EXTRACTION OF NEW BIOACTIVES FROM NEOCHLORIS OLEOABUNDANS USING PRESSURIZED TECHNOLOGIES AND FOOD GRADE SOLVENTS

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Abstract. In the last years there has been an increasing interest in functional food ingredients due to their capacity to promote our health. Thus, one of the main areas in Food Science and Technology is the extraction and characterization of new bioactive compounds of natural origin.

Considering the huge biodiversity of microalgae it is easy to understand that they represent one of the most important biological sources for novel biological compounds. In fact, it has been suggested that secondary metabolites produced by these organisms under certain extreme conditions (temperature, nutrients, UV-vis irradiation), provide with unique structures with important activities for human health such as antioxidant. As an example, β-carotene and astaxanthin produced by *Dunaliella salina* and *Haematococcus pluvialis* respectively, have important application in the nutraceutical market.

In the search of new bioactives from microalgae, another important aspect to be considered is the development of fast and environmental-friendly extraction processes to isolate the bioactive compounds.

The purpose of this work was to develop a methodology to extract and characterize the carotenoids of N. Oleoabundans to demonstrate for the first time the potential of this microalga as a source of antioxidants. Thus, pressurized liquid extraction (PLE) was optimized by means of an experimental design considering food grade solvents such as ethanol and limonene; the chemical characterization of the extracts was carried out by means of HPLC-MS. The main carotenoids accumulated by the microalgae were lutein, cantaxanthin, zeaxanthin, or monoester and diester of astaxanthin.

Keywords: Pressurized liquid extraction, carotenoids, *Neochloris oleoabundands*, limonene, experimental design

1. Introduction

The huge diversity of microalgae makes them an almost unlimited resource for bioactive compounds discovery. Depending on the particular growing conditions applied or through biotechnology approaches, these organisms are frequently able to stimulate the synthesis and accumulation of valuable compounds, among which are carotenoids [1]. Along with their well-known antioxidant properties, bioactivities like prevention of cancer [2], cardiovascular diseases [3] or macular degeneration [4] have been attributed to different carotenoids. The production of carotenoids has been possible due to the advance in microalga biotechnology, and for example *Dunaliella salina* has shown to be able to accumulate high amounts of β-carotene when subjected to particular growing conditions, whereas *Haematococcus pluvialis* is the major producer of astaxanthin under environmental stress.

The employment of advanced extraction techniques versus conventional techniques to carry out the extraction of bioactive compounds from natural matrices (as microalgae) is gaining increasing attention nowadays. In fact, the potential of pressurized liquid extraction (PLE) using GRAS (Generally Recognized As Safe) solvents to extract carotenoids from different microalgae such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella vulgaris*, and *Spirulina platensis* has already been demonstrated [5-8]. PLE is based on the extraction using temperature and pressure that maintain the extraction solvent in the liquid state during the extraction process. High pressure forces the solvent into de matrix facilitating the extraction whereas high temperature promote analyte solubility and decreases the viscosity and the surface tension of the solvents improving the extraction rate. In the liquid state, it is possible to obtain higher extraction yields in a shorter period of time, using significantly smaller volumes of extraction solvents compared to conventional extraction methods. In order to chemically characterize the compounds extracted, it is necessary the use of advanced analytical tools such as LC-MS.

Neochloris oleoabundans is a green microalga which has been mainly studied due to its ability to produce lipids suitable for biodiesel production. There are only two works described in the literature which provide some data related to the carotenoids content of this microalge. One of them describe the contribution of phenolic and carotenoids substances to antioxidant activity from different microalgae (being N. Oleoabundans one of them), estimating the carotenoid content spectrophotometrically [9], whereas the other presents a preliminary characterization of the carotenoids of *N. oleoabundans* among other microalge suitables for biodiesel production [10].

The aim of this work was to study for the first time *N. oleoabundans* as an alternative source of carotenoids. To do that, PLE using food grade solvent, such as ethanol and limonene, was optimized by means of an experimental design to extract carotenoids from *N. oleoabundans*. Besides, the carotenoids extracted were exhaustively characterized using a LC-MS methodology.

2. Materials and methods

2.1. Samples

Neochloris oleoabundans (UTEX#1185) was obtained from the culture collection of algae at the University of Texas (Austin, TX). Batch cultures were grown in 8 cm wide glass reactors containing 1L of modified Bold´s Basal Medium [11] supplemented with 0.3 g/L of KNO3 and subjected to continuous stirring by bubbling air at a constant flow rate. Pure CO_2 was supplied every 30 s at 10 min intervals to the air stream to provide inorganic carbon and to maintain the pH value at 8. This was achieved using an electronic gas-control valve (Wilkerson R03-C2). Reactors were maintained in a culture chamber at 24 ± 2 ºC, with a 16:8 h light:dark photoperiod supplied with fluorescent light (Philips TLD 58W) at a photosynthetic photon flux density of 400 µmol photons m^{-2} s^{-1}. After cells reached the late exponential phase, biomass was harvested by centrifugation (7000 rpm for 5 min at 10ºC), frozen at -20ºC and freeze-dried at -40 ºC for 48 h and stored under dry and dark conditions until further use.

Previous to the PLE extraction, 2.5 g of sample were treated by three cycles of cryogenic grinding using a Mixer mill CryoMill (Retsch, Haan, Germany) to breakdown the microalga cell wall and obtain the highest extraction yield.

2.2. Experimental design

The influence of extraction temperature and solvent composition (different % of limonene in the mixture) on the extraction yield and total amount of carotenoids was studied using a three-level factorial design. A total of 11 experiments (9 points of the factorial design (3^2) and 2 center points to consider the experimental errors) were carried out in a randomized order. The factors tested at three different levels in the design were: extraction temperature at 40, 100 and 160 ºC, and % limonene at 0, 50, and 100 %. The response variables selected were extraction yield and total amount of carotenoids. The quadratic model proposed for each response variable (Yi) was:

$$Yi = \beta_0 + \beta_1 T + \beta_2 S + \beta_{1,1} T^2 + \beta_{1,2} T*S + \beta_{2,2} S^2 + error \quad (1)$$

where T is the temperature, S is solvent composition (% limonene in the mixture), β_0 is the intercept, $\beta_{\square}$ and β_2 are the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are the quadratic coefficients, $\beta_{1,2}$ is the interaction coefficient and error is the error variable. The parameters of the model were estimated by multiple linear regression (MLR) using the Statgraphics Centurion XVI program (Statpoint Technologies, Inc.). The effect of each term in the model and its statistical significance, for each of the response variables, was analyzed from the standardized Pareto chart. The model adequacy was evaluated by the coefficient of determination (R^2), the residual standard deviation (RSD), and the lack-of-fit test for the model from the ANOVA table. From the fitted model, the optimum conditions, which maximize the extraction yield and the total amount of carotenoids response variables, were provided by the program. Surface plots were developed using the obtained fitted quadratic polynomial.

2.3. PLE Extraction

PLE extractions of *N. oleoabundans* were carried out using an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA, USA) equipped with a solvent controller. Extractions were performed at three different extraction temperatures and solvent composition (% of limonene in the mixture), according to the experimental design, and 20 min as extraction time. Prior to each extraction, an extraction cell heat-up step was carried out for a given time which is fixed by the system (i.e., 5 min when the extraction temperature was 40 ºC and 100 ºC, 8 min at 160 ºC). All extractions were done using 11 mL extraction cells at 1500 psi, containing 2 g of alga mixed homogeneously with 2 g of sea sand. The extracts obtained were protected from light and stored under refrigeration until analysis.

2.4. Liquid chromatography analysis

For the quantification of carotenoids an Agilent 1100 Liquid Chromatograph equipped with a DAD (Agilent Technologies, Palo Alto, CA, USA) and a YMC-C30 reversed-phase column (250 mm x 4.6 mm id, 5 μm particle size, YMC Europe, Schermbeck, Germany) were used. The mobile phase was a mixture of MeOH:MTBE:water (90:7:3 v/v/v) (A) and MeOH:MTBE (10:90 v/v) (B) eluted according to the following gradient: 0 min, 0 % B; 20 min, 30 % B; 35 min, 50 % B; 45 min, 80 % B; 50 min, 100 % B; 52 min, 0 % B. Flow rate was 0.8 ml/min, the injection volume was 10 μL, and detection was at 450 and 660 nm (recorded spectra from 240 to 770 nm by DAD).

For the calibration curve, six standard solutions of β-carotene and lutein (ranging from 1 to 0.025 mg/mL and from 0.04 to 1.25 x 10^{-3} mg/mL, respectively), and seven standard solutions of astaxanthin monopalmitate and dipalmitate (ranging from 0.04 to 6.25 x 10^{-4} mg/mL) were prepared by appropriate dilution from a stock solution (1-2 mg/mL). Lutein was dissolved in ethanol, astaxanthin monopalmitate and astaxanthin dipalmitate in hexane:acetone (1:1 v/v), and β-carotene in hexane.

The characterization of the obtained extracts was performed by LC–MS. The instrument employed was an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with a DAD and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. The LC conditions employed to carry out the analysis were the same that those described previously. Regarding MS analysis, it was carried out under APCI positive ionization mode using the following parameters: capillary voltage, -3.5 kV; dry temperature, 350 ºC; vaporizer temperature, 400 ºC; dry gas flow, 5 L/min; corona´s current (that sets the discharge amperage for the APCI-source), 4000 nA; nebulizer gas pressure, 60 psi. A range from m/z 150 to 1300 was acquired.

3. Results and discussion

A novel experimental design was used to optimize the extraction of carotenoids from *N. oleoabundans* using PLE with food-grade solvents such as ethanol and limonene. The ability of pressurized ethanol to extract carotenoids from different microalgae has been previously demonstrated [5-8]. Regarding limonene, it is a green biodegradable solvent (suggested as a good alternative to hexane since it possess a dielectric constant very close to this toxic organic solvent) which has been used to extract oils from microalgae [14], or carotenoids from tomatoes [15]. Taking that into account, it can be expected that a combination of both green solvents would favor the extraction of carotenoids from *N. oleoabundans*. Therefore, a three-level factorial design (3^2) was performed to optimize the extraction temperature and the solvent composition using as

responses variables the extraction yield (determined as dry weight/initial weight expressed in %) and total amount of carotenoids in the extract (expressed as mg carotenoids/g extract). In **Table 1** the experimental matrix design with the levels of the experimental factors along with the results obtained for the responses analyzed are shown.

Table 1. Experimental matrix design.

Exp.	Temperature (ºC)	Solvent composition (% limonene in the mixture)	Extraction yield (%)	mg carotenoids/g extract
1	160	0	33.7	88.8
2	40	100	31.0	84.4
3	100	0	30.1	97.8
4	40	50	33.4	85.1
5	40	0	23.4	120.2
6	100	50	36.1	72.7
7	100	50	35.3	74.8
8	100	100	34.1	84.6
9	100	50	35.6	75.0
10	160	100	26.5	74.8
11	160	50	33.3	57.4

To quantify the total amount of carotenoids, the *N. oleoabundans* extracts were firstly analyzed by LC-APCI-MS in order to identify tentatively the carotenoid. To do that, the information provided by the two detectors (DAD and MS), the use of commercial standards and the data found in the literature was combined. From the twenty peaks whose UV-Vis spectra pointed out to pigment compounds, two chlorophylls (chlorophyll a and chlorophyll b) and eleven (free or diester) carotenoids could be identified by MS. Among them, it was possible to identify β-carotene, lutein and violaxanthin that have been described as the major carotenoids in green algae, as well as other minor carotenoids, such as zeaxanthin. Along with these primary carotenoids other secondary metabolites such as canthaxanthin, echinenone and esterified forms of astaxanthin could be also identified in the extracts. Their presence could be related to the ability of microalgae to synthesize under unfavourable culture conditions certain amounts of a complex mixture of secondary carotenoids [16]. Regarding esterified forms of astaxanthin, typical fragmentation pattern of carotenoids fatty acids monoesters and diesters were obtained for several peaks since in their MS and MS^2 spectrums, it was possible to observe not only the quasimolecular ion ([M+H]+) but also the fragment corresponding to the loss of a fatty acid ([M+H-FA+H20]+).

Once the extracts were chemically characterized, the quantification of the identified carotenoids was carried out by LC-DAD. Figure 1 show the carotenoids profile obtained at 100 ºC using 100 % ethanol. It is important to highlight that important differences were observed in the chromatographic profile when 100 % of ethanol or limonene were used that basically depend on their distinct polarity. These differences were mainly observed in the first part of the chromatogram where a higher proportion of polar compounds were obtained when ethanol was used as extraction solvent, demonstrating the different selectivity of both solvents.

To overcome the limitation imposed by the lack of commercial standards for some carotenoids, their quantification was done using as standards those with spectral and structural similarities. Thus, violaxanthin, lutein, canthaxanthin, and echinenone were quantified using the calibration curve of lutein; zeaxathin, carotenoids monoester and β-carotene using the calibration curve of β-carotene; and monoesters and diesters were quantified using, respectively, astaxanthin monopalmitate and astaxathin dipalmitate calibration curves. Besides, a molecular-weight-corrections factor (determined by dividing the molecular weight of the carotenoid to be quantified by that of the standard) was applied to consider the difference in detector response.

Considering the results obtained for the total amount of carotenoids along with those obtained for the extraction yields (Table 1), the statistical treatment of the experimental design was performed. By analyzing the standardized Pareto charts it can be deduced that the interaction between temperature and solvent composition is the term that mostly influenced the extraction yield whereas the quadratic effect of the solvent composition was the most important term in the total amount of carotenoids. After excluding from the model those terms not significantly different from zero ($P > 0.05$), i.e the quadratic effect of temperature in the total amount of carotenoids, the mathematical model was refitted by MLR. Values of 0.944 for the yield and 0.970

for the amount of carotenoids were obtained for the determination coefficients (R^2 which indicates the variability of the response variable explained by the model), the RSD of the fit and RRSD (measure of the relative error of the fit and are expressed as percentage of the mean value of the response) for both response variables were below 1.3 and 1.6 % respectively. These results show that the estimated model was adequate enough to describe the data (P-value of lack-of-fit test higher than 0.05).

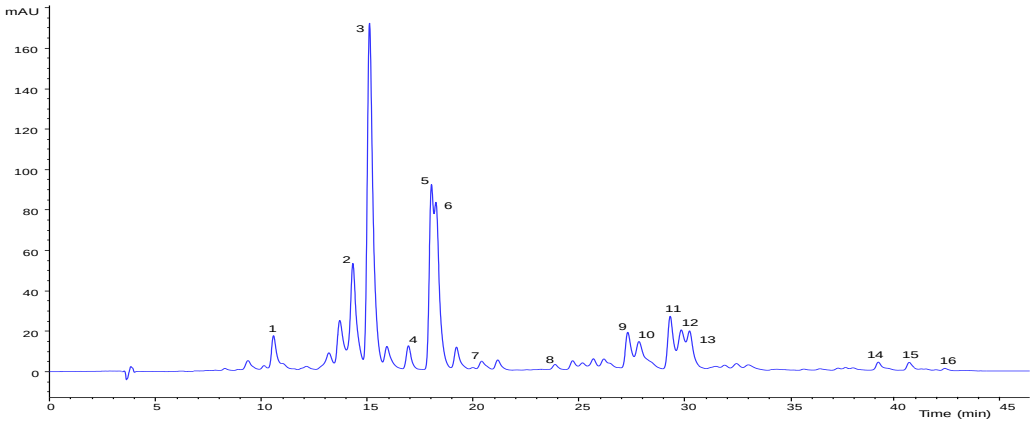

Figure 1. Chromatogram (450 nm) obtained from the LC-DAD analysis of *N. oleoabundans* extract obtained at 100 ºC using 100% ethanol as extracting solvent. Peak identification: 1, violaxanthin; 2, chlorophyll b; 3, lutein; 4, zeaxanthin; 5, cantaxanthin; 9, chlorophyll a; 7, related to cantaxanthin; 8, echinenone; 9, carotenoid monoester; 10, astaxanthin monoester $_{C18:4}$; 11, carotenoid monoester; 12, astaxanthin monoester $C_{18:3}$, 13, β-carotene; 14, astaxanthin diester $C_{16:0}/C_{18:1}$; 15, astaxanthin diester $C_{16:0}/C_{16:0}$; 16, carotenoid diester.

The surface plots obtained for both response variables as a function of temperature and solvent composition (as % of limonene in the mixture) are shown in Figure 2.

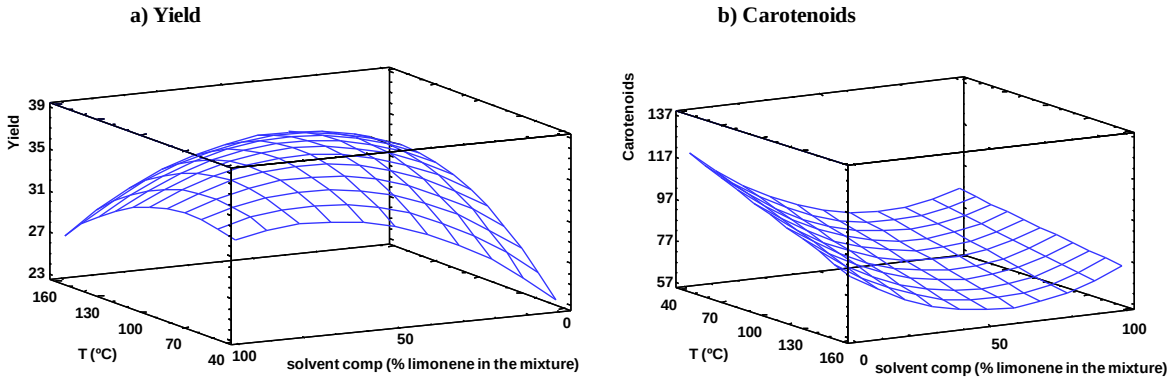

Figure 2. Response surface plots of the response variables depending on the extraction temperature and solvent composition (as % of limonene in the mixture).

The statistical software defined 109 ºC and 51 % of limonene as the optimum values for temperature and solvent composition to obtain the higher extraction yield (see extraction yield surface plot). Regarding the amount of carotenoids, the surface plot shows an increase in the response by decreasing both the temperature and the percentage of limonene, so the lower levels of the factors (i.e. 40 ºC and 0 % limonene) were predicted by the model as the optimum values to obtain the maximum amount of carotenoids. Bearing in mind the different values of temperature and solvent composition predicted to each response variable, a multiple response optimization was performed to find the values of both experimental factors which enabled to obtain simultaneously the maximum yield and amount of carotenoids considering both responses equally important. Thus, the optimum level factors were 112 ºC as extraction temperature and 0 % limonene (thus 100% ethanol) as solvent composition. Under these conditions, the values predicted by the model were around 32 % of extraction yield and 98.8 mg carotenoids/g extract with overall desirability value of 0.6542. Comparing these

results with those obtained under the experimental conditions closer to the optimum (see Table 1, run 3), it can be seen that the values predicted by the model and the experimental values were very close.

Considering both the extraction yield and the carotenoid content in the extracts, carotenoids present in *N. oleoabundans* were around 29 mg/g dry weigh, value that is in the range of those reported in the literature for other microalgae species which are considered valuable sources for obtaining carotenoids.

4. Conclusions

The use of food-grade solvents as ethanol and limonene has shown great potential to carry out the PLE extraction of carotenoids from *N. oleoabundands*. By means of an experimental design, the extraction temperature and solvent composition were optimized to obtain simultaneously the maximum extraction yield and total amount of carotenoids. Besides, the combination of the data obtained from the analysis of the extracts by LC-DAD and LC-MS enabled, for the first time, a tentative identification of different carotenoids present in the *N. oleoabundands* extracts under certain growing conditions. The results obtained show that the main carotenoids accumulated in this microalga are lutein, cantaxanthin, zeaxanthin, and mono and diester of astaxanthin, among others, demonstrating that *N. oleoabundands* can be considered as a novel potential source of natural carotenoids.

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