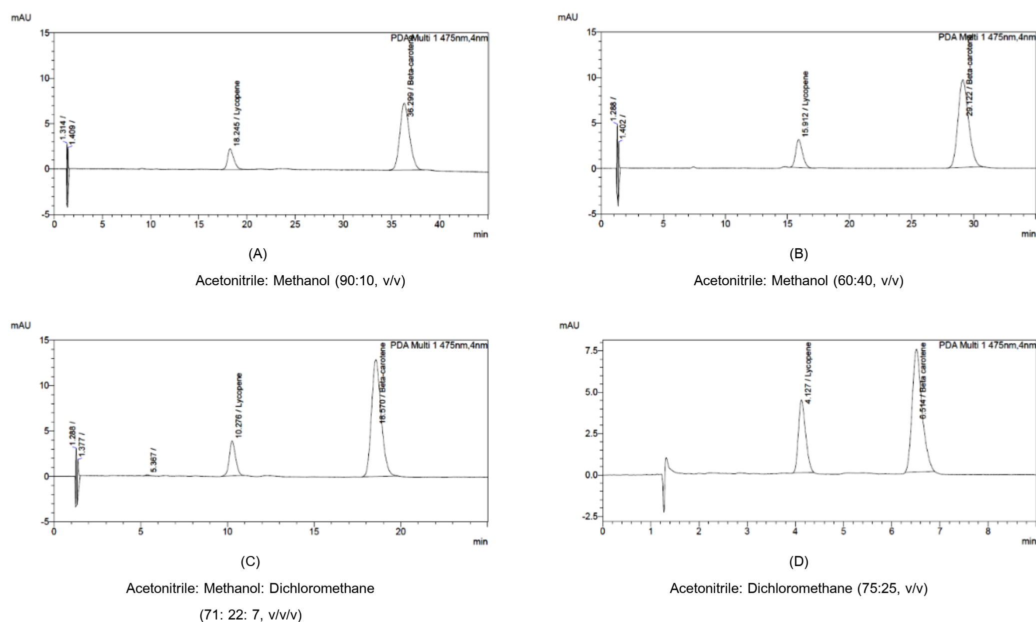


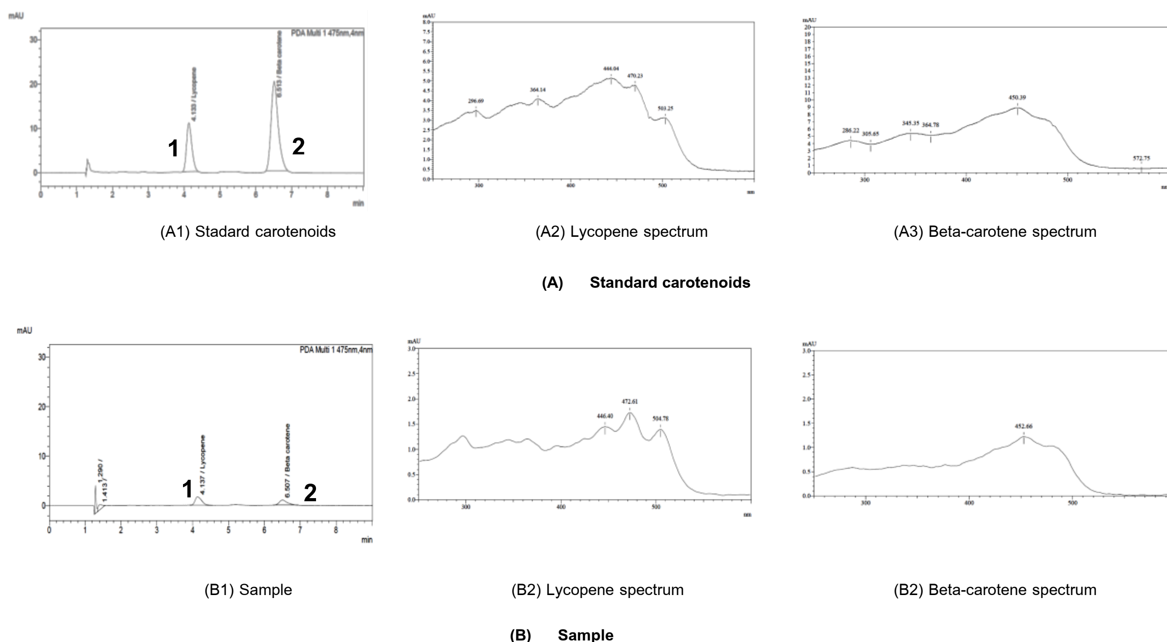
(A) Acetonitrile: Methanol (90:10, v/v)

(B) Acetonitrile: Methanol (60:40, v/v)

(C) Acetonitrile: Methanol: Dichloromethane (71: 22: 7, v/v/v)

(D) Acetonitrile: Dichloromethane (75:25, v/v)

Figure 1 Chromatogram of lycopene and beta-carotene standards in different mobile phases



(A1) Stadard carotenoids

(A2) Lycopene spectrum

(A3) Beta-carotene spectrum

(A) Standard carotenoids

(B1) Sample

(B2) Lycopene spectrum

(B2) Beta-carotene spectrum

(B) Sample

Figure 2 Chromatogram and spectrum of standard and sample run on acetonitrile: dichloromethane (75:25, v/v); 1: lycopene and 2: beta-carotene

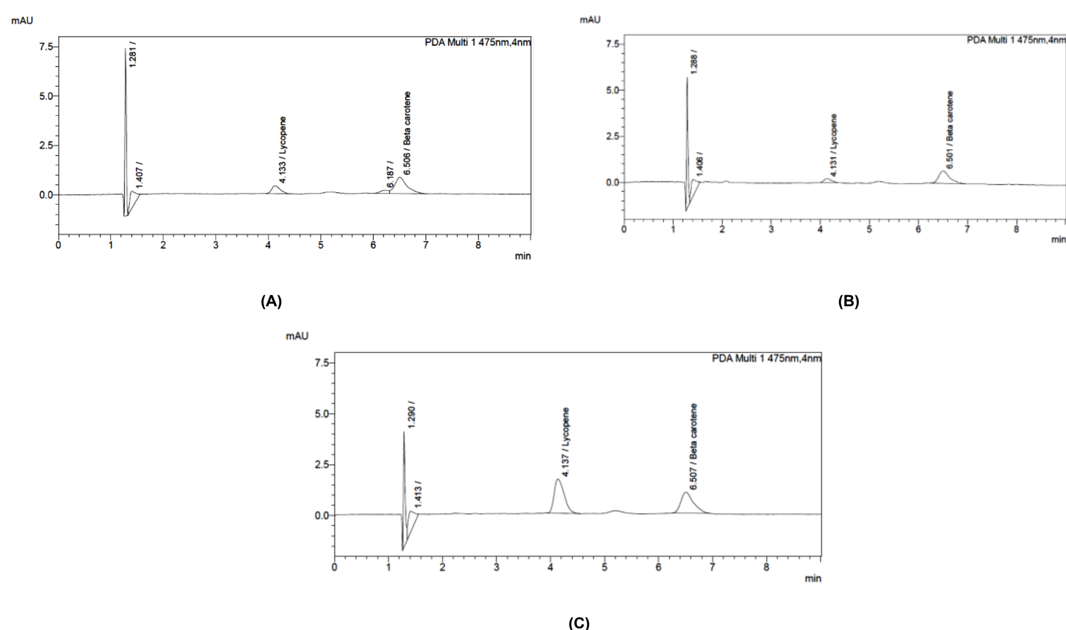


Figure 3 Chromatogram of lycopene and beta-carotene in each part of gac fruits; peel (A), pulp (B), and aril (C) at detection wavelength of 475 nm.

Table 1 System suitability data

Mobile phase	Retention time (min.)		Resolution	Tailing factor	
	Lycopene	beta-carotene		Lycopene	beta-carotene
Acetonitrile: Methanol (90:10 v/v)	18.25	36.32	12.03	1.31	1.17
Acetonitrile: Methanol (60:40, v/v)	15.90	29.14	10.31	1.23	1.17
Acetonitrile: Methanol: Dichloromethane (71: 22: 7, v/v)	10.33	18.63	9.78	1.11	1.22
Acetonitrile: Dichloromethane (75:25, v/v)	4.10	6.44	5.70	1.15	1.39

Table 2 Linearity and range of lycopene and beta-carotene standard.

Compound	Range (µg/mL)	Linear equation	R^2
Lycopene	17.31 – 46.15	y = 611.0064x + 1523.4690	0.9986
Beta-carotene	3.70 – 7.93	y = 27733.8336x – 58929	0.9976

Table 3 Accuracy and precision of lycopene and beta-carotene standards.

Carotenoids	Conc. (µg/mL)	% Recovery	Precision (% RSD)	
			Intraday	Inter-day
Lycopene	15	103.20 ± 1.87	0.88	1.81
	25	102.13 ± 3.95	0.62	3.87
	40	99.59 ± 2.85	1.50	2.86
Beta-carotene	3	97.02 ± 2.53	1.22	2.60
	5	99.96 ± 3.30	0.86	3.30
	7	100.14 ± 0.56	0.22	0.56

Table 4 Lycopene and beta-carotene content in each part of gac fruits.

Parts of gac fruit	Carotenoids content (mg/gDW)*	
	Lycopene	Beta-carotene
Peel	0.74 ± 0.07	0.21 ± 0.02
Pulp	1.15 ± 0.06	0.30 ± 0.02
Aril	9.45 ± 0.27	1.44 ± 0.07

* DW = dry weight.

Table 5 Comparison of concentrations of carotenoids in aril part of gac fruit (mg/g fruits) with other published resul

Reference	Sample preparation	Extraction solvent	Carotenoids content	
			Lycopene	Beta-carotene
Vuong et al[1]	fresh fruits	tetrahydrofuran and hexane	0.408	0.083
Aoki et al[6]	fresh fruits	acetone, diethyl ether and acetonitrile	0.380	0.101
Ishida et al[7]	dried fruits with vacuum centrifuge	hexane/propanol (8:2, v/v)	1.903 (*trans*) 0.128 (*cis*)	0.641 (*trans)* 0.170 (*cis*)
Kubola & Siriamornpun[9]	dried fruits with freeze-drier	hexane/acetone/ethanol (50:25:25 v/v/v)	7.02	1.6 - 5.9

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