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Composition of Carotenoids in *Scenedesmus protuberans*: Application of Chromatographic and Spectroscopic Methods

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Abstract This study aimed to identify and determine the carotenoids from green microalga, *Scenedesmus protuberans* using analytical techniques. Identification of carotenoids was realized by comparing their absorption and mass spectral data with those of reference standards available and reported values. Chromatographic data were then combined with the spectroscopic information. The separation of carotenoids was achieved by C_{30} column and high-performance liquid chromatography-diode array detection was used for their determination. In the present work, the carotenoid content of *S. protuberans* was found to be 1.45 mg/g of violaxanthin, 2.47 mg/g of all-*trans*-lutein, 0.15 mg/g of all-*trans*-α-carotene, 0.55 mg/g of all-*trans*-β-carotene, and 0.20 mg/g of 9 or 9′-cis-β-carotene. Due to lack of their standards, the amount of all-*trans*-loroxanthin and cis-isomers of other carotenoids could not be quantified. In order to validate the method, Certified Reference Material (BCR 485-Mixed vegetables) was used. In conclusion, this study can serve as a reference for the analysis of carotenoids in other microalgae.

Keywords *Scenedesmus protuberans* · Extraction · Carotenoids · Method validation · Chromatography and spectroscopy

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Introduction

There has been considerable attention for the isolation of bioactive compounds from marine resources, and microalgae play a significant role among them (Christaki et al. 2012). The microalga can be simply described as an organism that is small, generally unicellular, and found mainly in water. They are reported to be colorful due to their photosynthetic and some other pigments (Olaizola 2003). They contain many substances like fatty acids, tocopherols, vitamins, antioxidants, and pigments (Andersen 2005). These could be alternative neutraceuticals. One of the main important advantages of microalgae is their rapid growth over traditional plants (Spolaore et al. 2006). Probably, the most promising aspect of microalgal biotechnology is to develop microalgal products that have pharmaceutical properties (Borowitzka 1995).

Carotenoids are reported to be synthesized by plants and microorganisms but not the animals (Rao and Rao 2007). Carotenoids are liphophilic substances that are chemically classified in two groups: carotenes and xanthophylls (Britton et al. 2004). They are known to have the ability to quench singlet oxygen due to their conjugated double bond system. For this reason, carotenoids are powerful antioxidants, and they can lower the risk of developing certain human diseases like cardiovascular diseases, cancer, and age-related macular degeneration (Fraser and Bramley 2004; Kotake-Nara and Nagao 2011; Maiani et al. 2009).

Due to an extensive conjugated polyene chain, carotenoids show yellow, orange, and red colors. They have absorption maxima in the range of 400–500 nm. Therefore, carotenoids can exhibit unique molecular shape, chemical reactivity, and light-absorbing properties. The positions of the absorption maxima and the shape or fine structure of the ultraviolet-visible (UV–vis) spectrum are characteristic (Landrum 2010). The differences in the spectral characteristics of individual carotenoids are often small but are of great importance

in their identification. Based on these features, high-performance liquid chromatography (HPLC) using absorbance detectors are widely used. They can determine carotenoid profiles both qualitatively and quantitatively. However, many carotenoids are structurally similar. For this reason, it has been stressed that UV–vis spectrum alone is not sufficient to characterize a carotenoid but provides information about the chromophore (Britton 1995a). Therefore, identification of carotenoids using other detection methods is required. Among those, mass detectors have shown great advantages for the analysis of these substances, including the elucidation of their structure based on the molecular mass and their fragmentation pattern. The use of mass spectrometry (MS) data has been an important chemical approach to structure elucidation (Rivera and Canela-Garayoa 2012; Sivathanu and Palaniswamy 2012; van Breemen et al. 2012). Finally, the retention times of the carotenoid standards should be compared with those of carotenoids that are tentatively identified.

It can be said that carotenoid research is a multidisciplinary area that many researchers work like analytical chemists, organic chemists, food chemists, biologists, physiologists, medical doctors, and environmentalists. It shows that great demands have been placed on their identification and determination (Ladislav et al. 2005). Carotenoid analysis is a complex task, and therefore, new, fast, and easily applicable procedures must be developed. In addition, it has been emphasized that their identification and quantification require highly sensitive and selective analytical methods (Carmona et al. 2003; Inbaraj et al. 2008; Maoka et al. 2002; Rehbein et al. 2007; Tian et al. 2003; Van Breemen 1997).

Moreover, an important problem in carotenoid analysis lies in the unavailability of standard carotenoid compounds. In these past few years, more carotenoid standards have become available; however, their prices are extremely high, as they are unstable. For this reason, it has been thought that accumulated carotenoids in microalgae can also be an alternative source for standards apart from their roles in diseases.

The goal of this work was to identify and determine the concentration of carotenoids produced by selected microalga (*Scenedesmus protuberans*) from EGE-MACC (Ege-Microalgae Culture Collection). As photosynthetic organisms, all algae contain chlorophylls and carotenoids. Algal carotenoids exhibit wide structural diversity. Carotenoids are found in algae, and recently, taxonomic studies of algae have been developed. Many different kinds of carotenoids were found from the algal species studied. Some carotenoids are found only in some algal divisions or classes. The most important sources of microalgae are *Chlorella*, *Chlamydomonas*, *Dunaliella, Muriellopsis*, and *Haematococcus spp*, all of which belong to the Chlorophyceae family. Different types of carotenoids are present in these biomasses, and they are synthesized under certain cultivation conditions. *Scenedesmus* is also a genus of algae, specifically of the Chlorophyceae (Guedes and Amaro 2011; Takaichi 2011). *S. protuberans*, being a type of Chlorophyceae, is a novel one whose carotenoid content has not been studied yet in literature. That is why this microalga has been selected in the present study. The identification of carotenoids in *S. protuberans* was done using chromatographic/spectroscopic techniques. Their quantification was achieved by high-performance liquid chromatography-diode array detection (HPLC-DAD) using commercially available carotenoid standards.

Materials and Methods

Chemicals

All-*trans*-lutein, all-*trans*-violaxanthin, all-*trans*-neoxanthin, all-*trans*-β-carotene, all-*trans*-α-carotene, 9 or 9′-cis-β-carotene, and *trans*-β-apo-8′-carotenal were provided by CaroteNature (Switzerland) while triethylamine, and pyrogallol were purchased from Sigma-Aldrich. All the solvents used in this study were HPLC-grade obtained from Merck.

Cultivation and Preparation of *S. protuberans*

S. protuberans was obtained from the culture collection of Ege University Microalgae Culture Collection (Ege-MACC). It was obtained from Lake Eğridir in TURKEY and isolated by streak plate method. After that, it was grown in Bold's Basal Medium (Fabregas et al. 2000) under constant light (2,000 l×) and temperature (25±1 °C) since *S. protuberans* grows very quickly in this selected medium.

The microalgal cells in 2.0-L bottles were illuminated with the continuous light of 2,000 lux at 25.0 °C. Ventilation rates were 1.25 vvm. Cultures were prepared from 10 % inoculation from a stock of 4-day 10 % inoculation. Illumination was provided by using cool white fluorescent lamps (Philips, 18 W/54). All glassware and the medium were sterilized in an autoclave at 121.0 °C for 20.0 min prior to use.

When the microalgal cells were ready for harvesting, they were filtered by 0.45-μm cellulose acetate filter paper and washed with deionized water to remove the growing medium. Harvested cells were then lyophilized and stored at −20 °C for one night. After this process was completed, they were grounded using a mortar to reduce their size for extraction process. The morphology of this green microalga used in this study can be examined from Fig. 1.

Identification of *S. protuberans*

Microscopic and scanning electron microscope (SEM) analyses allowed preliminary identification of *S. protuberans*. Optical image was obtained under a trinocular light microscope (Olympus CH40, Japan). SEM characterizations were carried

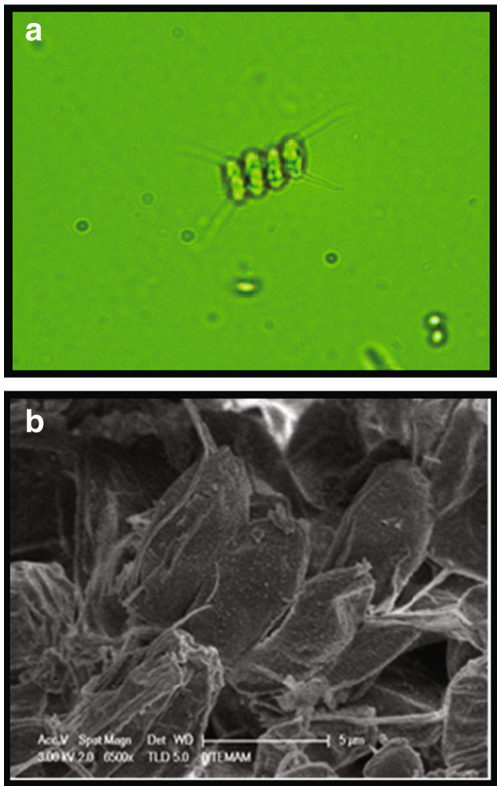

Fig. 1 **a** Optical image (×40) and **b** SEM image (×6,500) of *S. protuberans*

out using Philips XL-30S FEG (Holland). Moreover, the elemental composition of the microalga was determined with LECO-932 (USA) elemental analyzer.

Extraction of Carotenoids from *S. protuberans*

For the extraction of carotenoids from *S. protuberans*, a simple ultrasound-assisted extraction method was developed by using 37 kHz ultrasonic bath (Elmasonic S80H) with a power of 200 W. Then, the solvents were removed with the use of rotary evaporator (Heidolph Hei-VAP Advantage, Germany). Each microalgal powder (0.25 g) was added $CaCO_3$ (0.25 g) and extracted with 10.0 mL of THF/DCM (1:1) containing 0.010 % (*w/v*) pyrogallol. The mixture was placed in the ultrasonic bath for 15.0 min. After the ultrasonic extraction, the solution was centrifuged at 6,000 rpm for 10 min, and the supernatant was collected. The residue was repeatedly extracted with 10.0 mL extraction solvent until it was colorless. The supernatants were combined and evaporated by the rotary evaporator at 35.0 °C under vacuum. For the determination of carotenoids in green microalga, the extraction procedure was followed by the 2-h saponification in dark. As this time increases, it has been observed that most of the carotenoids degrade. All extraction and work-up procedures were conducted under yellow light to prevent photo-isomerization and degradation of carotenoids.

Saponification of the Extracts

Saponification is mainly reported to be effective for removing colorless contaminating lipids and for destroying the chlorophyll. It also hydrolyzes carotenoid esters. Carotenols esterified with one or more fatty acids are encountered more frequently than are carotenoid carboxylic acid esters. Saponification of carotenol esters is advantageous before submission to HPLC (Britton 1995b). This has the advantage due to concentrating the carotenoids by removal of neutral lipids. In addition, it is also easier to characterize and determine quantitatively the free carotenols than the fatty acid esters that occur (Bechtold and Mussak 2009).

The colored extracts obtained from green microalgae were then pooled in a separatory funnel containing diethyl ether. After gentle shaking, the phases were left to settle and the aqueous phase was removed. This operation should be repeated several times until the upper layer is colorless. The residue obtained was dissolved in 10.0 mL of diethyl ether for partition followed by the addition of 10.0 mL of 10.0 % methanolic KOH solution containing 0.01 % pyrogallol, and saponification was carried out for 2 h under nitrogen atmosphere. Carotenoids were extracted from the methanolic KOH phase with 10.0 mL diethyl ether and 10.0 mL 10.0 % Na_2SO_4 solution. Addition of the salt stops the reaction and helps the removal of excess alkali. The combined diethyl ether phase was filtered through 0.20 μm nylon membrane filter (Sartorius) and concentrated by rotary evaporator at 25.0 °C under 400 mbar. The remaining water was removed with the aid of nitrogen gas. The residue was then dissolved in 10.0 mL of dichloromethane. The samples were diluted ten times with mobile phase for all-*trans*-lutein, all-*trans*-violaxanthin, and 9 or 9′-cis-β-carotene. For the other carotenoids, direct analysis was performed without making any dilution.

As a sample volume, 20.0 μL was injected for HPLC analysis. The summary of the whole process was designated in Fig. 2.

Analysis of Carotenoids in *S. protuberans* by HPLC-DAD and LC-APCI-MS

Agilent 1,200 Series HPLC-DAD (USA) system was used for the determination of carotenoids. Prior to use, all HPLC-grade mobile phase solutions were degassed using an ultrasonic bath (Elmasonic S80H,). The reversed-phase column was a YMC Carotenoid C_{30} column, 250×4.6 mm, 5 μm (Waters, Milford, MA, USA). The column temperature was set at 25.0 °C. The separation was achieved by gradient elution at a flow rate of 1.0 mL/min. The injection volume was 20.0 μL, and detection was made by diode array detector with quantitation at the wavelength of maximum absorption for each analyte in order to improve the response factor. On the other hand, the

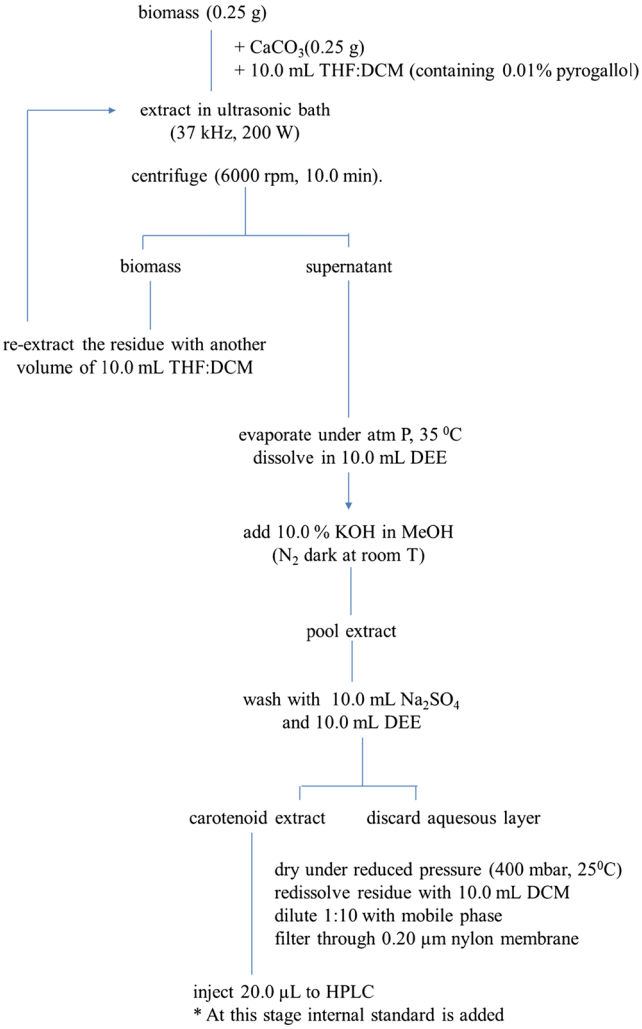

Fig. 2 Summary of the extraction method and saponification procedure for the analysis of carotenoids in the green microalga *S. protuberans*

chromatogram was obtained at 450 nm. The full spectrum from 300 to 600 nm was also recorded.

In order to obtain mass spectra, AB Sciex 4,000 Q-trap (USA) instrument was used. Positive ion mode (atmospheric pressure chemical ionization (APCI)) was used for the investigation of carotenoids. The total ion current scanning range was 500–700 *m/z*. The APCI vaporizer was 350 °C. The optimum corona current was 3 μA during positive ion APCI.

Calibration Curves

Due to the light-sensitive nature of carotenoids, stock standard solutions were prepared under yellow light (Philips lamp TLD 36 W/16 yellow, light transmission at 500–750 nm) at room temperature within the shortest possible time. For the preparation of stock carotenoid standards, 5.0 mg of all-trans carotenoid and, for internal standard, 5.0 mg of all-trans-β-apo-8′-carotenal were accurately weighed and dissolved in chloroform in separate volumetric flasks (50.0 mL). Calibration standards (0.020–10.0 mgL^{-1}) were prepared from the stock solution and contained a fixed concentration of internal standard (3.0 mgL^{-1}). Three replicate measurements were made for each standard solution. All standard and sample solutions were kept in amber-colored volumetric flasks wrapped with aluminum foil after nitrogen flush in order to avoid decomposition of carotenoids. Calibration curves were obtained by plotting the ratio of absorbances of each analyte and the internal standard as a function of analyte concentration. Absorbance values were measured in terms of peak area. It is a great problem that there is no available certified reference standard for each carotenoid. For this reason, in order to check the accuracy of calibration curve, certain concentration of a standard solution (within the limit of linearity) was injected after each sample introduction. Tolerance limit for concentration was under 5 %.

Determination of LOD and LOQ

The detection and quantification limits (limit of detection (LOD) based on 3 s and limit of quantification (LOQ) based on 10 s, respectively) were determined using the standard deviation of the lowest concentration of the calibration curve. Standard deviation of the response was based on the manually integrated peaks after ten successive injections.

Identification and Determination of Carotenoids in *S. protuberans*

For the separation of carotenoids in green microalgae, a gradient system was developed due to its resolving power and improved sensitivity as the isocratic elution took much more time. The mobile phase consisted of a gradient of methanol (A), methyl *tert*-butyl ether (B) and water (C). The gradient profile of the mobile phase (A/B/C) was set at 70:25:5 and changed linearly to 60:35:5 in 5 min, and then it was regulated to 45:55:0 from 5 to 10 min. Finally, the mobile phase was adjusted to 25:75:0 for the last 5 min (10 to 15 min). Internal standard calibration method was applied, and for this purpose, *trans*-beta-apo-8′carotenal was used. The developed LC-DAD method described is fast, specific, and precise for the determination of carotenoids in the green microalga. HPLC chromatogram for *S. protuberans* was shown in Fig. 3, and the absorption profiles for each carotenoid were depicted in Fig. 4.

In order to get information about the absorption profiles of carotenoids, spectroscopic data were used which were recorded between 300 and 600 nm using diode array detection (DAD). It presents the absorption spectra for the carotenoid standards and the carotenoids extracted from *S. protuberans* (supplementary information). In addition, Table 1 shows the DAD spectra required for tentative identification. In order to be sure of good chromatographic separation validation

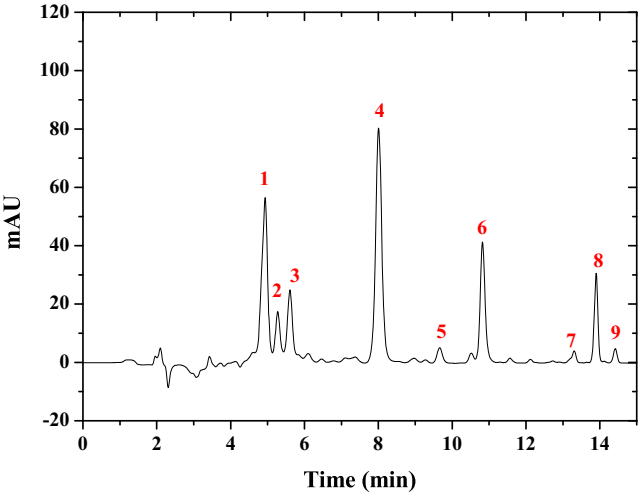

Fig. 3 HPLC chromatogram for *S. protuberans* obtained at 450 nm

parameters like LOD, LOQ, percent peak purity, capacity factor and selectivity factor should be checked for the proposed method. These values were presented in Table 2 for *S. protuberans*.

For the identification of carotenoids, spectroscopic data are not enough, and mass spectrum is generally required. For this reason, a mass spectrum of green microalgal extract was obtained. The liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) data, which have been used as complementary information of carotenoids, were summarized in Table 3 for *S. protuberans*.

Results and Discussion

Composition and Morphology of *S. protuberans*

Some of the important factors that may be responsible for the carotenoid contents of a microalga could be its morphology and composition. Chemical nature of substances may differ for each microalgae. For this purpose, the elemental analyses were realized, and according to the results obtained, *S. protuberans* involves 43.12 % C, 5.21 % N, 6.51 % H, and 0.73 % S by mass.

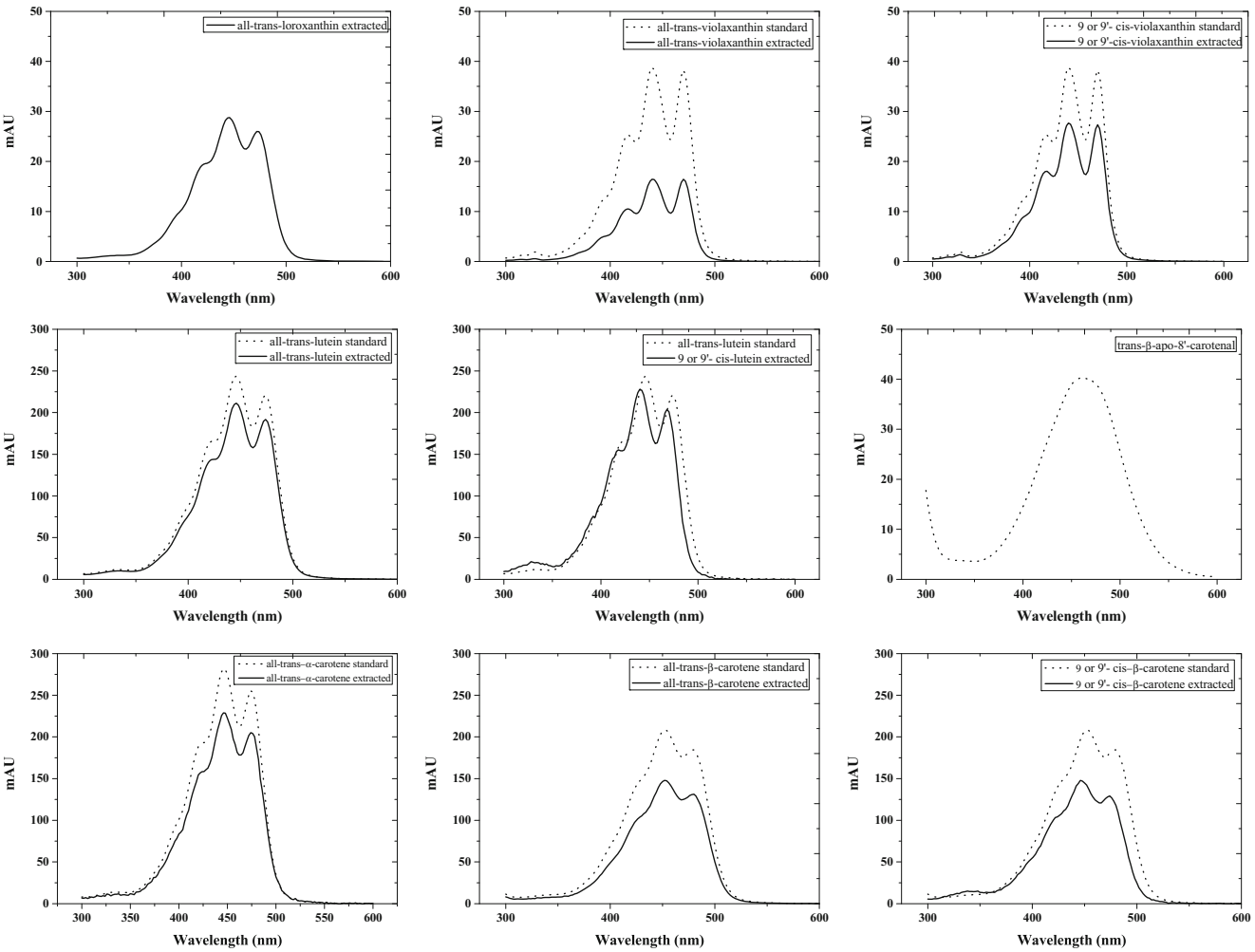

Fig. 4 DAD spectra for available carotenoid standards and carotenoids extracted from *S. protuberans*

Table 1 UV-visible absorption data for tentative identification of carotenoids in *S. protuberans*

Peak no.	Compound	λ (reported)[a]			λ (observed)			λ (standards)		
1	All-*trans*-loroxanthin		446	474		446	474			
2	All-*trans*-violaxanthin	419	440	470	417	440	470	418	440	470
3	9- or 9′-cis-violaxanthin	414	436	464	418	440	470			
4	All-*trans*-lutein	422	445	474	423	446	474	424	446	474
5	9- or 9′-cis-lutein	420	442	470	418	440	470			
6	Internal standard[b]		458			456			456	
7	All-*trans*-α-carotene	423	444	473	423	446	474	423	446	474
8	All-*trans*-β-carotene	428	450	478		452	478		452	478
9	9- or 9′-cis-β-carotene	422	448	474	423	446	474	423	446	474

[a] Britton et al. 2004 (Reported values are taken according to the results obtained in ethanol since initial mobile phase composition is mainly methanol)
[a] *trans*-beta-apo-8′-carotenal

The optical and scanning electron microscope images were designated in Fig. 1. The cell size and shape, in turn, affect the efficient extraction of biomolecules from the species. In order to obtain the carotenoids during the extraction process, the cell wall of the microalga should be destroyed and dissolved in the appropriate solvent. For its destructive power, ultrasound-assisted extraction was used for the extraction of carotenoids from the microalgae. Extraction procedure was followed by a saponification step to simplify the separation of carotenoids. The procedure was briefly presented in Fig. 2.

HPLC-DAD Analysis of Carotenoids in *S. protuberans*

According to the chromatogram obtained from *S. protuberans*, all the carotenoids were well separated from each other using a simple gradient elution method. Xanthophylls were eluted in the first 10 min, and then carotenes were observed.

Due to lack of some carotenoid standards, all-*trans*-loroxanthin, 9- or 9′-cis-violaxanthin, and 9- or 9′-cis-lutein could not be quantified. The presence of loroxanthin in *S. protuberans* has been considered to be relatively high based on its peak area. It has been reported that loroxanthin is produced only by specific green algae (Britton et al. 2004). Therefore, *S. protuberans* might be an alternative source for loroxanthin, and particularly, it could serve as a carotenoid standard.

According to the HPLC chromatogram obtained (Fig. 3), some of the peaks were tentatively identified based on the literature data (Britton 1995b). It has been known that three features of the UV–vis spectrum provide valuable information for structural characterization and quantitative analysis: (1) wavelength of maximum absorption, (2) spectral shape, and (3) intensity of absorption (Britton et al. 2004). Even small differences in their absorption profiles give us an idea about the identity of each carotenoid. For this reason, diode array detector throughout the analysis obtained the absorption spectra of each carotenoid. DAD spectra for available carotenoid standards and carotenoids extracted from *S. protuberans* were presented in Fig. 4. This data then combined with the values obtained by mass spectrometric data. Table 1 shows the characteristic absorption wavelengths of carotenoids identified for the microalga, which gives important clues about the identification. According to the data, values of carotenoid standards well match with the observed values. The employed chromatographic conditions seemed to separate many of the carotenoid compounds within 15.0 min. Finally, the validation parameters with the proposed LC method were calculated, and the results were shown in Table 2.

Mass Spectrometry of Carotenoids in *S. protuberans*

APCI has become the most widely used ionization technique for carotenoids, and most mass spectra of carotenoids have been acquired using positive ion mode. (Hao et al. 2005; Kurz et al. 2008; Rezanka et al. 2009; Rivera et al. 2011; Weller and Breithaupt 2003). The fragment pattern observed in the carotenoid mass spectra depends on the ionization technique and composition of the mobile phase used; characteristic carotenoid fragments have been observed with various ionization techniques. In addition, the removal of a hydroxyl group or a molecule of water $[M-17]^+$ or $[M+H-18]^+$ is characteristic of the presence of a hydroxyl group in the compound. These ions have been obtained through EI, ESI, and APCI (van Breemen 1995). Since the fragmentation in chemical ionization is less, the most abundant ions generated are usually the quasimolecular ions $[M+H]^+$ (Britton 1995b). In the present study, $[M+H]^+$, $[M+H-18]^+$ and $[M+H-36]^+$ ions were easily seen in the mass spectra of green microalga.

In order to help the identification of carotenoids, spectral data obtained from DAD detector were combined with the mass spectra. Hence, possible carotenoids were defined. The LC-APCI-MS data of carotenoids for *S. protuberans* were compared with the data given in literature as shown in Table 3. Analytical standards were provided for the ones whose mass

Table 2 Summarized validation parameters with proposed LC method (gradient elution with 70:25:5 MeOH/MTBE/H_2O at the wavelength of interest, flow rate, 1.0 mL/min) for *S. protuberans*

Carotenoids	LOD (μg/mL)	LOQ (μg/mL)	r^2	Peak purity %	Capacity factor (k')	Selectivity factor (α)	Content (mg/g)[a]
All-*trans*-loroxanthin		No available standard		98.9	1.43	1.00	ND
All-*trans*-violaxanthin	0.014	0.045	0.9998	98.7	1.59	1.11	1.45
9- or 9′-cis-violaxanthin		No available standard		97.6	2.15	1.21	ND
All-*trans*-lutein	0.0032	0.011	0.9998	99.5	3.15	1.47	2.47
9- or 9′-cis-lutein		No available standard		97.4	3.97	1.26	ND
All-*trans*-α-carotene	0.016	0.055	0.9997	98.6	5.68	1.27	0.15
All-*trans*-β-carotene	0.012	0.041	0.9995	99.2	6.05	1.08	0.55
9- or 9′-cis-β-carotene	0.015	0.051	0.9995	97.4	6.25	1.03	0.20

ND not detected

[a] RSD values<5 %

values, spectral data, and pattern matched. Finally, the spectral values and retention times were compared with the carotenoids in each chromatogram. However, some of the standards like loroxanthin and some cis-structures of many carotenoids are not available. Therefore, tentative identification has been made.

Method Validation for Extraction and Saponification of Carotenoids in *S. protuberans*

For the validation of extraction/saponification of carotenoids, the same procedure was applied by using Certified Reference Material (CRM) (BCR 485-mixed vegetables). The criterion to consider a good agreement was the accuracy of the process for extraction and saponification. Literature studies show that there are a few number of standard or certified reference materials for the method validation of carotenoids. Most of them are limited in terms of content and matrix (Dias et al. 2010; Kimura et al. 2007). In addition, some of them are no longer produced. Plant extract is thought to be the most relevant material with the microalgal extract, and therefore, a certified reference material, BCR 485 (CRM-mixed vegetables), was used for the validation of our method. According to the analyses performed, the results obtained are consistent with CRM values of the carotenoids present in the sample. The results can be examined in Table 4.

Conclusions

In recent years, there has been particular emphasis on obtaining accurate data on carotenoids that are reliable since they are important for various health and nutrition activities. On the other hand, significant attention has recently been drawn to the use of microalgae since they can produce carotenes and xanthophylls in rich amounts. For this reason, carotenoid production has become one of the most successful branches in biotechnology of microalgae.

Another problem associated with analysis of carotenoids is the difficulty in obtaining standard compounds. Therefore our

Table 3 LC-APCI-MS (positive mode) data of carotenoids in *S. protuberans*

Peak no.	Carotenoid	*m/z* (reported)[a]	*m/z* (observed)	(*m/z* standards)
1	All-*trans*-loroxanthin	584 $[M]^+$, 566 $[M\text{-}H_2O]^+$, 548 $[M\text{-}2H_2O]^+$	585.5 $[M+H]^+$, 567.4 $[M+H\text{-}H_2O]^+$, 549.5 $[M+H\text{-}2H_2O]^+$	
2	All-*trans*-violaxanthin	600 $[M]^+$, 582 $[M\text{-}H_2O]^+$, 508 $[M\text{-}92]^+$	601.5 $[M+H]^+$, 583.5 $[M+H\text{-}H_2O]^+$, 509.4 $[M+H\text{-}92]^+$	601.5, 583.5, 509.4
3	9- or 9′-cis-violaxanthin	600 $[M]^+$	601.5 $[M+H]^+$	
4	All-*trans*-lutein	569 $[M]^+$, 550 $[M-H_2O]^+$	569.5 $[M+H]^+$, 551.5 $[M+H-H_2O]^+$	569.5, 551.5
5	9- or 9′-cis-lutein	568 $[M]^+$	569.5 $[M+H]^+$	
7	All-*trans*-α-carotene	536 $[M]^+$, 518 $[M-H_2O]^+$	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	537.4, 519.4
8	All-*trans*-β-carotene	536 $[M]^+$, 518 $[M-H_2O]^+$	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	537.4, 519.4
9	9- or 9′-cis-β-carotene	536 $[M]^+$, 518 $[M-H_2O]^+$	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	537.4, 519.4

[a] Britton et al. 2004

Table 4 Method validation results for some carotenoids using BCR 485 (CRM-mixed vegetables)

Carotenoids	Reported carotenoid values in mixed vegetables BCR 485 (mg/kg)	Carotenoid values obtained using proposed method (mg/kg)[a]
All-*trans*-lutein	12.5±0.8	12.6±0.2
All-*trans*-α-carotene	10.5±0.6	10.5±0.5
All-*trans*-β-carotene	23.7±1.5	23.7±0.9

[a] Method validation was performed using BCR 485 according to the proposed extraction and saponification procedure (n=3)

study also emphasized that the identification and determination of carotenoids from new sources are required. Based on this study, it could be proposed that loroxanthin from *S. protuberans* can be a good alternative as an analytical standard.

In this work, a new extraction method was applied, and an HPLC-DAD method was developed to separate the carotenoids in *S. protuberans* by employing a C_{30} column with a simple gradient elution. The analytical results showed that the method was fast, relatively simple, and applicable. Spectroscopic and mass spectrometric data were linked to investigate the carotenoids. In conclusion, the future of microalgal biotechnology seems very promising, and the microalgae has great potential for carotenoid products.

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