Food Research International 100 (2017) 260–266

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity

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ARTICLE INFO

Keywords:
Algae
Blue-green algae
Carotenoids
Bioactive compounds
Antioxidant capacity
HPLC-PDA-MS/MS

ABSTRACT

Carotenoids from cyanobacteria *Aphanothece microscopica Nageli* and green microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* were identified. The total carotenoid content, based on dry weight of biomass, of *A. microscopica Nägeli*, *C. vulgaris* and *S. obliquus* were 1398.88 μg/g, 1977.02 μg/g and 2650.70 μg/g, respectively. A total of 23 different carotenoids were separated in all the extracts, the major ones being all-*trans*-β-carotene (29.3%) and all-*trans*-lutein (28.1%) in *Scenedesmus*; all-*trans*-echinenone (22.8%) and all-*trans*-β-carotene (17.7%) in *Chlorella*; all-*trans*-echinenone (28.3%) and all-*trans*-β-carotene (26.2%) in *Aphanothece*. The carotenoid extracts were shown to be a potent scavenger of peroxyl radical, with values of 31.1 (*Chlorella*), 14.0 (*Scenedesmus*) and 7.3 (*Aphanothece*) times more potent than α-tocopherol.

1. Introduction

The carotenoids have high commercial values, especially for their high demand as bioactive compounds. Thus, antioxidant pigments are currently the most marketed products from microalgae, renovating the interest in increasing the research and development of these compounds in microalgae biomass (Poojary et al., 2016). The global market of carotenoids was US$ 1.2 billion in 2010 and is expected to increase to $ 1.4 billion by 2018. The highest market shares are β-carotene and astaxanthin, with an average price close to USD 2500/kg (Suganya, Varman, Masjuki, & Renganathan, 2016).

The total number of reported carotenoids that has been fully characterized is about 1167, this number includes the enormous variety of carotenoids in microalgae (Britton, Liaaen-Jensen, & Pfander, 2004; Yabuzaki, 2017). Microalgae produce carotenoids with structural characteristics very different from those commonly found in fruits and vegetables, in particular, acetylenic carotenoids, ketocarotenoids and glycosylate carotenoids (Poojary et al., 2016; Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka, 2015; Crupi et al., 2013; Haugan & Liaaen-Jensen, 1994; Hertzberg, Liaaen-Jensen, & Siegelman, 1971).

Diatoxanthin, crocoxanthin, echinenone, canthaxanthin and myxoxanthophyll are secondary bioactive carotenoids of microalgal origin, which may have diverse industrial applications (Grama et al., 2014). Secondary carotenoids do not participate in photosynthesis and are characterized by extra-thylakoid localization. This metabolic characteristic contributed for the great structure variety of carotenoids from microalgae (Solovchenko et al., 2013). Echinenone and canthaxanthin are considered to be stronger antioxidants than β-carotene; astaxanthin shows high colorant potential (Papp et al., 2013).

Currently, microalgal carotenoids are mainly produced from *Dunaliella salina* and *Haematococcus pluvialis*. Nevertheless, there are inherent shortcomings for these strains, such as slow growth, insufficient yield, and strict nutritional requirements (Guo et al., 2016). By contrast, the microalgae *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* grow rapidly, have substantial carotenoid content, and perform robustly in bioreactors (Francisco, Neves, Jacob-Lopes, & Franco, 2010; Maroneze et al., 2016). Therefore, these microalgae can be considered as potential alternative producers of carotenoids.

Thus, the objective of our study is to identify the carotenoid composition, and determine the peroxyl radical scavenger capacity of the carotenoid extracts of three microalgae species. The results may draw attention to the importance of these species as alternative sources of bioactive compounds, with an elevated potential for industrial exploitation.

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http://dx.doi.org/10.1016/j.foodres.2017.06.069
Received 16 March 2017; Received in revised form 27 June 2017; Accepted 28 June 2017
Available online 29 June 2017
0963-9969/

2. Materials and methods

2.1. Microorganisms and culture media

Axenic cultures of *Aphanothece microscopica Nägeli* (RSMan92), *Chlorella vulgaris* (CPCC90) and *Scenedesmus obliquus* (CPCC05) were used in the experiments. Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The incubation conditions were 30 °C, photon flux density of 15 μmol/m^2/s and a photoperiod of 12 h were used.

2.2. Microalgal biomass production

The biomass productions were made in phototrophic conditions. The cultivations were performed in a bubble column photobioreactor (Maroneze et al., 2016) operating under a batch regime, fed on 2.0 L of BG11 medium. The experimental conditions were as follows: initial cell concentration of 100 mg/L, isothermal reactor operating at a temperature of 25 °C, photon flux density of 150 μmol/m^2/s and continuous aeration of 1VVM (volume of air per volume of culture per minute) with the injection of air enriched with 15% carbon dioxide. The biomasses were separated from the culture medium by centrifugation. It was subsequently freeze dried for 24 h at − 50 °C above − 175 μm Hg, and then stored under refrigeration until the time of analysis. The cultivations were performed twice, and in duplicate.

2.3. Carotenoid extraction

The carotenoids were exhaustively extracted from the freeze-dried sample (0.2 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500 × *g* (Mandelli, Miranda, Rodrigues, & Mercadante, 2012). The extraction procedure was repeated until the supernatant becomes colorless, which was reached approximately after 9 extractions with ethyl acetate and 5 with methanol. The homogenized sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator ($T < 30$ °C), suspended in a mixture of petroleum ether/diethyl ether [1:1 (*v*/v)], and saponified overnight (16 h) with 10% (*w*/*v*) methanolic KOH at room temperature. The alkali was removed by washing with distilled water, and each extract was once again concentrated in a rotary evaporator, flushed with N_2 and kept at − 37 °C in the dark until chromatographic analysis. All extractions were performed in triplicate.

2.4. HPLC-PDA-MS/MS analysis

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), online degasser, and injection valve with a 20 μL loop (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model AmaZon speed ETD, Bruker Daltonics, Bremem, Germany). The carotenoid separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007). Prior to HPLC–PDA–MS/MS analysis, the carotenoid extract was solubilized in methanol (MeOH): methyl-terbutyl-ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phase consisted in a mixture of MeOH and MTBE. A linear gradient was applied from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL/min. The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV–visible spectrum (λ max, spectral fine structure, peak *cis* intensity), and mass spectra characteristics (protonated molecule ($[M + H]^+$) and MS/MS fragments), compared with data available in the literature (Britton, 1995; De Rosso & Mercadante, 2007; Rodrigues et al., 2015; Van Breemen, Dong, & Pajkovic, 2012; Zepka & Mercadante, 2009).

The carotenoids were quantified by HPLC-PDA, using external calibration curves for all-*trans*-violaxanthin, all-*trans*-zeaxanthin, all-*trans*-lutein, all-*trans*-β-carotene and all-*trans*-α-carotene of five concentration levels. All-*trans*-luteoxanthin and 9-*cis*-neoxanthin were quantified using the curve of all-*trans*-violaxanthin; the isomers zeaxanthin, 13-*cis*-antheraxanthin, all-*trans*-antheraxanthin and all-*trans*-diatoxanthin were quantified using the curve of all-*trans*-zeaxanthin; the isomers lutein and all-*trans*-crocoxanthin were quantified using the curve of all-*trans*-lutein; and the isomers and epoxides of the β-carotene, all-*trans*-canthaxanthin, all-*trans*-myxoxanthophyll, all-*trans*-β-cryptoxanthin, all-*trans*-echinenone, 9-*cis*-echinenone were quantified using the curve of all-trans-β-carotene. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C30 column.

2.5. Peroxyl radical scavenging assay for lipophilic extracts

The antioxidant capacity assay of the lipophilic extracts was carried out according to Rodrigues, Mariutti, Chisté, and Mercadante (2012). The dry carotenoid extracts were suspended in dichloromethane and pooled together to compose the stock solution. Aliquots of the stock solution were taken to prepare the working solutions in five different concentrations (42, 57, 109, 204 and 321 μL). After evaporation under N_2 flow, they were dissolved in DMSO/MTBE (10:1, *v*/v) and homogenized. The assays were carried out in a microplate reader (Synergy Mx Biotek, Winooski-VT, USA). The ROO· scavenging capacity was measured by monitoring the effect of the carotenoid extract or α-tocopherol standard on the fluorescence decay resulting from the ROO· induced oxidation of the C_{11}-BODIPY$^{581/591}$ probe. ROO· was generated by thermal decomposition of AIBN at 41 ± 0.5 °C. The ROO· scavenging capacity was calculated as the ratio of the slop of the curve representing the sample concentration against the net area under the curve, and the slope of the curve representing α-tocopherol concentration against the net area under the curve.

2.6. Statistical analysis

Descriptive statistics, analysis of variance (one-way ANOVA) and Tukey's test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software Statistica 7.0 (StatSoft, Tulsa-OK, USA).

3. Results and discussion

A total of 23 different carotenoids were separated in the extracts of *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopica Nägeli* (Fig. 1). The separated carotenoids were identified or tentatively identified based on the combined information obtained from chromatographic elution, co-chromatography with standards, UV/visible, and mass spectra characteristics (Table 1). Since a detailed description of carotenoid identification using the above information was already reported by Rodrigues et al. (2015), Rodrigues et al. (2014) and De Rosso and Mercadante (2007) only considerations regarding the carotenoids not identified in these previous reports were discussed below.

Diatoxanthin (peak 7) is an acetylated carotenoid mainly found in microalgae with low spectral fine structure (III/II = 9%). The molecular mass of diatoxanthin was confirmed by the protonated molecule ($[M + H]^+$) at *m/z* 567 and by consecutive losses of two hydroxyl groups, at *m/z* 549 $[M + H - 18]^+$ and 531 $[M + H - 18 - 18]^+$, verified in the MS/MS spectrum. In addition, a fragment at low abundance was observed at *m/z* 475, resulting from the loss of toluene [M