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Article

Generation of Volatile Compounds from Carotenoids of *Dunaliella bardawil* Algae by Water Bath Heating and Microwave Irradiation

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The volatile compounds formed by thermal degradation of the carotenoids present in *Dunaliella bardawil* were investigated by microwave irradiation (MW) and water bath heating (WB) in different conditions of temperature and time using central composite design. Volatiles extraction by solid phase microextraction (SPME) was optimized and performed at 40 °C for 15 minutes, and those in greater amount were quantified by a validated method of gas chromatography coupled to a quadrupole mass spectrometer (GC-qMS). Employing WB, 10, 12 and 120 ng mL^{-1} of β-cyclocitral, α-ionone e β-ionone, respectively, were obtained between 60 and 87 °C for 30-75 min, while by MW, 5, 5, 50 ng mL^{-1} were obtained between 75-107 °C for 1.5-2.8 min. Considering the shorter time of MW, it can be concluded that if the time necessary to obtain the best yield by WB is employed in multiple MW cycles, an amount 10 times greater of those compounds would be obtained by MW than by WB. The results suggest a new biotechnology application for the carotenoids of the microalgae of the *Dunaliella* genus.

Keywords: *Dunaliella bardawil*, β-cyclocitral, ionone, carotenoid, microwave, water bath

Introduction

Microalgae are photosynthetic microorganisms widely distributed in marine, freshwater and terrestrial ecosystems.[1,2] They produce a great diversity of compounds with high commercial value, such as polyunsaturated fatty acids, carotenoids, phycobilins, polysaccharides, vitamins and sterols.[3-7] The production of microalgae supplies the cosmetic and food supplement industries, and boosts the development of natural food products, an emerging and promising field for industrial application.[8] Microalgae have properties typical of higher plants (efficient oxygenic photosynthesis and simplicity of nutritional requirements), but also biotechnological attributes of microbial cells (rapid growth in culture medium and capacity to accumulate or secrete metabolites of interest),[4] supporting large scale applications.

Among the microalgae used to obtain carotenoids, two species of the genus *Dunaliella*, *D. bardawil* and *D. salina* stand out for the production of β-carotene in their chloroplasts.[9] The accumulation capacity of β-carotene in *Dunaliella* is influenced by stress factors in culture, such as high light intensity and salt concentration, low temperatures and/or nitrate deficiency.[10] Studies reported in the literature aimed at increasing the production of carotenoids in *Dunaliella* showed that its concentration varies in a range between 4 and 38 μg mL^{-1}.[11-14]

Herrero *et al.*[15] evaluated the chemical composition and antimicrobial activity of the pressurized extract of *D. salina*. Norisoprenoids such as α-ionone, β-ionone and β-cyclocitral, besides neophytadiene and phytol were observed, as well as antimicrobial activity associated to volatiles. Previously, Larrouche *et al.*[16] showed that β-ionone inhibits the respiratory chain, preventing microbial oxygen consumption.

Norisoprenoids can be formed from carotenoids by external agents such as temperature, light, and acidity change, or by enzyme/microbial action.[17-20] The oxidation of β-carotene results in epoxides, apocarotenals and apocarotenones, which in later stages of oxidation produce lighter carbonyl compounds such as norisoprenoids.[21] However, the production of aroma compounds from plants is a low-yield process, and the main source is from synthesis.[22,23] On the other hand, biotechnological processes

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usually involve less environment damage than chemical processes, and can favor stereospecific flavors.

A preliminary evidence that *Dunaliella* might be useful in biotechnological processes for aroma production was obtained by Donadio *et al.*[24] investigating the sea salt known as "Fleur de sel", the solar salt of Saint-Armel (Brittany, France), a valuable spice in international cuisine. The authors suggested that, in addition to its pale orange color influenced by the carotenoid content of the sea algae, the flavor of the salt could be correlated to the norisoprenoids formed from the *Dunalliela* carotenoids.

Microwave irradiation is widely known as an alternative tool to conventional methods of synthesis and extraction, for instance, microwave-assisted extraction (MAE) can substitute Soxhlet extraction,[25] ultrasound and simple heating reflux. In food processing, microwave irradiation has been successfully applied in pasteurization, decontamination and disinfection.[26,27]

Thus, the aim of this study was to investigate the formation of volatile compounds from the controlled degradation of carotenoids present in *D. bardawil* microalgae by microwave irradiation *versus* water bath heating.

Experimental

Cultivation conditions of *Dunaliella bardawil*

Microalgae *D. bardawil* (UTEX LB 2538, University of Texas) cultivation was performed using the culture medium developed by Shaish *et al.*[28] at pH 8.5 and agitation at 180 rpm in an orbital shaker table at 25 °C (± 3). Cultivation was carried out in two stages, in order to obtain a biomass rich in carotenoids, according to previous studies of our group (data not shown). To maximize cell growth, cultivation was carried out with a constant light intensity of 150 μE m^{-2} s^{-1} (fluorescent lamps of 59 W). After the culture achieved the stationary growth phase (6×10^{-5} cells mL^{-1}), it was centrifuged at 3500 rpm, at 15 °C for 15 min. The supernatant was discarded and the green cell pellet (GP) resuspended in 300 mL of culture medium (without $NaNO_3$). In the second stage performed to promote the accumulation of β-carotene, the culture remained for 72 h under stirring and light intensity of 400 μE m^{-2} s^{-1}. Then, aliquots of 10 mL of biomass rich in carotenoids (BC1) were stored in 15 mL tubes with Shaish medium in a freezer until the moment of analysis, when the biomass was thawed and centrifuged at 3500 rpm, at 15 °C for 15 min. The supernatant (Shaish medium) was discarded and the orange cell pellet (OP) resuspended in 10 mL of sterile distilled water, and then transferred to 40 mL vials with cover and silicone septum. The biomass rich in carotenoids with distilled water (BC2) was used to evaluate the controlled degradation of carotenoids in water bath and microwave controlled conditions.

Solid phase microextraction (SPME)

The experimental conditions of solid phase microextraction (SPME) were optimized to monitor the production of volatile compounds in the biomass (BC2) process. A central composite factorial design with five levels was employed (Table 1). The volatile compounds were trapped on a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber and analyzed by gas chromatography coupled to a quadrupole mass spectrometer (GC-qMS).

Table 1. Experimental design matrix for SPME

Experiment	Temperature / °C	time / min
1	30	30
2	40	15
3	40	45
4	60	9
5	60	51
CP	60	30
CP	60	30
6	80	15
7	80	45
8	88	30

CP: central point.

Statistica software version 7 (2004) was used to process the analyses results. Each experiment was performed in triplicate.

GC-qMS analysis

GC-qMS was conducted on an Agilent chromatograph (6850) coupled to a mass detector (5975C) system, equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm), operated in electron ionization (EI) mode at 70 eV over a 50-550 *m/z* range. Selective ion monitoring (SIM) of α-ionone, β-ionone and β-cyclocitral (*m/z* 41, 91, 93, 121, 136, 137, 152, 177, 192) was performed. Helium was used as carrier gas at 1.5 mL min^{-1}. Oven temperature ranges were 35-130 °C (6 °C min^{-1}), 130-160 °C (3.5 °C min^{-1}) and 160-250 °C (35 °C min^{-1}, kept for 2 min at 250 °C). All analyses were performed in splitless injection mode. The injector temperature, source of quadrupole and transfer zone were: 250, 230 and 150 °C, respectively.

Linear retention index (LRI) of the compounds,[29,30] standards co-injection and comparison with NIST 2008 library database were used to confirm compound identity.

Quantification of β-cyclocitral and ionones

The parameters evaluated in the determination of the concentration of the major volatiles, were: selectivity, linearity, range, limit of detection (LOD) and quantification (LOQ), accuracy and precision (repeatability), in accordance to Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO).[31] The analytical curves of β-cyclocitral and α-ionone were built by using aqueous solutions fortified with standards.

To determine selectivity, matrix effect was investigated for β-cyclocitral and α-ionone by comparing their curves in solution (water and ethanol) and matrices (cell suspension of *D. bardawil* added with β-cyclocitral and α-ionone, in both solvents) and the slopes of two regression lines.[32] After determining the matrix effect, linearity was verified by the presence of outliers, by Grubb's test, and homoscedasticity by Cochran's test. The determination coefficient and the residual plot were then obtained.

From a stock solution of β-cyclocitral and α-ionone (36.7 and 35.7 μg mL^{-1}, respectively), aqueous standard solutions were prepared with the following concentrations: 1, 3, 5, 10, 15 ng mL^{-1}; 1, 30, 60, 90, 150 ng mL^{-1}, respectively. Each solution was analyzed by HS-SPME/GC-qMS. First, the cell suspension of *D. bardawil* in water was placed in a 40 mL vial and standard solutions added (final volume of 10 mL). Then, SPME fiber was manually exposed in the vial headspace under the conditions determined by the central composite factorial design. All measurements were made in three replicates, each replicate being the analysis of a different aliquot of each aqueous solution.

Accuracy was determined through the recovery of β-cyclocitral and α-ionone and precision through repeatability. LOD and LOQ were obtained by injecting β-cyclocitral and α-ionone until a signal to noise ratio equal to 3 and 10, respectively.

Quantification of carotenoids

Total carotenoid content was measured in the β-carotene-rich biomass (BC2). The biomass was extracted with ethanol (95%) and spectrophotometrically measured by the method of Lichtenthaler.[33] Carotenoid profile was obtained by HPLC-UV (460 nm) after separation in a Nova Pak C-18 analytical column according to Brotas and Plante-Cuny[34] related to standards.

Controlled degradation of carotenoids from *D. bardawil* by water bath heating and microwave irradiation

In order to evaluate the influence of time and temperature on the degradation of the carotenoids present in *D. bardawil* biomass (BC2), a central composite design with two variables was used, in which five levels of temperature were employed for both water bath heating (WB) and microwave irradiation (MW) processes (Table 2). Each experiment was made in triplicate, using 10 mL of sample BC2. Statistica software version 7 (2004) was used for data analysis.

Table 2. Matrix of experiments for water bath heating (WB) and microwave irradiation (MW) controlled degradation of *D. bardawil* biomass (BC2)

Experiment	Temperature / °C	WB time / min	MW time / min
1	70	15.0	0.5
2	70	75.0	2.5
3	100	15.0	0.5
4	100	75.0	2.5
5	64	45.0	1.5
6	106	45.0	1.5
7	85	2.5	0.08
8	85	87.0	2.9
9	85	45.0	1.5
10	85	45.0	1.5

Results and Discussion

SPME optimization

In the SPME analyses of *D. bardawil* biomass, seven volatiles were identified: α-ionone, β-ionone, β-cyclocitral, dihydroactinidiolide, 7,8-dihydro-β-ionone, 5,6-epoxy-β-ionone and *trans*-geranyl acetone, among which β-cyclocitral, α-ionone and β-ionone were the major compounds in all experiments performed (Supplementary Information section, Figure S1). Taking into account that carotenoid degradation depends on temperature and heating time,[24] and also that SPME conditions can influence volatile production, a central composite design for SPME conditions was carried out monitoring β-cyclocitral and the ionones. The response surface graphs are shown in the Supplementary Information section (Figure S2).

A considerable increase of the absolute areas of ionones occurred when SPME was carried out up to 60 °C and 40 min. For β-cyclocitral, the increase was observed between 50-75 °C and 35-50 min. With the

aid of standards, Nonier *et al.*[35] showed that from room temperature up to 40 °C (40 min), the concentration of β-carotene suffered a reduction of only 2.5%, while at 70 °C (20 min) it was completely degraded. Therefore, the increase in α- and β-ionone areas with time and temperature does not necessarily indicate that a more effective extraction occurred, as suggested for β-cyclocitral (Supplementary Information section). In fact, higher temperatures may promote the degradation of carotenoids in *D. bardawil*.

Based on this, softer conditions for SPME extraction were used. At 30 °C/30 min and 40 °C/15 min, the GC profile was equivalent for α- and β-ionone, suggesting that the degradation of carotenoids in *D. bardawill* biomass had not yet started. So, the last condition of 40 °C for 15 min was chosen due to the shorter time.

Norisoprenoids quantification

As no quantitative method for the determination of norisoprenoids obtained from carotenoids of *D. bardawil* has been described in literature, the parameters of validation were defined as shown in Table 3. Evaluation of the matrix effect was performed by comparing the slope of the straight line and, as $t_{calculated}$ was greater than $t_{tabulated}$ for β-cyclocitral (Table 3),[32] a matrix effect was accepted for this compound, and extrapolated for α-ionone.

The analytical curves and residue profile for β-cyclocitral and α-ionone are shown in the Supplementary Information section (Figure S3). The method was successfully implemented, allowing more accurate data for the experiments aimed at the production of norisoprenoids.

Optimization of volatiles formation from *D. bardawil* biomass (BC2) by water bath heating and microwave irradiation

HPLC-UV of the carotenoids present in *D. bardawil* biomass showed that 90% was composed by β-carotene and 10% by lutein (Supplementary Information section, Figure S4) and total carotenoids in BC2 biomass was of 15 μg 10 mL^{-1}. The controlled degradation of carotenoids and the formation of β-cyclocitral, α- and β-ionones were evaluated in a controlled water bath heating (WB) and by microwave irradiation (MW) (Table 2, Experimental section). SPME-GC-qMS (40 °C for 15 min) was performed, as previously discussed.

The influence of temperature and incubation time on the response variable (concentration of β-cyclocitral, α-ionone and β-ionone from BC2 biomass), using MW and WB, is shown in the contour plots of Figure 1 (response surface graphs are displayed in the Supplementary Information section, Figure S5). The concentration (ng mL^{-1}) of β-cyclocitral, α-ionone and β-ionone at the central point is shown in the Supplementary Information section, Table S1.

In SPME from *D. bardawil* (BC2) WB treatment, the response variable (concentration) was greater for β-cyclocitral when temperature was between 60-87 °C for 30-70 min; for α-ionone, it was between 65-85 °C for 30-55 min and for β-ionone, between 60-62 °C for 45-75 min, leading to the production of 10, 12 and 120 ng mL^{-1} of β-cyclocitral, α-ionone and β-ionone, respectively. For MW, the response variable (concentration) was greater for β-cyclocitral between 92-107 °C for 2.3-2.8 min, while for α-ionone and β-ionone the best conditions were between 75-85 °C for 1.5-1.8 min and

Table 3. Validation parameters for β-cyclocitral and α-ionone in GC-qMS (SIM)

Parameter	β-Cyclocitral[a]	α-Ionone[a]
Linearity		
Concentration levels / (ng mL^{-1})	1, 3, 5, 10, 15	1, 30, 60, 90, 150
Correlation coefficient (r)	0.9916	0.9947
Cochran's test	homoscedasticity C_{cal} = 0.37 C_{tab} = 0.68	homoscedasticity C_{cal} = 0.63 C_{tab} = 0.68
Selectivity		
m/z	41, 81, 137	43, 93, 121
Matrix effect	present t_{cal} = 2.71 t_{tab} = 2.05	absent t_{cal} = 1.30 t_{tab} = 2.05
LOQ / (ng mL^{-1})	1.0	0.6
LOD / (ng mL^{-1})	0.3	0.2
Accuracy		
Mean recovery / %	80.87	89.91
Precision		
Repeatability / %	13-23	12-15

[a]All results were obtained from triplicate injections; cal: calculated; tab: tabulated; LOQ: limit of quantification; LOD: limit of detection.

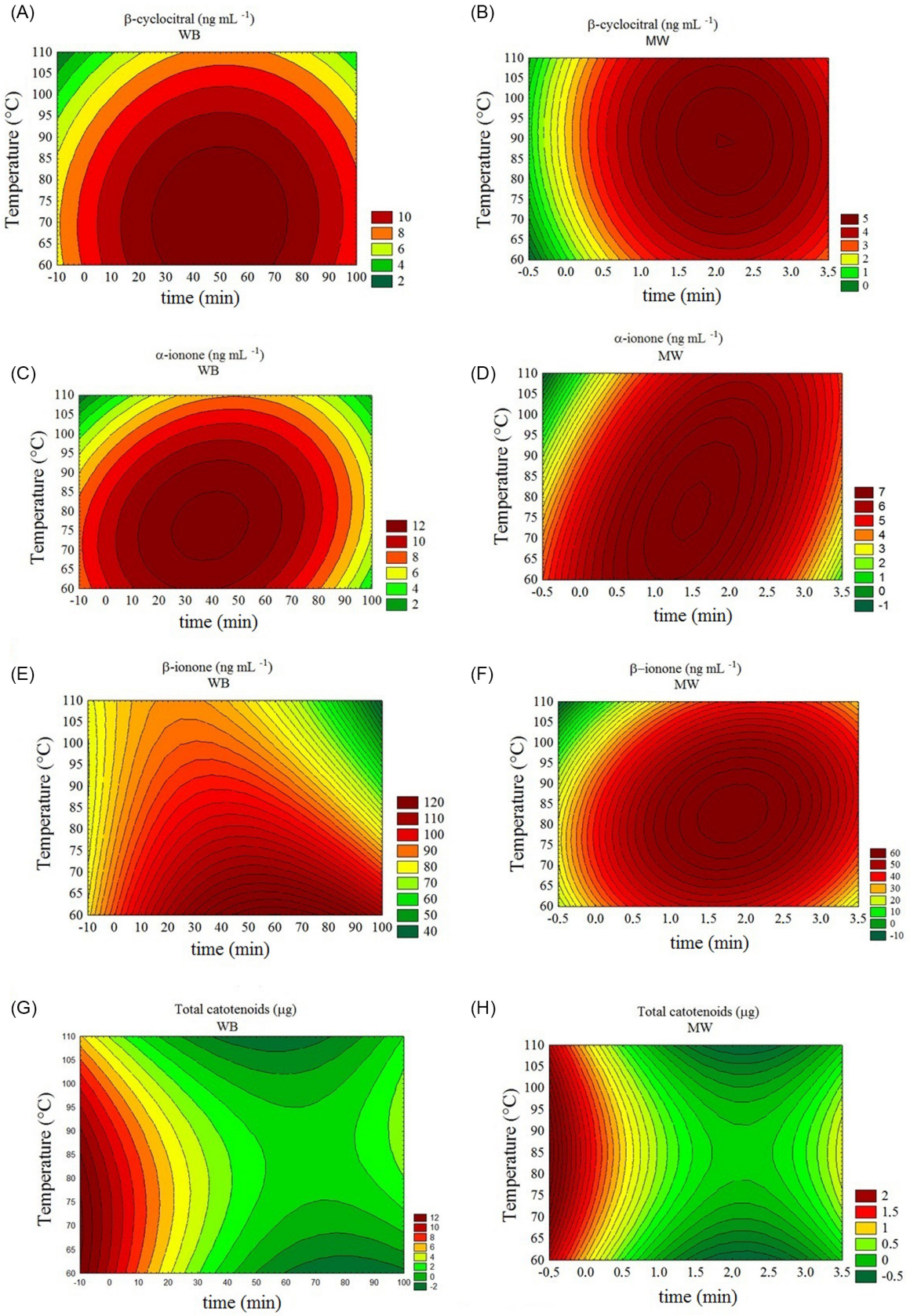

Figure 1. Contour plots for (A) β-cyclocitral WB; (B) β-cyclocitral MW; (C) α-ionone WB; (D) α-ionone MW; (E) β-ionone WB; (F) β-ionone MW; (G) total carotenoids WB; (H) and total carotenoids MW as a function of temperature and time employed in the controlled degradation of carotenoids from *D. bardawil*.

75-88 °C between 2.0-3.5 min, respectively, achieving 5, 5 and 50 ng mL^{-1} of β-cyclocitral, α-ionone and β-ionone, respectively. Previous study[36] on the production of norisoprenoids from carotenes in potato chips revealed contents of the same order of magnitude. Related to the carotenoid content, WB process imparts a complete degradation at temperatures between 75-95 °C for 50-85 min, while in MW, the complete degradation occurs between 80-95 °C for 1.7-2.5 min.

Kanasawud and Crouzet[37] studied the kinetic degradation of β-carotene in water under sonication and saturated by oxygen. Twenty-one volatiles were observed, dihydroactinolide included, postulated to be formed from β-ionone, as well as other volatiles. A diversity of works investigated the radical oxidation of carotenoids at different conditions. More recently, Dongho *et al.*[38] investigated carotenoid degradation caused by sunlight exposure and heating conditions in crude palm oil, revealing a significant decrease of carotenoids in accelerated heating. Interestingly, they also observed a high increase of peroxide value related to lipid oxidation, where the reactive oxygen species (ROS) produced in the process could also impact carotenoid degradation. In fact, ROS react with the polyene chain of carotenoids to generate apocarotenals and, later, norisoprenoids.[21] As *D. bardawil* is rich in tri- and diacylglicerols,[39] both direct heating and ROS mechanisms may contribute to norisoprenoid production.

For the time variable (minutes), WB process suggests 45 to 55 min for the simultaneous production of the three compounds. For MW, it was not possible to observe a common area for the production of the three compounds, but only for β-cyclocitral and β-ionone (2.3-2.8 min).

It is also interesting to observe that MW process can lead to a greater yield of volatiles when the time is the same as in the WB process. As an example, for β-cyclocitral at the center point, 1.5 min were necessary for MW and 45 min for WB (Table S1), to give 4.46 ± 0.34 and 12.83 ± 0.11 ng mL^{-1}, respectively. Therefore, 30 cycles of 1.5 min in MW would yield 10 times more β-cyclocitral at the central point conditions in both methods.

Conclusions

To the best of our knowledge, this is the first work using carotenoids from *D. bardawil* microalgae to produce volatile compounds, employing microwave irradiation and water bath controlled heating. This proposal suggests a sustainable production or enrichment of volatiles commonly present in food products, since water and mild conditions of time and temperature were employed.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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