RESEARCH PAPER

Quantitative Estimation of Beta-Carotene in Spirulina Species by HPTLC

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ABSTRACT

Currently, Spirulina microalgae has become the most popular nutraceutical food product. It is rich in macro as well as micronutrients. In the present study, among three species of spirulina, we have used two species (Spirulina platensis and Spirulina maxima). The objective of research was to develop and validate a quick High Performance Thin Layer Chromatography method to determine beta carotene (BC) in both Spirulina species. Method validation was performed using the parameters like linearity, detection limit (LOD), quantification limit (LOQ), precision, accuracy, and robustness with help of ICH Q2 (R1) guidelines. A good separation and sharp peak of BC from both species of acetone extracts were obtained with mobile phase n-hexane: acetone: acetic acid (9: 1: 0.1, v/v/v). The retardation factor (Rf) of BC was observed 0.700 ± 0.2. The peak area was determined at wavelength 448nm. Good linearity was obtained in the range of 1000 - 3500 ng/band with correlation coefficient (R2) of 0.9991. Detection limit and quantitation limit was found to be 381.11 ng/band and 976.89 ng/band respectively. Developed HPTLC method was found to be precise, accurate and robust. The marketed product was then analyzed using the method, and the % content for BC in the formulation was found to be 95.77 % w/w.

Keywords: Spirulina platensis, Spirulina maxima, Beta carotene, HPTLC, Method development. Analytical.

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INTRODUCTION

In recent years, health-conscious people have shifted their attention from synthetic supplements to herbal supplements. However, they are now focusing on microalgae, an unexplored area of alternative healthcare. In African as well as East Asian Countries this microalga has become a more popular food product in recent years. *Haematococcus pluvialis, Chlorella*[1]*, Spirulina, and Dunaliella salina*[2], are some examples of microalgae that are consumed as food by humans for good nutritional value. Among these Spirulina microalgae have gained much more popularity and significant effects. This Spirulina is also known as the blue-green algae and the genus is *Arthospira*[3]. Three species of Spirulina have therapeutic activity and are safe to use as food i.e. *Spirulina maxima, Spirulina platensis*[4], and *Spirulina fusiformis*[5]. The structure and chemical constituent concentrations of *Spirulina maxima* and *Spirulina platensis* differ from one another. *S. maxima* have a spiral shape, but *S. platensis* has a cylindrical shape [6-7].

Spirulina possesses various therapeutic activities such as antilipidemic, antihypertensive, antioxidant, antidiabetic, and antibacterial activity as well [8-9]. It additionally possesses a high nutritional content[10]. These various activities of Spirulina are due to its good chemical composition and nutritional profiles. Bioactive molecules like phycocyanin[11], lutein, caffeic acid, quercetin, cinnamic acid, and many more are present in Spirulina[12-13]. Beta carotene is one of the active constituents present in the Spirulina. Also, it is a precursor of Vitamin A[14]. It is mainly known for its antioxidant activity and holds activities like anti-inflammatory, antibacterial, and immunomodulatory [15-16]. The estimation of Beta carotene from various plant sources by High Performance Liquid Chromatography has already been performed[17]. Even carotenoids present in other microalgaes also estimated by High Performance Thin Layer Chromatography (HPTLC) methods[18]. HPLC method to estimate beta carotene from spirulina has been developed by certain researchers[19].

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In this work, we proposed an HPTLC method that is easy to use, precise, accurate, and inexpensive to estimate beta carotene from Spirulina species (*Spirulina platensis* and *Spirulina maxima*). For validation of developed method ICH Q2 (R1) guideline were followed[20].

MATERIALS AND METHOD

Algae powder collection

For the study, among different species of spirulina microalgae, the two species were taken into consideration. The *Spirulina platensis* (SP) microalgae dried powder was procured from Nutrigen Agro Tech, Pune, Maharashtra; while *Spirulina maxima* (SM) dried powder was taken from Chandan Agro Tech, Kolhapur, Maharashtra, India.

Procurement of the reference standard

The reference material beta-carotene (BC) having purity of 99% was bought from Sigma Aldrich Pvt, Ltd. in Bengaluru, India.

Chemicals

Acetone, n-hexane, and acetic acid (99.5%) were used for the mobile phase and acquired from Loba Chemicals, Pvt. Ltd. Mumbai. Water utilized in the research work is double distilled and it was taken from an in-house water system by Rephile.

Extraction Process

The solvent system applied was acetone to extract the BC from SP and SM. The weighed 25 g of spirulina powder of both species was extracted with 150 mL of acetone for 72 hr, with the cold maceration extraction technique. After 72 hr, the liquid extracts were filtered and then dried under the Rota evaporator. The dried extract was stored under a cool temp (2 to 8 ºC) [21].

Sample Preparation

For achieving a concentration of 1000 µg/mL, 10 mg of standard beta carotene (Std. BC) were precisely measured and dissolved in 10 mL of acetone. As BC is a light-sensitive drug the solution was made in an amber-colored volumetric flask and then further vortexed for 1-2 mins. For the calibration curve, the same stock solution was used. To achieve the concentration of 5000 µg/mL of both species extracts; weighed 10 mg of dried extracts and dissolved in 2 mL of acetone. This solution was filtered using syringe filters of size 0.45 µm and then used for HPTLC.

Chromatographic conditions

Coated silica gel plate 60 F_{254} with dimension of 20 × 10 cm, having 250 µm thickness made by E. Merck, Germany was used for HPTLC analysis. The plates were preactivated for 25 mins at 100ºC. The BC and both species (SM and SP) extracts were banded 2 µL and 5 µL respectively, through the sample applicator linomat 5 autosampler. The band size was kept at 6 mm. Saturation time was kept at 25 min with solvent system n-hexane: acetone: acetic acid in a ratio 9:1:0.1 v/v. TLC plates were developed under CAMAG made a twin-trough chamber having dimension of 20 cm × 10 cm × 4 cm in ascending position. The development distance of plates was done up to 80 mm. Developed plates were visualized under TLC visualizer 3 at white light and fluorescent lamp. Further plates are scanned at the wavelength of 448 nm under Scanner 3. The complete setup was controlled by WinCATS software version 3.15 by CAMAG.

Validation of Developed Method

Validation of developed method was performed by considering ICH guideline Q2 (R1). Parameters like specificity, linearity, detection limit, quantification limit, accuracy, precision, repeatability, and robustness were checked for the developed HPTLC method[20].

Specificity

By proving that the presence of other compounds has no effect on the identification and/or quantification of an analyte, specificity can be demonstrated. In order to determine the methods specificity, samples taken from extracts of spirulina species and standard BC were checked. The Rf of the extracts and the standard were compared in order to confirm the spots for BC.

Linearity and Range

To achieve a concentration of 1000 - 3500 ng/band, different volumes like 1 µL to 3.5 µL of stock solutions (1000 µg/mL) were banded on the TLC plate. These concentrations are plotted against the area equivalent of concentrations for the calibration curve. Correlation coefficient (R^2) was obtained.

LOD and LOQ

The LOD and LOQ for beta carotene was calculated. Mentioned formulas were used for determination of LOD and LOQ form calibration curve[22].

$$LOD = 3.3 \times \frac{\sigma}{s} \qquad LOQ = 10 \times \frac{\sigma}{s}$$

Recovery (Accuracy):

Study was performed to assess the developed method's accuracy. It was evaluated via the standard addition method. For this study 80%, 100%, and 120% of standard drug (BC) were spiked in the assay sample solution. It was replicated three times and recovered accuracy in the percentage was determined[23].

Precision

To confirm that the method is precise, an intraday and interday precision analysis was carried out. Three replicates were banded on the same day at three distinct conc. levels of 1500, 2500, and 3500 ng/band to determine the intraday precision. The interday precision is determined by performing the same measurement three times on three consecutive days. Relative standard deviations (% RSD) were calculated for intraday and interday precision.

Repeatability

Repeatability was evaluated to check the developed method can reproduce same results. It was performed

by repeating the same concentration (2500 ng/band) for six replicates at a time. The % RSD was taken.

Robustness

For analytical methods, robustness is much essential parameter. The robust method determines that the developed method is stable, robust, and unaffected by purposeful minute changes in parameters. Parameters like change in Mobile phase by ± 0.2 mL v/v, without any change in the acetic acid, changes in UV wavelength, saturation time, and development distance by ± 2 nm, ± 5 mins, and ± 0.5 cm respectively. The concentration utilized in it was 2500 ng/band. Retention factor (Rf) and present accuracy were calculated with optimized method[24].

Assay of Marketed Formulation (Capsule)

The Grenera Spirulina Capsule (500 mg spirulina powder per capsule) was used for performing the assay. After accurately weighing 20 capsules, the average weight was calculated. In a dried mortar and pestle, the granules in the capsule were ground into a fine powder. In 10 mL of acetone spirulina powder corresponding to 500 mg was dissolved within a volumetric flask. Dilution was made to get the concentration of 1500 µg/mL and applied on the TLC plate in triplicate.

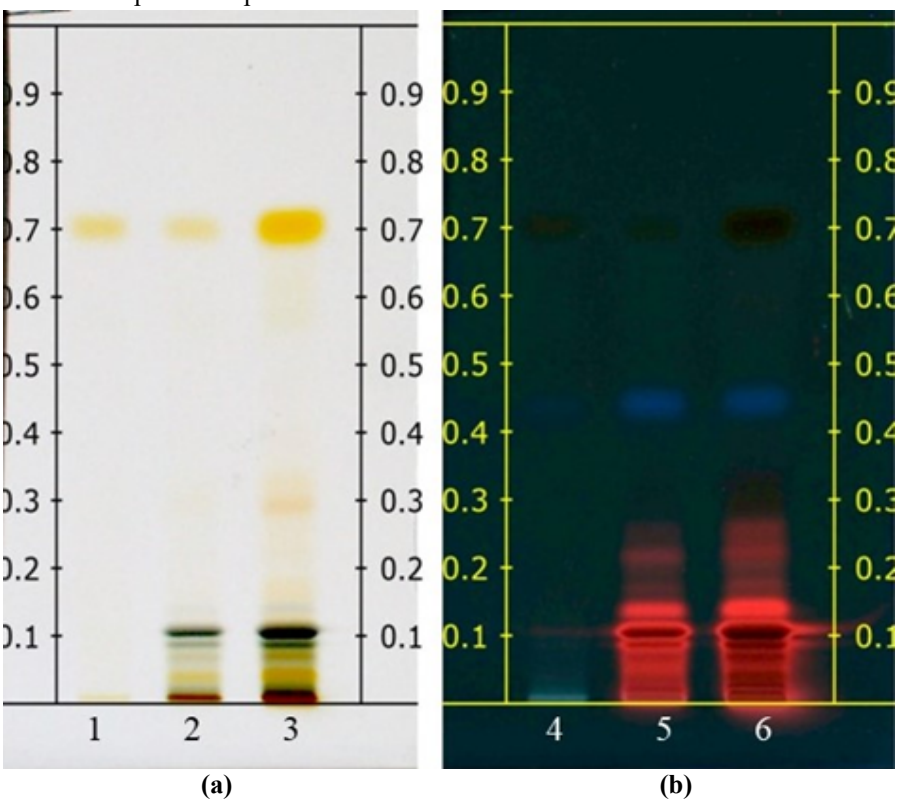
RESULTS AND DISCUSSION

Mobile phase Optimization:

After various trials of different combinations of mobile phases; the resolved & sharp peak of standard BC and BC in extracts of spirulina species (SM and SP) were acquired with a mobile phase containing of n-hexane: Acetone: Acetic acid with the ratio of 9:1:0.1 (v/v/v). A development distance of 8 cm was selected for the TLC plate. A 25-minute chamber saturation time and 25-minute plate activation time at 100ºC were maintained.

Specificity

In order to determine the method specificity, samples taken from extracts of spirulina species (SM and SP) and standard BC were checked. BC was visualized with the distinct band under white light and at fluorescent lamp (Figure 1 a, b). Scanning under optimized chromatographic conditions, at wavelength of 448 nm, the retention factor (Rf) was found to be 0.700 ± 0.2 for standard BC and BC in both extracts. Densitograms of Std. BC and extracts (SM and SP) are shown below (Figure 2 a, b, c).



(a) **(b)**

Figure 1: (a) Image under white light, (b) Image under fluorescent lamp-Track 1, 4 is Std. BC; Track 2, 5 is SP extract while track 3, 6 is SM extract.

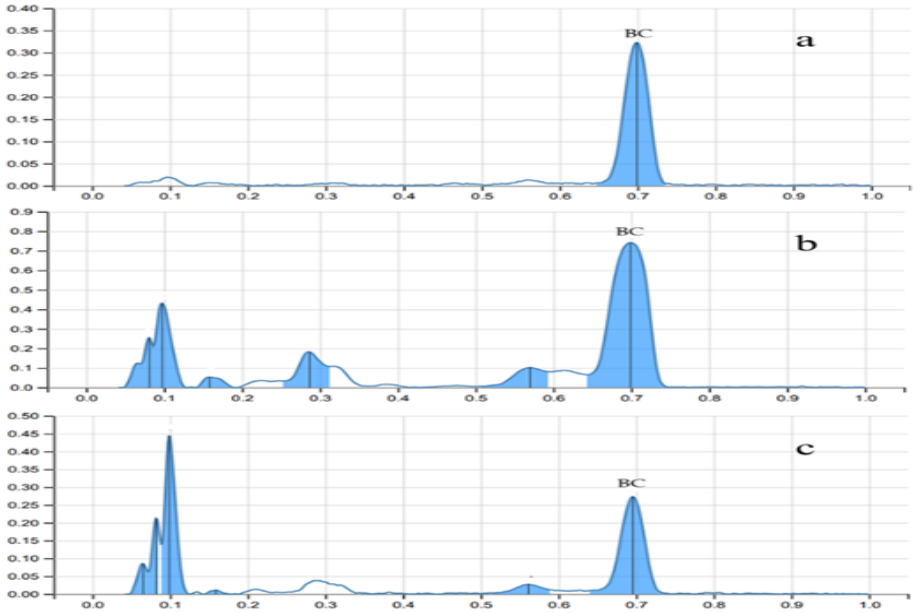


Figure 2: Representative Densitogram of standard Beta carotene (a), extract of SM (b) and extract of SP (c) respectively.

METHOD VALIDATION

Linearity and Range

A relationship across area responses and concentrations (ng/band) can be seen in the linearity range. In concentration range of 1000 - 3500 ng/band developed method was found to be linear. Regression equation obtained was y = 0.3343x + 65.895 with R^2 = 0.9991. (Figure 3).

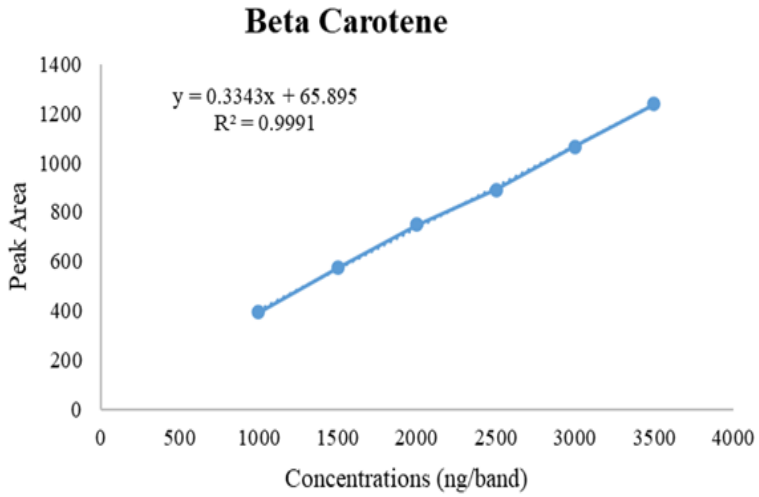


Figure 3. Calibration curve of beta carotene

LOD and LOQ

Using the equation technique, the developed methods detection limit and quantification limit were determined. LOD and LOQ were found to be 381.11 ng/band & 976.89 ng/band respectively.

Recovery (Accuracy) studies

By spiking the standard drug, the proposed method's recovery was assessed with the conventional standard addition method. The recovery of BC for 80%, 100%, and 120% was found to be 94.6-98.5 % respectively. Data is represented in Table 1.

Table 1: Result of recovery studies (n=3)

Grenera Spirulina Capsule	Standard addition (%)	Total Amount (mg)	Amount found (mg)	% Recovery	Mean % recovery
Spirulina powder 500 mg	80	900	857.88	95.32	96.49
	100	1000	956.7	95.67	
	120	1100	1083.4	98.49	

Precision

An interday and intraday precision study was conducted to assess the developed method's reproducibility. The % RSD of triplicate concentrations of 1500, 2500, and 3500 ng/ band was found to be less than 2.0 %. Table 2 determines the mean area, SD, and an intraday and interday precision study was conducted to assess the developed method's reproducibility. % RSD for interday and intraday precision.

Table 2: Precision results of proposed method (n = 3)

Concentration ng/band	Intraday precision			Interday precision		
	Avg. area	SD	% RSD	Avg. area	SD	% RSD
1500	597.66	7.2	1.2	491.66	7.09	1.4
2500	1018.67	16.8	1.6	882.66	11.59	1.3
3500	1552.33	27.3	1.7	1483	17.69	1.1

Repeatability:

The methods repeatability was investigated, and the average area of the same concentration (n=6) and its percentage RSD were calculated; and it was found to be less than 2.0 %. Repeatability data is represented in Table 3.

Table 3: Repeatability study (n = 6)

Concentration	Area	Avg. area ± SD	% RSD
2500 ng/band	1029	1026.167 ± 3.66	0.36
	1028		
	1022		
	1024		
	1023		
	1031		

Robustness

By making changes in the mobile phase composition, UV wavelength, saturation time, and development distance the robustness of the method was analyzed. % RSD and percent accuracy of Rf of all parameters were calculated which are revealed in Table 4.

Table 4: Results of robustness of method (n=3)

Conditions	BC		
	Rf	Accuracy (%)	% RSD
UV wavelength: 448 nm ± 2 nm			
446	0.689	98.56	1.3
450	0.687	98.28	1.8
Mobile phase composition: n-hexane: acetone: acetic acid (9: 1: 0.1 v/v/v) ± 0.2 mL			
n-hexane: acetone: acetic acid (8.8: 1.2: 0.1 v/v/v)	0.724	103.5	1.8
n-hexane: acetone: acetic acid 9.2: 0.8: 0.1 v/v/v	0.681	97.42	1.3
Saturation time: 25 min. ± 5 min.			
20	0.712	101.85	1.2
30	0.691	98.85	1.2
Development distance: 8 cm ± 0.5 cm			
7.5	0.689	98.56	1.6
8.5	0.695	99.42	1.5

Assay of Marketed Formulation (Capsule)

Using Grenera Spirulina capsule as a marketed formulation, an assay was perform to determine the content of beta carotene in capsule (each capsule contains 500 mg of spirulina powder). Three replicate determinations of beta carotene were performed, 95.77 ± 0.5 % was found as percent content for BC.

CONCLUSION

Since, spirulina is generally regarded as safe (GRAS), it is a superfood for mankind. In that *Spirulina platensis* and *Spirulina maxima* is most widely used due to its great nutritional and therapeutic activity. In the current research work, easy and rapid HPTLC method has been developed to estimate beta carotene from extracts of the spirulina species (*Spirulina maxima* and *Spirulina platensis*). After validation in compliance with the requirements of the ICH Q2 (R1) standard, the developed method was confirmed to be precise, robust, and accurate for usage. Also, it can be useful as starting point for bioanalytical application and for stability indicating degradation studies of BC.

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