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Research Article

Developing a Robust Method for Quantification of *β*-Carotene in *Dunaliella salina* Biomass Using HPLC Method

Mohammad Hossein Morowvat[1,2*], Younes Ghasemi[1,2]

[1]*Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, P.O. Box 71345-1583, Shiraz, Iran.*
[2]*Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, P.O. Box 71468-64685, Shiraz, Iran.*

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ABSTRACT

Current analytical methods to determine β-carotene, a naturally occurring carotenoid pigment, such as spectrophotometry or color assay lack the appropriate selectivity and sensitivity and sometimes with significant fluctuations in the obtained results. In this study, a robust reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed from the photosynthetic unicellular green microalga *Dunaliella salina*. β-carotene extraction was performed using acetone and chloroform method (2:1 v/v). The extracted β-carotene specimens were chromatographed using a C_{18} chromatography column with methanol: chloroform (85:15 v/v) and 2.0 mL min^{-1} flow rate. Two distinct standard curves with different concentrations (0.2-50 µg mL^{-1} and 100-500 µg mL^{-1}) were provided for β-carotene determination. The observed accuracy, precision and limits of detection and quantitation were quite within the satisfactory levels. The limit of detection (LOD) and quantitation (LOQ) of method were found to be 0.1 µg mL^{-1} and 0.2 µg mL^{-1}, respectively. The obtained values for β-carotene determination through HPLC method were in agreement with the conventional colorimetric routes (R^2=0.9587). The suggested analytical method might be magnificently exploited for β-carotene quantification in *D. salina* and also other related microalgal strains.

Keywords: β-carotene, Biomass, *Dunaliella salina*, Functional foods, HPLC analysis.

INTRODUCTION

Carotenoids, are organic pigments occurring in the chloroplast and other organelles in plants and some photosynthetic unicellular microorganisms such as bacteria, fungi and microalgae. This pigments could not be synthetized in humans and other animals. Therefore, there is an essential need for external sources of carotenoids in human diet, to meet the nutritional demands. Another reason for the growing importance of carotenoids, is their antioxidant and free radical scavenging activity and also their biological function as vitamin A precursory role. Many studies have shown a significant correlation between the consumption of carotenoids and reduction in the incidence of some diseases, including cancer[1], heart and cardiovascular diseases[2,3], prevention of osteoporosis[4], early-aging of the skin[5,6], soft tissue calcification[7], visual and neural impairments[8]. Industrial production of carotenoids from natural resources, is not restricted unique to plant species and nowadays significant efforts have been performed to identify and extract the potential resources of carotenoids in some species of bacteria and microalgae[9,10]. So far, various methods have been presented for analyzing carotenoid content in various food and pharmaceutical products. In this context, exploitation of the HPLC method, spectrophotometry and simple color reading assay has shown more efficiency[11]. Although, spectrophotometric and color reading methods show suitable features for rapid assessment of carotenoid content in a sample, but for a reliable and reproducible experiment a more robust method with accuracy and higher sensitivity, such as HPLC, which has a selective and flexible function should be employed. β-carotene instability during the extraction process, materials shipments or organic solvents removal steps, has hampered the β-carotene production as sample for analytical purposes. Hence, in most cases, the exploitation of additional processes is necessary to ensure complete extraction of carotenoids. In addition, due to low solubility of β-carotene in some common solvents like methanol for HPLC analysis, and this fact that substitution of another solvent instead of methanol, causes possible interference in the mobile phase used for the extraction of β-carotene. Therefore, in the context, all protocols for analysis of carotenoids, cannot be used for β-carotene measurement. As a result, due to the chemical instability of β-carotene, its analysis via HPLC, requires high precision and subtle manipulation methods. For this reason, access to reliable and fast way to use the analysis HPLC, to determine the amount of β-carotene is necessary. In addition, finding new and more efficient methods for the extraction and isolation of β-carotene from more diverse samples, including microalgae, seems vital. This study aims to solve common problems in the field of identification and quantification of β-carotene via HPLC, and to develop a simple, rapid and reliable protocol for

Author for Correspondence: mhmorowvat@sums.ac.ir

extraction, separation and quantification of β-carotene in various biological samples including microalgae. The halophilic microalga *Dunaliella salina*, known to produce high levels of carotenoid pigments, was used for production and extraction of β-carotene. Besides, the obtained results were compared with the results of conventional spectrophotometry-based approaches.

MATERIALS AND METHODS

Chemicals and reagents

β-carotene (C4582, type II synthetic, HPLC grade with ≥95% purity) was purchased from Sigma-Aldrich, St. Louis, Missouri, United States. The HPLC grade methanol and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). The HPLC grade triethylamine (TEA) and tetrahydrofuran (THF) stabilized with <0.025% butylated hydroxytoluene were obtained from Fluka (Sigma-Aldrich, St. Louis, Missouri, United States). Besides, the employed solvents for liquid-liquid extraction were obtained from Merck (Darmstadt, Germany). Double distilled water was attained obtained from a Milli-Q System (Millipore, Bedford, MA, USA). Mobile phase used in HPLC was filtered using a 0.2 µm membrane filter (Eilite Analytical Instruments Co., Dalian, China).

Strain, media and culture condition

D. salina microalgal strain belonging to Chlorophyceae class and Chlorophyta division was obtained from the Microalgal Culture Collection of Shiraz University of Medical Sciences (MCCS), Shiraz, Iran. For cultivation and preservation of the microalgal strain the Johnson medium[10] with 12% salinity was employed. The microalgal growth was carried out for 28 days prior to HPLC analysis. The study was conducted in 500 mL Erlenmeyer flasks containing 200 mL of culture medium[12]. The Erlenmeyer flasks were incubated at 25 °C in an orbital incubator shaker (PECO, Iran), agitated at 130 rpm for 96 h under the light intensity of 60 mol m^{-2} s^{-1}, to obtain a final cell concentration of around 30×10^4 cell mL^{-1}. The specified inoculum was employed as the standard culture for all future experiments in shake flask studies.

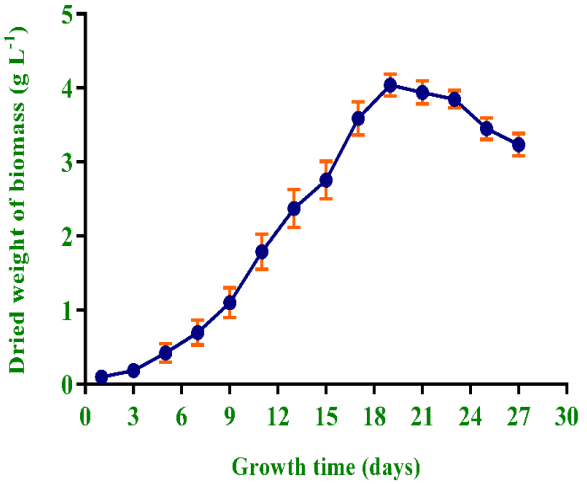

Figure 1: Growth trend of *D. salina* in shake flask scale during 28 days of study using dry cell weight method.

Cell growth measurement

The microalgal growth rates were monitored evert two days during 28 days of study using dry cell weight method[13]. *C. reinhardtii* cells (5 mL) were isolated aseptically and then centrifuged for 5 min at 6000 *g*. The obtained cell pellets were washed twice with an isotonic normal saline solution (0.09 g NaCl L^{-1}) and centrifuged again to remove the possible impurities. After it, the microalgal pellets were dried at 88°C overnight for cell dry weight determination.

β-carotene extraction

β-carotene concentration was quantified using colorimetric and HPLC methods. Before β-carotene detection, the sample preparation step was carried out as follow: after 28 days of cultivation, a sample of *D. salina* (1 mL) was taken from the culture that had been mixed thoroughly. After 5 min of centrifugation at 4000 rpm the upper phase was decanted. The obtained samples were lyophilized using freeze-drying method. Then 2 mL of dichloromethane was added to the biomass. Each sample was mixed by vortex for 1-2 min to reach the complete extraction. Samples then were centrifuged again for another 5 min at 4000 rpm for separation of the biomass (now colorless) and solvent phase. The extracted pigments in the solvent phase were quantified through colorimetric and HPLC method.

HPLC method development

The reversed-phase HPLC method was consisted as the mobile phase of an isocratic system of methanol: chloroform (85:15 v/v). The flow rate was 2.0 mL min^{-1} all over the steps. A C_{18} analysis column (25 × 4.6 mm; particle size 5 µm; Shimadzu, VP-OSD, Japan) was carried out to separate the analytes. A UV-detector compatible to the 450 nm wavelength (model 746, Waters, MA, USA) was exploited to detect the processed outputs and after it an appropriate integrator (model 486, Waters, MA, USA) was used to record the obtained information. To inject the analyzed samples a loop injector system with 50 µL loop, offered by Rheodyne® (Cotati, CA, USA) was used. An increase of retention time after 20 injections of samples could be reversed by washing the column with methanol:

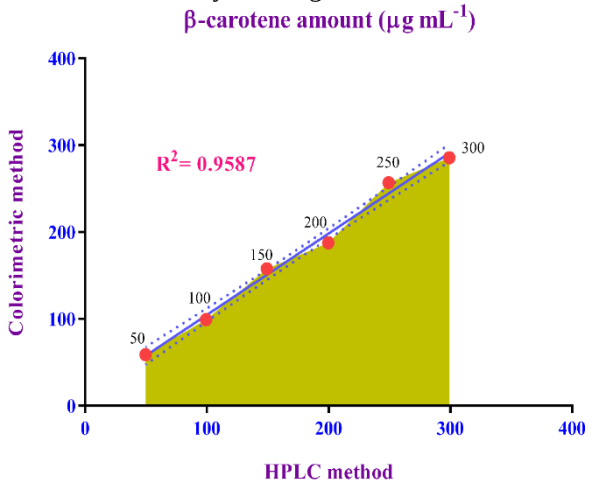

Figure 2: Correlation between the measured different amounts of β-carotene standard (50-300 µg mL^{-1}) using colorimetric and HPLC methods.

acetonitrile: methylene chloride (80:10:10 v/v/v). A stock solution of β-carotene with the concentration of 1 mg mL^{-1} was prepared in dichloromethane and the concentrations of 500, 200, 100, 50, 20, 10, 5, 2, 0.5, and 0.2 µg per mL were arranged using serial dilution method with the proper amounts of dichloromethane.

β-carotene standard curve

The obtained samples, obtained from the previous section, were injected directly to chromatograph. Nominal concentrations of β-carotene were used to perform the linear regression analyses. Besides, the corresponding peak heights, the intercept and measured slope for the depicted calibration curves and the regression coefficient (r), were also investigated.

Analytical validation tests

For precision and accuracy validation studies, within run and between run variations, absolute recovery (accuracy) and the limits of detection and quantitation were carried out.to test the within run and between run variations, in one run, 4 samples with β-carotene concentrations of 500, 100, 10, and 0.2 µg per mL, collected from the lowest, mid and highest regions of the produced standard curve were organized for three times and then investigated by optimized HPLC analysis. Moreover, for each studied concentration of β-carotene, the requested coefficient of variations was measured. Besides, for preparing the standard curve, the sampling procedure was performed in triplicate from three different regions located in lower, middle and higher places. The operative β-carotene concentrations were as same as within-run study. Continuing our analyses, the equivalent CV% for each run of study was measured. The accuracy (absolute β-carotene recovery), was measured using the identified β-carotene concentration with the prepared standard curve and nominal levels of β-carotene. The least concentration of β-carotene which could bring the signal to noise ratio of 3 was considered as the limit of detection (LOD). Moreover, the least concentration of β-carotene which was capable for quantification with acceptable precision and accuracy was regarded as the limit of quantitation (LOQ).

β-carotene determination using colorimetric method

β-carotene content was determined at 450 nm using a UV/Visible spectrophotometer (PG instrument Ltd.). The β-carotene levels were measured using spectrophotometry method using the below equation (1):

$$\beta\text{-carotene } (\mu g/mL) = 25.2 \times A_{450} \quad \text{(Eq. 1)}$$

Statistical analysis

To assess the significance of the obtained results, IBM SPSS software version 22.0 (Armonk, NY: IBM Corp.) and GraphPad prism version 6.00 (GraphPad Software, La Jolla California, USA) were employed. In ANOVA analysis, the statistical different levels at 5% were considered as significant.

RESULTS AND DISCUSSION

Cell growth, biomass production and kinetics

Dry cell weight measuring method was employed to observe the *D. salina* growth rate. The growth condition was conducted in 500 mL Erlenmeyer flasks containing 200 mL of Johnson culture medium (12%). Fig. 1 demonstrates the biomass production rate of *D. salina* during 28 days of experiment. The four initial days were regarded as the lag phase. After that, the logarithmic growth phase was noticed in 4^{th} to 10^{th} day of culture. It entered to the stationary growth phase at the 10^{th} day. The microalgal biomass was found to be 0.097 g L^{-1} at the first day of experiment. After finishing the cultivation experiment at 28^{th} day of study, *D. salina* dry cell weight was achieved to the maximal point of 4.042 g L^{-1} at 19^{th} day. The ultimate growth pattern displayed a typical sigmoidal pattern which was in agreement with previous reports on *D. salina* growth patterns[14]. Moreover, the growth parameters, maximum biomass productivities and cumulative β-carotene concentration from the studied microalgal strain during 28 days of study is presented in Table 1. As it has been indicated, the maximum biomass concentration (X_{max}) for the studied strain was found to be 4.042 g L^{-1} and the maximum specific growth rate (μ_{max}) was 0.22 d^{-1}. Besides, the maximum cell number of *D. salina* was 0.942 x 10^6 cell mL^{-1} and the day with the maximum N_{max} (D_{max}) was the 19^{th} day of cultivation. Moreover, the maximum biomass productivity (P_{max}) was determined to be 0.24 g L^{-1} d^{-1} and the maximum concentration of β-carotene (C_{max}) was observed as 9.11 mg g^{-1}.

HPLC validation tests

Regarding the broad range of β-carotene levels in *D. salina* biomass during cultivation study, two distinct standard curves with different concentrations were provided. The first one for samples with the analytic concentration of 0.2-50 µg mL^{-1} and the other one for the samples ranging from 100-500 µg mL^{-1}. The exploited method provided two independent linear responses through the β-carotene concentrations of 0.2-50 mL^{-1} with R^2, slope and intercept of 0.9981, 4123.4, and -289.47, respectively, and through the concentration range of 100-500 µg mL^{-1} with R^2, slope, and intercept value of 0.9965, 2773.20, and 14782, namely. The mean values for absolute recovery, and also the within-and between run variations for the presented method for β-carotene determination were presented in Table 2 and Table 3. Moreover, the limit of detection (LOD) for the developed method was 0.1 µg mL^{-1} and the limit of quantitation (LOQ) was 0.2 µg mL^{-1}. During this study, initially an isocratic solvent system composed of methanol: acetonitrile (90:10 v/v) with 1 mL min^{-1} flow rate was employed. In mentioned situation, the observed retention time for β-carotene found to be 85 min. The amounts of used methanol for this experiment was about 90 mL for each sample. Hence, we tried to find a more appropriate method which could ably detect the β-carotene levels in shorter times. The solvent ratio was then changed to 95:5 v/v of methanol: acetonitrile and the retention time was improved to 53 min. With isocratic solvent system of 1 mL min^{-1} methanol: acetonitrile (98:2 v/v) the retention time of 50 min was obtained. In the next step, we only used methanol with flow rate of 1 mL min^{-1} and the retention time of 39 min was observed. After that, the isocratic solvent of 1 mL min^{-1} methanol: chloroform (95:5 v/v) was used. In this way, retention time was 95': 12". So we

Table 1: The growth parameters, maximum biomass productivities and cumulative β-carotene concentration in *D. salina* during 28 days of study.

Culture medium	Growth parameters*		Cell number		Biomass productivity	Cumulative β-carotene concentration
	X_{max}	μ_{max}	N_{max} (x 10^6 cell mL^{-1})	D_{max}	P_{max}	C_{max}
Johnson (12%)	4.042	0.22	0.942	19	0.24	9.11

*X_{max}= maximum biomass concentration (g L^{-1}); μ_{max} = maximum specific growth rate (d^{-1}); N_{max}= maximum cell number; D_{max}= the day with maximum N_{max}; P_{max} = maximum biomass productivity (g L^{-1} d^{-1}); C_{max}= maximum concentration of β-carotene (mg g^{-1}).

Table 2: Within-run variations of the HPLC method for quantization of β-carotene (*n*=3).

Nominal added concentration (μg mL^{-1})	Sample number	Measured concentration (μg mL^{-1})	Mean ± SD	CV%	Accuracy[a]	Mean ± SD
500	1	498.45	498.26±0.27	0.054	99.69 %	99.65± 0.055
	2	498.38			99.68 %	
	3	497.94			99.59 %	
100	1	95.78	97.02±1.098	1.132	95.78 %	97.02± 1.098
	2	97.86			97.86 %	
	3	97.43			97.43 %	
10	1	9.41	10.16±0.684	6.732	94.1 %	101.6±6.842
	2	10.32			103.2 %	
	3	10.75			107.5 %	
0.2	1	0.21	0.20± 0.01	5.00	105.0 %	100± 0.00
	2	0.19			95.0 %	
	3	0.20			100.0 %	

[a]Accuracy= (Measured concentration /Nominal concentration) x 100

Table 3: Between-run variations of the HPLC method for quantization of β-carotene (*n*=3).

Nominal added concentration (μg mL^{-1})	Run number	Measured concentration (μg mL^{-1})	Mean ± SD	CV%	Accuracy[a]	Mean ± SD
500	1	497.89	497.72±0.459	0.370	99.58 %	99.54±0.091
	2	498.07			99.61 %	
	3	497.20			99.44 %	
100	1	97.18	97.69±0.451	4.56	97.18 %	97.69±0.452
	2	98.03			98.03 %	
	3	97.87			97.87 %	
10	1	9.59	9.79±0.795	5.32	95.9 %	97.93±7.947
	2	9.12			91.2 %	
	3	10.67			106.7 %	
0.2	1	0.20	0.207±0.006	2.90	100.0 %	103.33±2.887
	2	0.21			105.0 %	
	3	0.21			105.0 %	

[a]Accuracy= (Measured concentration /Nominal concentration) × 100

changed the solvent ratio to 90:10 (methanol: chloroform) with flow rate of 1 mL min^{-1}. The retention time changed to 53': 22". This time, the flow rate was changed up to where the pressure allowed (2.0 mL min^{-1}) and retention time was 21': 03". As the last step, the solvent ratio was changed to methanol: chloroform 85:15 (v/v) with flow rate of 2.0 mL min^{-1} and the retention time of 13': 54" was obtained. So, the HPLC method was set on isocratic solvent of 2.0 mL min^{-1} methanol: chloroform (85:15 v/v). To compare the robustness of our developed HPLC method with conventional colorimetric methods for β-carotene determination, we examined a range of β-carotene standard from 50-300 μg mL^{-1}. As it has been depicted in Fig. 2, there was a noticeable agreement between the quantified concentrations of β-carotene with two mentioned methods (R^2=0.9587). Each experiment was conducted in triplicate and the average values were reported. Normally, carotenoids analysis by HPLC, is performed using reverse phase chromatography columns of C_{18} or C_{30} and using isocratic or gradient washing conditions. A vast range of solvents or solvent mixtures with different proportions have been used as the mobile phase. The applied detectors routinely have been UV-visible detectors (UV-Vis) at a wavelength of 450 nm, photodiode array (PDI) or mass spectrometry (MS)[15-20]. In some cases, the column has been heated, to improve the separation capability of pigments and standardization of separation conditions[11]. Various systems such as liquid-liquid extraction, solid phase extraction or supercritical fluid extraction have been used for carotenoids extraction[16,19,21,22]. Union official analytical chemists (AOAC), have recommended a mixture of methanol and

tetrahydrofuran (THF), at a ratio of 50:50 (volume-volume) for extracting carotenoids[23]. While other researchers have examined other solvents such as ethyl acetate (100 %), or different mixtures of ethanol-hexane, acetone-ethanol-hexane, ethyl acetate-hexane, or acetone-hexane for extraction[17,18]. In the current study, for the first time we developed another solvent system with an improved working conditions which could detect the β-carotene content in *D. salina* biomass in a robust and reproducible manner with great sensitivity, accuracy and precision. Further improvement studies might be needed for this protocol to be used for another microalgal strains or determining the β-carotene in another biological samples.

CONCLUSION

Microalgae are effective synthesizers of several interesting carotenoids. Among these, *D. salina* is the best source of β-carotene. In the present study, a new method for analysis of β-carotene by HPLC was developed. A notable advantage of this method over the previously reported HPLC methods for β-carotene was its considerable shorter run times as well as availability and popularity, both of which are of a great important in practice, particularly when a high number of samples are to be analyzed. The results of validation tests were, collectively, indicative for a method with relatively wide linear range, acceptable precision and accuracy both within and between analytical runs, and practically reliable sensitivity. This method can provide a simple and easy way to assay β-carotene in many sources like food, drugs, etc.

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