# CAROTENOIDS AS BIOMARKERS IN *BOTRYOCOCCUS BRAUNII* ALGAE

# CAROTENOIDE - BIOMARKERI ÎN ALGA *BOTRYOCOCCUS BRAUNII*

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***Abstract:*** *This paper resumes some experimental results regarding the possibility of using carotenoids as biomarkers in the Botryococcus braunii green algae. High performance liquid chromatography was used as analytical technique, revealing twelve carotenoids as biomarkers: two carotenes (α - carotene, β - carotene) and ten xanthophylls (violaxanthin, 5,6-epoxy-lutein, anteraxanthin, lactucaxanthin, lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, echinenone and 5,6-epoxy-β - carotene).*

***Rezumat:*** *Lucrarea de faţă expune rezultatele unor studii care vizează posibilitatea utilizării carotenoidelor ca biomarkeri în alga verde Botryococcus braunii. Ca tehnică analitică a fost utilizată cromatografia de lichide de înaltă performanţă, aceasta relevând doisprezece carotenoide ca biomarkeri: două hidrocarburi (α-carotenul şi β-carotenul) şi zece xantofile (violaxantina, 5,6-epoxi-luteina, anteraxantina, lactucaxantina, luteina, zeaxantina, α-criptoxantina, β-criptoxantina, echinenona şi 5,6-epoxi-β-carotenul).*

***Key words*****:** *carotenoids, chromatography, HPLC, algae, biomarkers, Botryococcus braunii*
***Cuvinte cheie***: *carotenoide, cromatografie, HPLC, biomarkeri, Botryococcus braunii*

## INTRODUCTION

The green *B. braunii* micro algae can be considered as a potential source of fuel due to its ability to produce relatively high amounts of hydrocarbons (up to 75% from its dry mass can be represented by hydrocarbons, depending of culture conditions and race). The chemical nature of hydrocarbons depends highly of the algal race. Three races of *B. braunii* were identified: the A race produces n-alcadienes and trienes with uneven number of carbon atoms, race B produces triterpenes, apparently of isoprenoidic origin, while race L produces licopadiene – a tetraterpene.

The green algae *Botryococcus braunii* was relatively little studied up to the present from the point of view of carotenoid composition. In 1994, seven carotenoids were identified in the L. variety of *B. braunii:* echinenone, (3S)-3-hydroxiechinenone, cantaxanthin, (3S, 3'S)-astaxanthin, (3S, 3'R)-adonixanthin, lutein and zeaxantin [GRUNG – 1994. This paper is in fact a continuation of an older research started in 1989 in which two other strains of *B. braunii* were studied comparatively [GRUNG – 1989]. Okada and his co-workers accomplished a more detailed research, studying three strains of *B. braunii* [OKADA – 1996, 1997, 1998], discovering five new carotenoids containing etheric bonds: botryoxanthin A, α-botryoxanthin and botryoxanthin B (identified in two strains - BERKELEY and KAVAGUCHI-1) and braunixanthins 1 and 2 (isolated from Kawaguchi-1 strain).

Studying the conditions which influence the growth of *B. braunii* algae in India, Dayananda and his co-workers [DAYANANDA – 2007] discovered that lutein and β-carotene are the major carotenoids. For carotenoid analysis, the authors dried the algal biomass, then extracted the final product with acetone; the absorbance of the acetonic extract was measured at 474 nm then the total carotenoid content was determined according to an older method [LICHTENTHALER - 1987]. Carotenoid separation was accomplished using high performance liquid

chromatography (figure 1), using an RP column $C_{18}$ (25 cm x 4.6 mm), isocratic elution with acetonitrile/methanol/dichlormetane (7:1:2), using a flow rate of 1 ml/ min and detection at 450 nm.

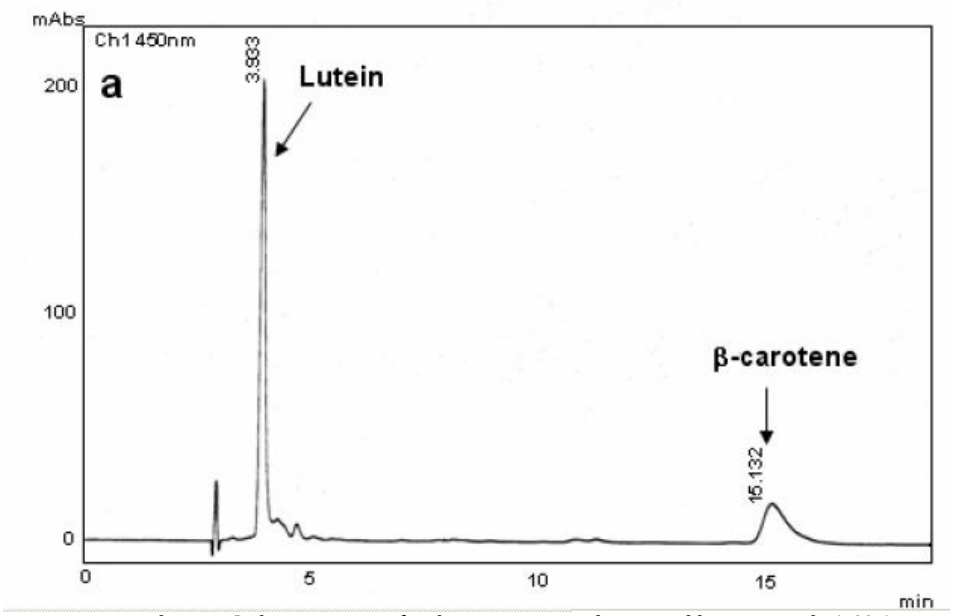


Figure 1. The HPLC chromatogram of B. braunii carotenoids reported by Dayananda (2007)

B-carotene and lutein were also reported as major carotenoids by Ranga Rao and his co-workers [Ranga Rao – 2006, 2007] in studies concerning the effects of salinity on the *B. braunii* development; they reported an improvement in the extraction method, as the algal biomass was initially frozen, then extracted with acetone, minimizing in this way the effect of oxygen, heat and light exposure. The quantitative determination of carotenoids was accomplished according to the Davies' procedure [Davies – 1976], measuring the absorbance of the acetonic extract at 450 nm; the chromatographic analysis lead to the separation of three more carotenoids (violaxanthin, astaxanthin and zeaxanthin), using Danayanda's system.

The subject of the actual research is some *B. braunii* algal strains available in the collection belonging to the Institute of Biological Researches Cluj-Napoca. The main purposes are: a.) to develop a much selective chromatographic separation system for carotenoids; b.) to establish the carotenoid biomarkers for *B. braunii* strains.

## MATERIALS AND METHOD

The green algae *B. braunii* strains belong to the Collection of Algae Cultures of the Institute of Biological Researches Cluj-Napoca. These strains were grown in BW nutritive solution [Schlosser – 1982], in 250 ml vessels, with continuous air stirring, under continuous illumination with white fluorescent light (2500 lux), at 22$^{o}$C. The algal development was monitored by daily measurement of absorbance at 668 nm, for 15 days.

Carotenoid extraction from the algal suspension samples was accomplished by direct saponification with a solution of 30% KOH in methanol, at room temperature, for 24 hours. Carotenoids were then extracted using diethyl ether; the etheric layer was separated and washed repeatedly with brine, then with distilled water until free of alkali. The aqueous layers were re-

extracted with small volumes of diethyl ether until colourless, then the organic layers were combined, washed several times with distilled water and evaporated to dryness under reduced pressure. The saponified extract was dissolved in 5 ml ethyl acetate, being then subjected to high performance liquid chromatography (HPLC) analysis.

Qualitative HPLC analysis was performed on a system consisting in two Altex 110 A pumps, an Altex mixing chamber, an Altex gradient controller, a Waters 990 photodiode-array detector. The Waters 990 software was used for data acquisition and processing. Samples were injected into the column via a Rheodyne 7125 injector using complete loop filling (20 µl).Separations were performed using a Nucleosil 120-5$C_{18}$ column and the following mobile phases: A - acetonitrile : water (9 : 1) and B - ethyl acetate. The flow rate of the mobile phase was 1 ml/ min., and the solvent gradient was as follows: from 0 to 20 min. - 10 to 70% B, then from 20 to 30 min.: 70 to 10% B. Carotenoids identification was completed based on HPLC co-chromatography with authentic standards and by comparison of the on-line visible absorption spectra with those of reference carotenoids [Britton - 1996; Haugan - 1995]; for this, authentic carotenoid standards were provided by F. Hoffmann - La Roche, Basel, Switzerland. Quantitative HPLC analysis was performed using an Agilent 1100 system, consisting in a degasser, an Agilent G1311A quaternary pump system, a Rheodyne injector equipped with a 20 µl loop, an UV/VIS Agilent G1314A detector, using the same column and mobile phase gradient. The Chemstation Agilent software was used for data acquisition and processing. The quantitative analysis of carotenoids was accomplished using the external standard method [Hart, 1995].

## RESULTS AND DISCUSSION

HPLC analysis revealed a complex chromatographic pattern for all the studied B. braunii algal strains, dominated usually by lutein. A representative HPLC chromatogram is presented in figure 2, in which 14 carotenoids were identified: four carotenes (α - carotene, β - carotene, 9Z – β - carotene and 15Z - β - carotene) and ten xanthophylls (violaxanthin, 5,6-epoxy-lutein, anteraxanthin, lactucaxanthin, lutein, zeaxanthin, α-criptoxanthin, β-criptoxanthin, echinenone and 5,6-epoxy-β - carotene). Their concentrations are summarized in table 1, together with that of the other studied strains.

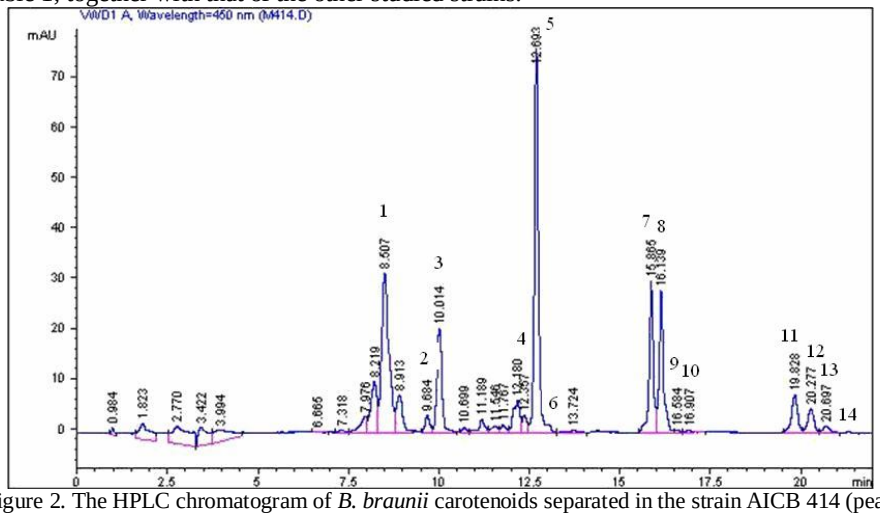

Figure 2. The HPLC chromatogram of *B. braunii* carotenoids separated in the strain AICB 414 (peak identification in table 1)

The obtained results demonstrates that the carotenoid HPLC pattern of the studied *B. braunii* strains is a complex one, dominated by lutein – which is by far the major carotenoid in all strains; this conclusion is in agreement with the previous published studies.

The other reported major carotenoid – β-carotene is a major one only in three strains (AICB 445, AICB 462 and AICB 464) , but this is never alone, being accompanied by the structure-related α-carotene, as well as two isomers 9Z – β - carotene and 15Z - β - carotene; this later three carotenoids were newer reported in other studies. It is highly possible that 9Z – β - carotene and 15Z - β - carotene are isolation artefacts.

Among xanthophylls, only violaxanthin and antheraxanthin were present in all cases in quantifiable amounts.

*Table 1*

The concentration of carotenoid markers in the studied B. braunii strains
(concentrations in μg/ ml algal suspension)

| Nr. | Carotenoid | AICB 414 | AICB 441 | AICB 445 | AICB 462 | AICB 464 |
|---|---|---|---|---|---|---|
| 1 | Violaxanthin | 0.72 | 0.18 | 0.60 | 0.47 | 0.91 |
| 2 | 5,6-epoxy-lutein | traces | traces | traces | traces | traces |
| 3 | Anteraxanthin | 0.32 | 0.02 | 0.05 | 0.03 | 0.08 |
| 4 | Lactucaxanthin | traces | traces | traces | traces | traces |
| 5 | Lutein | 0.81 | 0.80 | 2.55 | 2.09 | 4.04 |
| 6 | Zeaxanthin | traces | traces | 0.05 | 0.06 | 0.05 |
| 7 | α-criptoxanthin | 0.32 | traces | 0.04 | 0.04 | 0.05 |
| 8 | β-criptoxanthin | 0.31 | traces | traces | traces | traces |
| 9 | Echinenone | traces | traces | traces | traces | traces |
| 10 | 5,6-epoxy-β - carotene | traces | traces | traces | traces | traces |
| 11 | α - carotene | 0.11 | traces | 0.21 | 0.21 | 0.51 |
| 12 | β - carotene | 0.08 | 0.02 | 0.41 | 0.33 | 0.87 |
| 13 | 9Z – β - carotene | traces | traces | 0.11 | 0.09 | 0.23 |
| 14 | 15Z - β - carotene | traces | traces | 0.03 | 0.02 | 0.05 |

## CONCLUSIONS

From the fourteen separated carotenoids, twelve can be considered valuable biomarkers for *B. braunii* algae: two carotenes (α - carotene, β - carotene) and ten xanthophylls (violaxanthin, 5,6-epoxy-lutein, anteraxanthin, lactucaxanthin, lutein, zeaxanthin, α-criptoxanthin, β-criptoxanthin, echinenone and 5,6-epoxy-β - carotene).

As a final conclusion, using the proposed chromatographic system is possible to obtain a much higher selectivity than using the previous published ones, this aspect being evident comparing figures 1 and 2. The proposed extraction method using direct saponification of the algal suspension eliminates the exposure to air, oxygen and heat, minimizing thus the degradation of carotenoids.

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