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3B QUANG TRUNG - HOAN KIEM - HANOI - VIETNAM
TEL: (+84-24) 38247058 FAX: (+84-24) 39348740
Email: tapchiduoclieu@nimm.org.vn; tapchiduoclieu@gmail.com

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DEVELOPING HPLC-DAD METHODS FOR SIMULTANEOUSLY DETERMINING CONTENT OF LYCOPENE AND *β*-CAROTENE IN GAC SEED ARIL USING EXTERNAL STANDARDS AND QAMS

Pham Thi Hien[1], Nguyen Thi Lan Hoa[1], Ta Thi Thao[2], Le Thi Thu Hang[1], Ho Hoai Thuong[2], Pham Thi Khuyen[2], Nguyen Thi Ha Ly[1,*]

[1]*National Institute of Medicinal Materials, Hanoi, 11022, Vietnam;*

[2]*University of Science, Vietnam National University, Hanoi, Vietnam*

*Corresponding author: nguyenthihaly.chem@gmail.com

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Summary

Momordica cochinchinensis (Lour.) Spreng., commonly known as Gac, is a plant native to Vietnam with a range of reported health benefits, including cardioprotective, antioxidant activity, improving eyesight, nourishing skin, and cancer prevention. This study developed and validated two methods suitable for the quality control of its seed aril. First, a high-performance liquid chromatography-diode array detection (HPLC-DAD) method was developed and validated according to ICH guidelines. Linearity was demonstrated with correlation coefficients (R^2) ranging from 0.9989 to 0.9996. Second, a Quantitative Analysis of Multi-components by Single-Marker (QAMS) method was developed, using *β*-carotene as an internal standard and calculating a relative correction factor (RCF) for lycopene determination. Comparison of the QAMS method with the external standard method (ESM) showed a bias of < 5%. The developed methods are suitable for quality control of Gac seed aril.

Keywords: *Momordica cochinchinensis; Lycopene; β-Carotene; Quantitative analysis; QAMS; HPLC-DAD.*

1. Introduction

Gac (*Momordica cochinchinensis* (Lour.) Spreng.) has been cultivated in Vietnam for a long time, and it has two cultivars: red-ripe fruit and yellow-ripe fruit. The yellow-ripe fruit is currently cultivated in some areas of Lai Chau and Son La provinces. There are two types of red-ripe fruit: large and small; both grow mainly in the midlands and the Northern Delta [1],[2]. Gac seed aril (GSA) also contains a variety of other carotenoids and bioactive compounds, such as vitamin E (*α*-tocopherol), flavonoids, and phenolic compounds [3],[4]. GSA has a fatty acid content of 22%, predominantly comprising palmitic acid (29%), linoleic acid (28%) and oleic acid (2%). Because of its vibrant red color, GSA has long been used in Asia as a natural colorant for traditional foods. Additionally, Gac seeds are traditionally used to treat several conditions like mastitis, boils, and pyoderma [5, 6]. In Vietnam, the aril and oil of Gac fruits are frequently used to add to foods similar to sticky rice with Gac fruits to help treat eye conditions caused by vitamin A insufficiency and ameliorate sight. Gac seed aril and products from Gac fruit have veritably high antioxidant activity due to the high content of carotenoids, especially lycopene and *β*-carotene. They bring numerous health benefits by having anti-cancer, cardioprotective, and anti-inflammatory effects [7],[8].

In addition to traditional applications, commercial products like Gac powder and oil have been developed as natural colorants and nutraceuticals. The Vietnamese Pharmacopoeia V (VP V) currently includes three separate monographs: Gac (seeds), Gac (seed aril), and Gac (oil). The Gac (seed aril) monograph specifies several quality control parameters for the medicinal material: description, powder, fragmentation rate, extractives, water content, and TLC identification (using *β*-carotene as a reference standard). However, it lacks quantitative categories for quality assessment. Current quality control relies on traditional quantification methods, which require numerous reference standards and are therefore both costly and time-consuming. To address this limitation, this study proposes a novel method for the simultaneous determination of lycopene and *β*-carotene in GSA using HPLC-DAD, coupled with a QAMS approach. This approach aims to provide a more efficient assessment of the quality of GSA.

2. Experimental

2.1. Samples

Momordica cochinchinensis seed aril samples, harvested from various locations in Vietnam, were provided by the National Institute of Medicinal Materials and used as analytical samples.

After harvesting gac fruits, the seed arils and seeds were separated from the pulp under darkroom conditions. They were then wrapped in aluminum foil and dried at 50°C for 4 hours. Following drying, the seed arils were separated from the seeds and dried to a moisture content of

10%. These dried seed arils were stored in vacuum-sealed bags shielded from light using an external black bag until needed for analysis. The moisture content of the sample was determined using the solvent distillation method according to Appendix 12.13 of VP V.

2.2. Chemicals

β-Carotene standard were purchased from Chemfaces, China (CAS: 7235-40-7, Lot: CFS202202, purity 98%), and lycopene (CAS: 502-65-8, Lot: 000220023, purity 98%) from Sigma-Aldrich.

Solvents and chemicals used for HPLC analysis (acetonitrile and methanol) were purchased from Merck. All reagents used in sample extraction and preparation were of analytical grade (P.A.).

2.3. Instruments

- The HPLC-1 system (Shimadzu, Japan) used for the analysis consisted of a binary pump LC-20AD, a SIL-20A HT autosampler, a CTO-10AS column oven, and a DAD (SPD-M20A).

- The HPLC-2 system (Shimadzu, Japan) used for the analysis consisted of a binary pump LC-30AD, a SIL-30AC autosampler, a CTO-10AS column oven, and a DAD (SPD-M20A).

- Agilent Eclipse Plus C_{18} column (250 x 4.6 mm, 5 µm).

- Shimadzu Shim-pack Solar C_{18} Analytical column (250 x 4.6 mm, 5 µm).

- TOSOH TSKgel ODS-100Z C_{18} column (250 x 4.6 mm, 5 µm).

2.4. Methods

According to the literature review, *n*-hexane - acetone - ethanol (2:1:1; *v/v/v*) is a frequently used extraction solvent [4, 9-11]. Consequently, this study utilized *n*-hexane - acetone - ethanol (2:1:1; *v/v/v*) as the extraction solvent.

2.4.1. Preparation of standard solutions:

- *β*-Carotene standard solution 1 mg/mL: accurately weigh about 5 mg of *β*-carotene standard into a 5 mL volumetric flask, add about 3 mL of *n*-hexane - acetone - ethanol (2:1:1; *v/v/v*) solvent mixture, shake until completely dissolved, and make up to the mark with the solvent mixture. From this *β*-carotene stock solution with a concentration of 1 mg/mL, dilute with the solvent mixture system in different ratios to obtain *β*-carotene standard solutions with concentrations of about 1.95 µg/mL - 250.00 µg/mL.

- Lycopene standard stock solution 1 mg/mL: prepare similarly to *β*-carotene standard solution with a concentration range from 1.41 µg/mL - 180.00 µg/mL.

2.4.2. Preparation of sample solution:

The sample preparation followed a procedure based on references [9, 10]. The details are as follows: Weigh accurately about 1 g of the sample powder (crushed and sieved through a 250 mesh screen) into a 100 mL ground-glass-stoppered conical flask. Add 25 mL of the solvent *n*-hexane - acetone - ethanol (2:1:1; *v/v/v*), shake for 30 min, filter through filter paper into a 25 mL volumetric flask, add up to the mark with the solvent mixture *n*-hexane - acetone - ethanol (2:1:1; *v/v/v*), shake well to obtain the extract. Filter the extract through a 0.45 µm filter to obtain the sample solution for HPLC analysis.

2.4.3. Chromatographic conditions:

The mobile phase was a mixture of acetonitrile and methanol (70:30, *v/v*). The solvent flow rate was kept at 1.5 mL/min. The injection volume was 10 µL. Detection was set at a wavelength of 475 nm.

2.4.4. External standard method (ESM):

The analytical method was developed and validated for system suitability, specificity, calibration curve, accuracy, precision, detection limit, and quantitation limit following the current ICH guideline [12].

2.4.5. QAMS Method:

Step 1: Identify the two analytes.

Step 2: Calculate the concentrations of the two analytes using the calibration curves established with the ESM.

Step 3: Calculate the relative correction factor (RCF) and relative retention time (RRT) using formulas (1) and (2) for different injection volumes, different columns, and different instruments.

Step 4: Determine the retention time and the lycopene content using the *β*-carotene content, the RRT, and the RCF obtained.

Step 5: Compare the QAMS method with the ESM to assess accuracy (paired comparison was performed using Minitab 16 software).

The content of the multi-marker components measured by QAMS was compared with results from ESM to validate the methods of QAMS. *β*-Carotene was chosen as a standard for the quantitative analysis of lycopene. The RCF and the RRT are a constant of proportionality in a computational formula and can be calculated as follows:

$$RRT_x = \frac{T_{Rx}}{T_{Rs}} \quad (1) \qquad RCF = \frac{C_s \times A_x}{C_x \times A_s} \quad (2)$$

Where

T_{Rs}, As, and Cs are the retention time, peak area, and concentration of the standard (*β*-carotene);

T_{Rx}, A_x, and C_x are the retention time, peak area, and concentration of the analyzed compound (lycopene).

2.5. Calculation

The content of the measured component in GSA samples by the ESM method was calculated as follows:

$$\boldsymbol{X_{ESM}}(\%) = \frac{\boldsymbol{C_t \times V \times 100}}{\boldsymbol{M \times (100 - a)}} \times \mathbf{100} \quad (3)$$

where C_t: the concentration of analyzed compounds in the sample solution from the calibration curve equation (mg/mL);

V: volume of the sample solution (mL);

m: weight of sample taken to prepare the sample solution (mg);

a: the moisture of the sample (%).

The concentration of the measured component by QAMS was calculated as follows:

$$\boldsymbol{C_x} = \frac{\boldsymbol{RCF \times A_x \times C_S}}{\boldsymbol{A_s}} \quad (4)$$

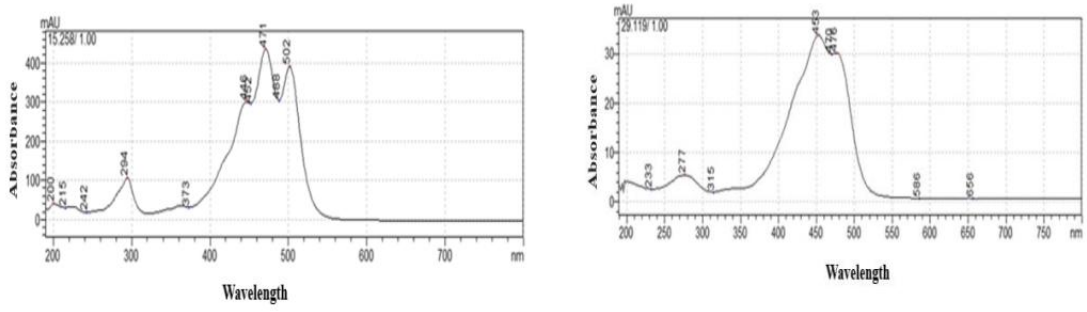
Where: A_x and C_x are the peak area and the concentration of the analyte in the test sample; A_s và C_s are the peak area and the concentration of the internal standard substance.

3. Results and discussion

3.1. Optimization of HPLC-DAD conditions

3.1.1. Wavelength selection for quantification:

Maximum absorption wavelengths of lycopene and *β*-carotene were determined by acquiring UV spectra of each standard solution individually. The resulting spectra are displayed in **Fig. 1**. As 475 nm is commonly selected for quantifying lycopene and *β*-carotene in the references [4], we also chose this wavelength for the simultaneous quantification of these compounds in GSA in this study.

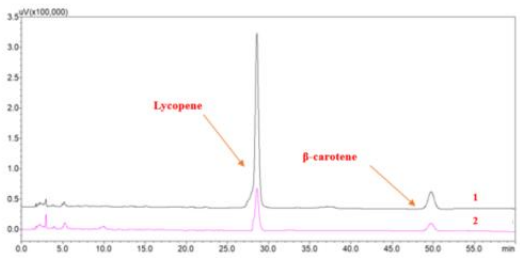


Lycopene **β-Carotene**

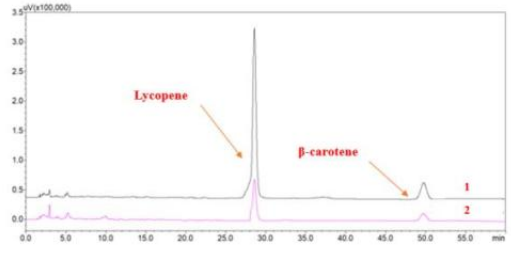
Fig. 1. UV absorption spectra of lycopene and *β*-carotene

3.1.2. Mobile phase optimization:

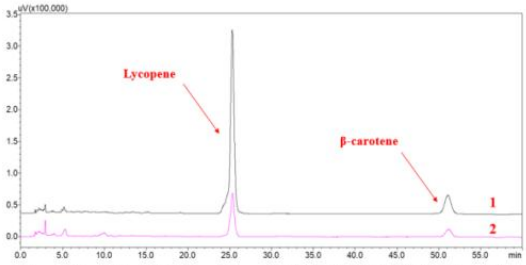
To obtain suitable chromatographic properties, various HPLC parameters, including mobile phase and elution procedure (MeOH and ACN with different procedures), were tested and compared.



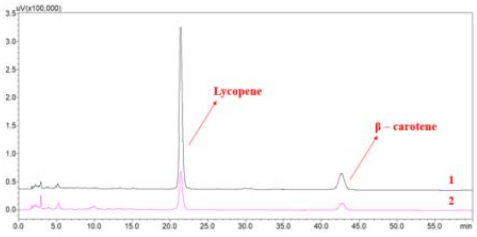
Condition 1: Mobile phase ACN - MeOH (30:70, v/v), flow rate v = 1.0 mL/ min



Condition 2: Mobile phase ACN - MeOH (55:45, v/v), flow rate v = 1.0 mL/ min



Condition 3: Mobile phase ACN - MeOH (70:30, v/v), flow rate v = 1.0 mL/ min



Condition 4: Mobile phase ACN - MeOH (70:30, v/v), flow rate v = 1.5 mL/ min

Fig. 2. Chromatograms of different mobile phase conditions.
(1 - Sample solution, 2 - Standard solution; Detection wavelength: 475 nm; Injection volume: 10 µL; Column temperature: room temperature).

Table 1. The results of the mobile phase survey

Conditions	Lycopene				*β*-Carotene			
	T_R (min)	R_s	T_f	N	T_R (min)	R_s	T_f	N
1	28.914	1.145	1.106	5022	49.758	22.181	1.109	9569
2	27.598	2.246	1.176	4404	48.785	19.326	1.078	14672
3	25.045	12.115	0.743	9755	51.124	2.469	1.046	13589
4	**21.410**	**8.012**	**0.866**	**12125**	**42.656**	**7.120**	**0.984**	**13689**

Note: T_R: Retention time; R_s: Resolution; T_f: Tailing factor; N: Number of theoretical plates.

The results showed that condition 1 provided insufficient lycopene resolution (<1.5). Condition 2 had asymmetrical, sharp peaks and a long analysis time. Condition 3 offered good separation, but its peak symmetry was unsatisfactory (<0.8). However, condition 4 met all requirements, with a short analysis time, good separation, and acceptable peak symmetry. Therefore, we chose condition 4 for further investigation.

3.1.3. Column temperature optimization:

Temperature influences the elution process of compounds from the separation column. When the column temperature changes, the eluting strength of the mobile phase is altered, which in turn affects the retention time of the analytes on the column, consequently impacting the separation efficiency. Therefore, it is necessary to investigate the effect of column temperature on the analytical conditions. The results of this investigation are presented in **Table 2.**

Table 2. The results of the column temperature survey

Temperature	Lycopene				*β*-Carotene			
	T_R (min)	R_s	T_f	N	T_R (min)	R_s	T_f	N
25°C	21.410	8.012	0.866	12125	42.656	7.120	0.984	13689
30°C	20.552	6.674	0.820	12295	40.652	2.570	1.004	14984
40°C	**15.266**	**6.507**	**0.887**	**11437**	**29.305**	**2.306**	**1.035**	**14661**
45°C	13.046	5.959	0.904	9441	24.517	2.177	1.102	17500
50°C	11.155	5.451	0.962	10891	20.579	2.015	1.088	12673

Note: T_R: Retention time; R_s: Resolution; T_f: Tailing factor; N: Number of theoretical plates.

The column temperature profoundly affected the retention time, resolution, and peak symmetry of both lycopene and *β*-carotene. Raising the temperature from room temperature to 40°C decreased the retention time of lycopene from 21.41 minutes to 15.27 minutes and that of *β*-carotene from 42.66 minutes to 29.31 minutes. Although increasing the temperature further to 50°C showed little change in retention, good peak shape, symmetry, and separation, to ensure column longevity, we selected a column temperature of 40°C.

3.1.4. Injection volume optimization:

In addition to mobile phase composition and column oven temperature, the injection volume in chromatography also significantly influences the separation results. Large injection volumes can cause peak broadening, while excessively small injection volumes may lead to low analyte signals, increasing the method's detection limit and

reducing sensitivity. To optimize the analytical conditions, different injection volumes were tested (5 µL, 10 µL, 20 µL, 30 µL, and 50 µL). The results of this study are presented in **Table 3**.

Table 3. The results of the injection volume survey

Injection volumes (µL)	Lycopene					*β*-Carotene				
	T_R (min)	R_s	T_f	N	S (mAu.s)	T_R (min)	R_s	T_f	N	S (mAu.s)
5	15.543	7.208	0.845	11731	4931394	29.810	2.558	1.018	15720	1031769
10	**15.727**	**6.507**	**0.887**	**11437**	**10760504**	**29.217**	**2.306**	**1.035**	**14661**	**1967452**
20	15.518	5.565	0.842	8075	20650229	29.810	2.271	1.005	14576	4440617
30	15.398	3.593	0.819	6123	15156454	29.554	2.197	0.992	14511	5450896
50	15.398	2.862	0.815	4750	23730976	29.570	2.136	0.971	12940	8589550

Note: T_R: Retention time; R_s: Resolution; T_f: Tailing factor; N: Number of theoretical plates; S: Area of peak

The results obtained revealed that the injection volume influences peak parameters. For both analytes, as the injection volume increased, the resolution (Rs) and the number of theoretical plates decreased. For lycopene, injection volumes of 20 - 50 µLresulted in a number of theoretical plates <10,000. For *β*-carotene, the number of theoretical plates was >10,000 across the injection volume range of 5 - 50 µL. Based on these results, injection volumes in the range of 5 - 10 µL provided satisfactory peak parameters. Therefore, we selected an injection volume of 10 µL for subsequent studies.

3.2. HPLC-DAD method validation

❖ *Selectivity*

The following solutions were subjected to analysis: a blank solution, a lycopene standard solution, a *β*-carotene standard solution, and a sample solution of GSA. The corresponding results are detailed in **Figs. 3** and **4.**

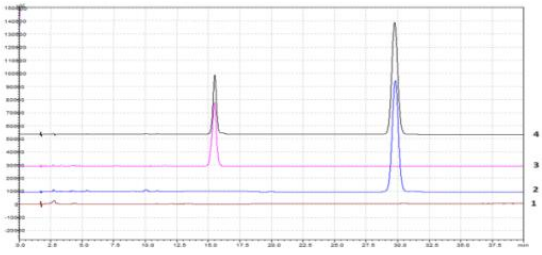
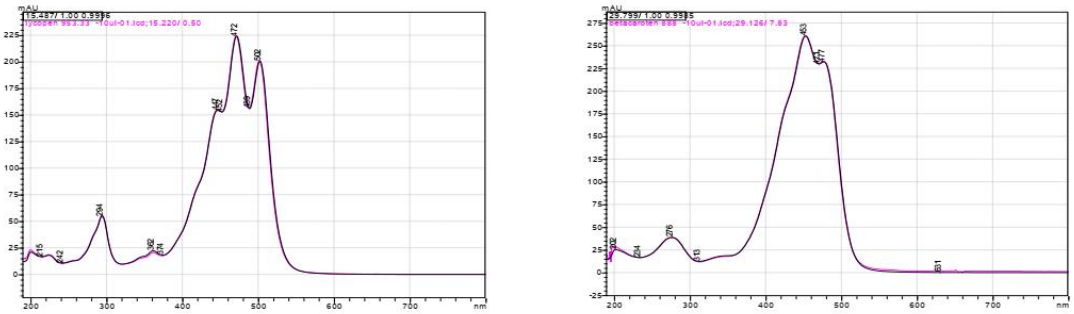


Fig. 3. The chromatogram assesses the selectivity of the method
(1 - Blank, 2 - β-carotene, 3 - lycopene, 4 - GSA sample)



Lycopene **β-Carotene**

Fig. 4. Compare the spectra of *β*-carotene and lycopene in the sample and standards

The lycopene and *β*-carotene peaks were completely separated in the chromatograms of both the standard solution and the GSA sample solution. Furthermore, the matching ratio of *β*-carotene and lycopene in the sample compared to the standards was greater than 0.99 (**Fig. 4**), indicating that the lycopene and *β*-carotene peaks in the sample chromatogram are pure. These results demonstrate that the developed method meets the required specificity for the simultaneous quantification of lycopene and *β*-carotene in the GSA sample.

❖ *Linearity*

Prepare standard solutions at appropriate concentrations by diluting the standard solution (1000 µg/mL) with the solvent mixture to

establish the calibration. Perform HPLC-DAD analysis of these standard solutions, record the slope values, and construct calibration curves for lycopene and β-carotene (**Fig. 5**). The calibration curves exhibited $P_{\text{-values (intercept)}} > 0.05$ and correlation coefficients (R^2) > 0.99, indicating the strong linear correlation between β-carotene (lycopene) concentration and peak area. The bias was less than 15%, and no evidence of systematic error was observed. Therefore, the proposed method can be used for the quantitative analysis of β-carotene and lycopene.

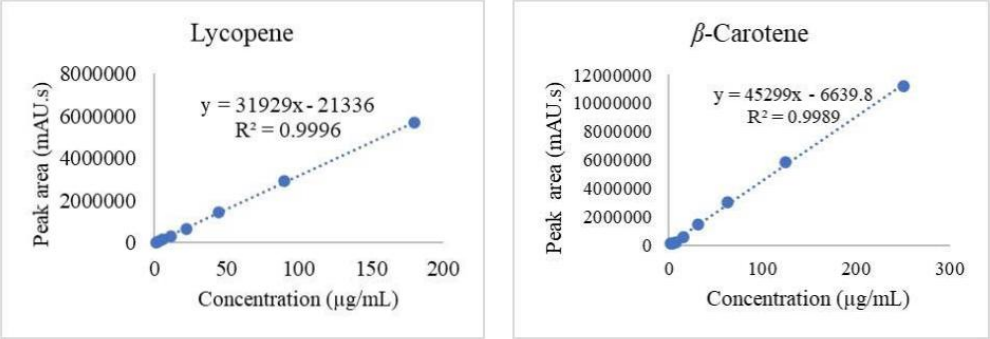


Fig. 5. Calibration curves of lycopene và β-carotene

Repeatability precision conduct research the trial 6 times with the same sample and determine the relative standard deviation (RSD) of the content. Reproducibility precision arranges the trial analogous to the repetition part of the system, but conducted at different times and analyzed on the same HPLC system. Conduct the quantification 6 separate times on a sample and calculate the relative standard deviation, according to AOAC conditions, RSD ≤ 3.7% for analytes with content in the sample ≥ 0.1%.

The sample was analyzed to determine the concentration of β-carotene and lycopene, then gradually diluted until on the chromatogram of the test solution, at the retention time of β-carotene and lycopene, the S/N ratio = 3 - 4 and S/N = 10 were determined. From there, the LOD and LOQ were found. Accuracy was evaluated using standard addition. β-Carotene and lycopene standards were added to the GSA sample, which had been pre-analyzed to determine its content. Recovery was assessed at multiple spiking levels using varying concentrations of β-carotene and lycopene standards. These results are presented in **Table 4**.

Table 4. Results of HPLC-DAD method validation for lycopene and β-carotene analysis in GSA

No.	Parameter	Compounds	
		Lycopene	***β*-Carotene**
1	System suitability	RSD % (t_R) = 0.042% RSD % (S) = 1.409%	RSD % (t_R) = 0.042% RSD % (S) = 1.643%
2	Intra-day precision	Mean = 0.097%	Mean = 0.021%
		RSD_r (%) = 3.931%	RSD_r (%) = 3.695%
3	Inter-day precision	Mean = 0.099%	Mean = 0.022%
4		RSD_R (%) = 4.995%	RSD_R (%) = 5.068%
5	LOD (mg/g)	0.15	0.12
6	LOQ (mg/g)	0.51	0.38
7	Accuracy	93.91 - 102.79%	92.27 - 103.64%

The standard addition method was used to evaluate the recovery efficiency of the method. Standard solutions of β-carotene and lycopene were added to the sample (GSA), for which the content had already been determined. The evaluation was conducted at different spiking levels of β-carotene and lycopene. The spiked samples were then processed and analyzed according to the selected conditions. The results in **Table 4** show that the recovery rates of β-carotene and lycopene were relatively high, ranging from 92.2 - 103.6%. Therefore, it can be concluded that the sample preparation method is reliable.

3.3. Quantitative Analysis of Multicomponent by Single Marker

Relative correction factors (RCF) were

calculated at different concentrations, different columns, and instruments, as described in formulas 1 and 2 of section 2.3.5. The results are presented in **Tables 5** and **6**.

Table 5. The values of RCF calculated under different injection volumes

Injection volumes (µL)	Lycopene			*β*-Carotene	
	Peak area (mAu.s)	Concentration (µg/mL)	RCF	Peak area (mAu.s)	Concentration (µg/mL)
4	961152	13.61	**1.44**	978707	21.75
6	1536693	30.77	**1.43**	1635250	36.25
10	2751502	48.80	**1.43**	2932787	64.89
16	4613215	86.84	**1.42**	4952063	109.47
20	5811759	145.15	**1.42**	6403474	141.51
Mean			**1.43**		
RSD%			**1.20**		

Table 6. The values of RRT and RCF calculated with different columns and instruments

Instruments	Columns	T_R (min)		RRT	RCF
		β-Carotene	Lycopene		
HPLC -1	Agilent C_{18}	38.892	20.418	0.52	1.43
		38.477	20.063	0.52	1.43
		38.972	20.264	0.52	1.43
	Shimadzu C_{18}	29.360	15.789	0.54	1.42
		28.935	15.705	0.54	1.43
		28.989	15.594	0.54	1.43
	TOSOH C_{18}	31.859	17.785	0.56	1.43
		32.078	18.047	0.56	1.43
		31.825	17.776	0.56	1.43
HPLC -2	Agilent C_{18}	32.891	17.324	0.53	1.43
		33.052	17.361	0.53	1.43
		33.133	17.417	0.53	1.43
	Shimadzu C_{18}	24.333	13.218	0.54	1.43
		24.329	13.212	0.54	1.43
		24.306	13.184	0.54	1.43
	TOSOH C_{18}	23.244	13.152	0.57	1.43
		23.386	13.269	0.57	1.43
		23.391	13.273	0.57	1.43
Mean				**0.54**	**1.43**
RSD%				**3.02**	**0.04**

Note: T_R: retention time; RRT: relative retention time; RCF: relative correction factor

The results of determining the RRT and RCF values of lycopene (RRT = 0.54; RCF = 1.43) compared to the *β*-carotene standard showed good repeatability (RSD values < 3%). This proves that the research method is stable and has good repeatability.

3.4. Application of ESM and QAMS for quantification of β-carotene and lycopene in GSA samples

The proposed method was used to evaluate the *β*-carotene and lycopene content in several GSA samples collected in Vietnam in 2023. The results, calculated using QAMS and ESM, are presented in **Table 7**.

Table 7. Content of lycopene and *β*-carotene in 10 samples of GSA determined by QAMS and ESM (n=3)

Code	Sampling location	Content (%)		
		β-Carotene	Lycopene	
			ESM	QAMS
M1	Duc Tho, Ha Tinh	0.059	1.111	1.114
M2	Cu Jut, Dak Nong	0.080	1.248	1.253
M3	Cu Jut, Dak Nong	0.081	0.296	0.294
M4	Son Tay, Ha Noi	0.117	0.778	0.779
M5	Son Tay, Ha Noi	0.114	0.622	0.621
M6	Son Tay, Ha Noi	0.046	0.541	0.54
M7	Vinh Yen, Vinh Phuc	0.019	0.183	0.179
M8	Luc Nam, Bac Giang	0.013	0.437	0.435
M9	Phu Thuong, Ha Noi	0.022	0.152	0.148
M10	Quynh Luu, Nghe An	0.085	0.579	0.578
P-value*			**0.649**	

3.5. Discussion

This study optimized the chromatographic conditions using an HPLC-DAD system with the following parameters: a C_{18} column, a mobile phase of ACN - MeOH (70:30, *v/v*), a flow rate of 1.5 mL/min, an injection volume of 10 µL, and a column temperature of 40°C. The method was validated according to ICH guidelines, demonstrating acceptable selectivity, system suitability, intra-day precision, inter-day precision, and recovery. In 2010, Tran Thi Huyen Nga's dissertation examined the biological activity of various carotenoids in some Vietnamese plants, reporting *β*-carotene content of about 0.022% in GSA [13]. Kubola J. and Siriamornpun S. analyzed the active compounds in GSA and found a maximum of 0.702% lycopene [4]. The lycopene and *β*-carotene content in the GSA samples analyzed in this study were found to range from 0.152% to 1.117% (higher than in other studies) and from 0.013% to 0.117%, respectively [4, 13]

This research established a QAMS method for the simultaneous quantification of lycopene and *β*-carotene in GSA samples using a single standard (*β*-carotene), with RCF of 1.43 and RRT of 0.56. The analysis revealed no statistically significant difference between the two methods ($P > 0.05$), indicating that the established approach provides accurate and dependable results.

4. Conclusion

This study employed HPLC-DAD with an external standard method (ESM) as the reference method. The chromatographic conditions were optimized, and the method was validated. Furthermore, a QAMS method was developed to simultaneously analyze both compounds using a single standard, thereby reducing analysis time and cost and contributing a novel approach to the quality standardization of Gac seed aril. Ten samples of Gac seed aril were determined, and the results showed that the mean contents of 0.013 - 0.117% and 0.152 - 1.248% for *β*-carotene and lycopene, respectively. There were no significant variations between the results of the two different determination methods QAMS and ESM ($P > 0.05$). The obtained results indicated that the established QAMS method can be accurately, economically, simply, and quickly applied to the multicomponent analysis of Gac seed aril.

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