**OPTIMIZATION OF CLEAN EXTRACTION METHODS TO ISOLATE
CAROTENOIDS FROM NEOCHLORIS OLEOABUNDANS. CHEMICAL
CHARACTERIZATION BY LIQUID CHROMATOGRAPHY TANDEM
MASS SPECTROMETRY.**

**María Castro-Puyana¹, Miguel Herrero¹, I. Urreta², Jose A. Mendiola¹, Alejandro
Cifuentes¹, Elena Ibáñez^{1*}, Sonia Suárez-Alvarez²**

¹Laboratory of Foodomics. Bioactivity and Food Analysis Department. Institute of Food
Science Research (CIAL-CSIC); Nicolás Cabrera 9, Campus UAM Cantoblanco, 28049
Madrid, Spain.

²Neiker Tecnalia, Biotechnology Department, Arkaute's Agrifood Campus, 01080 Vitoria-
Gasteiz, Alava, Spain.

Corresponding author: Prof. Elena Ibáñez, elena@ifi.csic.es

Tel: +34 910 017 956

Fax: +34 910 017 905

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oleoabundans*. experimental design, limonene.

Abstract

In this work, an experimental design has been used to optimize the extraction of carotenoids from *Neochloris oleoabundans* using pressurized liquid extraction with food-grade solvents such as ethanol and limonene. To the best of our knowledge, this is the first application of carotenoids extraction from this innovative green microalga. Experimental factors such as extraction temperature and solvent composition (different % of limonene in ethanol) were optimized by means of three-level factorial design using as responses variables the extraction yield and total amount of carotenoids in the extract. The statistical analysis of the results provided mathematical models to predict the behavior of the responses as a function of the factors involved in the process. Thus, the optimum conditions predicted by the model to reach simultaneously the maximum values of both response variables pointed out 116 °C as extraction temperature and 100 % ethanol as extracting solvent. Moreover, the chemical characterization of the obtained extracts was carried out by means of high-performance liquid chromatography-tandem mass spectrometry. Results obtained demonstrated that, under certain cultivation conditions, *N. oleoabundans* is able to accumulate different amount of carotenoids, mainly lutein, cantaxanthin, zeaxanthin, and mono and diester of astaxanthin, among others.

1. Introduction.

Nowadays, one of the main interests in Food Science and Technology is the extraction and characterization of new bioactive compounds that can be used as functional ingredients able to promote our health. These ingredients are preferred to have a natural origin, such as plants, algae or microalgae. In this sense, the potential of microalgae as source of compounds with functional properties has been already demonstrated [Herrero M, Cifuentes A, Ibáñez E (2006) Food Chem 98:136-148***Chacón-Lee TL, González-Mariño GE (2010) Comprehensive Rev Food Sci and Food Safety 9:655-675***Plaza M, Cifuentes A, Ibáñez E (2008) Trends in Food Sci and Technol 19:31-39*** Plaza M, Herrero M, Cifuentes A, Ibáñez E (2009) J Agric Food Chem 57:7159-7170].

Microalgae comprise a complex and heterogeneous group of organisms characterized by being photosynthetic organisms that possess simple reproductive structures. Their huge diversity in terms of number of different species makes the microalgae an almost unlimited field of application in the search for bioactive compounds. Their sometimes unique chemical structures and their ability to work as natural bioreactors potentiating the synthesis of valuable compounds depending on the cultivation conditions or through biotechnology approaches [Miguel Herrero, Jose A. Mendiola, María Castro-Puyana, Elena Ibáñez, Extraction and characterization of bioactive compounds with health benefits from marine resources: Macro and Micro Algae, Cyanobacteria and Invertebrates. In Marine Bioactive Compounds, M. Hayes, Ed., Springer Science+Business Media, LLC, USApp. 55-98, ISBN: 978-1-4614-1246-5]. Thus, marine microalgae constitute a natural source of a high variety of compounds which encompass carotenoids [Guedes AC, Amaro HM, Malcata FX (2011) Mar Drugs 9:625-644]. These are a family of pigmented compounds whose structure is formed by eight isoprenoid units constituting a symmetrical skeleton

with a long chain with conjugated double bonds. The importance of carotenoids is not only limited to their well-known antioxidant properties, but also it is due to the beneficial health properties. Bioactivities like prevention of cancer [Silberstein JL, Parsons JK (2010) *Curr Nutr Food Sci* 6:2-12], cardiovascular diseases [Riccioni G, Mancini B, Di Ilio E, Bucciarelli T, D'Orazio N (2008) *Eur Rev Med Pharmacol Sci* 12:183-190] or macular degeneration [Snodderly M (1995) *Am J Clin Nutr* 62:S1448-S1461] have been attributed to different carotenoids. Microalgal biotechnology has advanced considerably and it is possible to produce some carotenoids commercially through aquaculture. For instance, *Dunaliella salina* is able to accumulate high amounts of β -carotene when submitted to particular growing conditions [Zhu YH, Jiang JG (2008) *Eur Food Res Technol* 227:953-959] and *Haematococcus pluvialis* is the major producer of astaxanthin, being able to selectively accumulate this carotenoid up to 5% of its dry weight [Yuan JP, Chen F (2000) *Food Chem* 68:443-448]

An important aspect to be considered when dealing with the extraction of compounds from natural matrices such as microalgae is the development of appropriate, fast, cost-effective and environmental-friendly extraction processes able to isolate the compounds of interest. To this aim, the use of advanced extraction techniques is very interesting compared to conventional methodologies. In this sense, 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 been already demonstrated [Jaime L, Rodríguez-Meizoso I, Cifuentes A, Santoyo S, Suarez S, Ibáñez E, Señorans FJ (2010) *LWT-Food Sci Technol* 43:105-112***Herrero M, Jaime L, Martín-Álvarez PJ, Cifuentes A, Ibáñez E (2006) *J Agric Food Chem* 54:5597-5603***Plaza M, Santoyo S, Jaime L, Avalo B, Cifuentes A,

Reglero G, García-Blairsy Reina G, Señorans FJ, Ibáñez E (2012) LWT-Food Sci Technol 46:245-256***Denery JR, Dragull K, Tang CS, Li QX (2004) Anal Chimica Acta 501:175-181***Jaime L, Mendiola JA; Herrero M, Soler-Rivas C, Santoyo S, Señorans FJ, Cifuentes A, Ibáñez E (2005) J Sep Sci 28:2111-2119]. This extraction technique is based on the extraction at temperature and pressure high enough to maintain the extracting solvent in the liquid state during the whole process [Mendiola JA, Herrero M, Cifuentes A, Ibáñez E (2007) J. Chromatogr. A 1152:234-246]. It enabled to obtain higher extraction yields in a shorter period of time and using a significant lower amount of solvent than conventional extraction techniques.

Another important aspect that has to be closely considered is the chemical characterization of the compounds obtained after extracting. In this regard, it is necessary the use of advanced analytical tools able to identify each one of the compounds obtained in a chromatographic profile. Among the analytical techniques, High-performance liquid chromatography hyphenated to mass spectrometry (LC-MS) has been successfully employed to carry out the identification and structural characterization of different carotenoids extracted from *Haematococcus pluvialis* [Miao F, Lu D, Li Y, Zeng M (2006) Anal Biochem 352:176-181***Frassanito R, Cantonati M, Flaim G, Mancini I, Guella G (2008) Rapid Communications in Mass Spectrometry 22:3531-3539***Holtin k, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K (2009) 395:1613-1622].

In this work, *Neochloris Oleabundans* is studied for the first time as an alternative source of carotenoids. Most existing literature related to this microalga is focused on its ability to produce lipids. In fact around 80 % of its total lipids are triglycerides, and the most of its fatty acids are saturated fatty acids in the range of 16-20 carbons what it is ideal for biodiesel production [Tornabene TG, Holzer G, Lien S, Burris N (1983) Enzyme Microb

Technol 5:435-440***Gouveia L, Oliveira AC (2009) J Ind Microbiol Biotechnol 36:256-
274***Beal CM, Webber ME, Ruoff RS, Hebner RE (2010) Biotechnol and Bioeng
106:573-583***Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008) Appl Microbiol
Biotechnol 81:629-639*** Gatenby CM, Orcutt DM, Kreeger DA, Parker BC, Jones VA,
Neves RJ (2003) J Appl Phycol 15:1-11]. Lately, Goiris et al. have investigated the
contribution of phenolic and carotenoids substance to antioxidant activity in a series of
extract from different algal sample, being *N. Oleabundans* one of them. In this work, the
carotenoid content was estimated spectrophotometrically and the chemical characterization
of the extract was no carry out [Goiris K, Muylaert K, Fraeye I, Foubert I, De Brabanter J,
De Cooman L (2012) J Appl Phycol in press (DOI: 10.1007/s10811-012-9804-6)].
The aim of this work was to optimize, by means of an experimental design, the PLE
extraction of carotenoids from *N. Oleabundans* using GRAS solvent under different
extraction temperature. Besides, the different carotenoids extracted were characterized by
the application of a LCMS methodology. To best of our knowledge, this is the first time
that PLE and LCMS have been used to extract and characterize the carotenoid profile of *N.*
Oleabundans.

2. Material and methods.

2.1 Samples and chemicals.

Sodium hydroxide and ethanol were obtained from Panreac Quimica S.A (Barcelona,
Spain). Hydrochloric acid was acquired from Merck (Darmstadt, Germany). Methyl tert-
butyl ether (MTBE), methanol, acetone, and hexane were from LabScan (Gliwice, Poland).
Sea sand was supplied by VWR (Leuven, Belgium). Butylated hydroxytoluene (BHT),
limonene and standard samples of β -carotene, lutein, chlorophyll a (from *anacystis*

nidulans algae), and chlorophyll b (from spinach) were obtained from Sigma-Aldrich (St Louis, MO, USA). Astaxanthin monopalmitate and astaxanthin dipalmitate were obtained from CaroteNature (Lupsingen, Switzerland). The water used was Milli-Q Water (Millipore, Billerica, MA, USA).

Neochloris Oleoabundans (UTEX#1185) was obtained from the Culture Collection of Algae at the University of Texas (Austin, USA). Batch cultures were grown in 8 cm wide glass reactors containing 1 litre of modified Bold's Basal Medium [Andersen RA, Berges JA, Harrison PJ, Watanabe MM (2005) in: Andersen RA (ed) *Algal Culturing Techniques*. Elsevier, Amsterdam, pp. 429-538] supplemented with 0.3 g l⁻¹ of KNO₃ and subjected to continuous stirring by bubbling air. Pure CO₂ was supplied each 30s every 10 min to the air stream in order to provide inorganic carbon and keep the pH value below 8, using an electronic gas-control valve (Wilkerson R03-C2). Reactors were maintained in a culture chamber at 24 ± 2 °C, with a 16:8 h light: dark photoperiod supplied with fluorescent light (Philips TLD 58W) at a photosynthetic photon flux density of 400 mmol photons m⁻² s⁻¹. After cells reached the late exponential phase biomass was harvested by centrifugation (7000 rpm for 5 min at 10°C), pre-frozen at -20°C and freeze-dried at -40 °C for 48 hours and stored under dry and dark conditions until use.

2.2 Treatment of alga previous extraction.

Four different pretreatments of the microalga to breakdown the cell wall and obtain the highest extraction yield were studied:

- (a) 3 g of sample were suspended in water (8 mL), followed by three freezing-thawing cycles carried out in a – 20 °C freezer.

(b) 3 g of sample were suspended in 0.1 N HCl (10 mL), followed by mixing at 450 rpm in a Thermomixer comfort (Eppendorf Ibérica, Madrid, Spain) during 10 min at 70 °C.

(c) 3 g of sample were suspended in 0.1 M NaOH (10 ml), followed by mixing at 450 rpm in a Thermomixer comfort (Eppendorf Ibérica, Madrid, Spain) during 15 min at 25 °C.

(d) 2.5 g of sample were treated by three cycles of cryogenic grinding using a Mixer mill CryoMill (Retsch, Haan, Germany). Three steps were carried out in each cycle: pre-cooling (frequency 1/s = 5 during 2 min), grinding (frequency 1/s = 20 during 3 min) and intermediate cooling (frequency 1/s = 5 during 1 min).

In procedures a, b, and c, the samples were centrifuged afterwards at 5200 rpm for 5 min at 5 °C. The supernatants were removed and the residual samples were frozen at – 20 °C and dried by freeze drying.

2.3 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 and 2 center points to consider the experimental errors) were carried out in randomized order. The two factors tested at three different levels in this design were: extraction temperature at 40, 100 and 160 °C, and % limonene at 0, 50, and 100 %. The response variables selected were extraction yield (determined as dry weight/initial weight expressed in %) and total amount of carotenoids (expressed as mg carotenoids/g extract). The quadratic model proposed for each response variable (Y_i) was:

$$Y_i = \beta_0 + \beta_1 \text{Temp} + \beta_2 \text{lim} + \beta_{1,1} \text{Temp}^2 + \beta_{1,2} \text{Temp} * \text{lim} + \beta_{2,2} \text{lim}^2 + \text{error} \quad (\text{equation 1})$$

where β_0 is the intercept, β_1 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 Plus v. 5.1 program which permits both the creation and the analysis of experimental designs. 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 goodness of fit of the model 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.

2.4 Extractions methodologies.

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 above 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 °C and 100 °C, 8 min at 160 °C). 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.

The procedure of conventional extraction from *N. Oleoabundans* was carried out taking into account the protocols described previously by Cha *et al.* [Cha KH, Koo SY, Lee D-U (2008) J Agric Food Chem 56:10521-10526] and Sarada *et al.* [Sarada R, Vidhyavathi R, Usha D, Ravishankar GA (2006) J Agric Food Chem 54:7585-7588] with some modifications. Briefly, 200 mg of sample was diluted with 20 mL acetone containing 0.1 % (w/v) BHT. Then, the sample was shaken for 3h (at 20 °C and 452 rpm), and centrifuged for 10 min at 5000 rpm (4 °C) to precipitate the solids. The supernatant was collected, filtrated and evaporated to dryness using nitrogen purging. For LC analysis, the residue was redissolved in ethanol.

2.5 Quantification of carotenoids by LC-DAD.

HPLC analyses of the extract were carried out employing an Agilent HP 1100 series (Agilent Technologies, CA, USA) equipped with a DAD, and using a YMC-C₃₀ reversed-phase column (250 mm x 4.6 mm id, 5 µm particle size, YMC Europe, Schermbeck, Germany). 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 amounts 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 amounts of astaxanthin monopalmitate and dipalmitate (ranging

from 0.04 to 6.25×10^{-4} mg/mL), chlorophyll a and chlorophyll b (ranging from 0.2 to 3.13×10^{-3} mg/mL) were injected into the LC-DAD instrument. Each standard was dissolved from a stock solution (1-2 mg/mL) with different solvents, i.e. lutein, chlorophyll a, chlorophyll b, were dissolved in ethanol, astaxanthin monopalmitate and astaxanthin dipalmitate in hexane:acetone (1:1 v/v), and β -carotene in hexane. The linear regression equation for each standard curve was obtained by plotting the amount of standard compound injected against the peak area. The regression equation and the correlation coefficient (r^2) were obtained and results are shown in **Table 1**.

2.6 LC-MS characterization of *N. Oleoabundans* extracts.

The instrument employed to chemically characterize the extracts obtained at the different extraction conditions tested was an Agilent 1200 liquid chromatograph (Agilent Technologies, CA, USA) equipped with a DAD and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. To carry out the analyses, a YMC-C30 reversed-phase column (250 mm x 4.6 mm id, 5 μ m particle size, YMC Europe, Schermbeck, Germany) was used employing as mobile phases 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 μ L, and detection was at 450 and 660 nm and the DAD recorded the spectra from 240 to 770 nm. Regarding MS analysis, it was carried out under APCI positive ionization mode using the following parameters: capillary voltage, -3.5 kV; dry temperature, 350 $^{\circ}$ C; vaporizer temperature, 400 $^{\circ}$ C; dry gas flow, 5 L/min; corona's current, 4000 nA; nebulizer

gas pressure, 60 psi. A range from 150 to 1300 m/z was acquired.

3. Results and discussion.

3.1 Selection of the pretreatment to induce cell-wall lysis.

The first step to achieve an efficient extraction from the microalgae was to break the cell wall since it can hinder the extraction and availability of compounds. Thus, the effect of four different pretreatments, such as acid and basic hydrolysis, cryogenic grinding, and freezing-thawing, to induce cell-wall lysis was investigated in order to obtain not only the highest possible extraction yields but also the maximum amount of carotenoids extracted. To compare the results obtained from each treatment, the extraction conditions under PLE conditions were fixed to 100 % ethanol at 100 °C (1500 psi, 20 min) according to a previous work from our research group [Jaime L, Rodríguez-Meizoso I, Cifuentes A, Santoyo S, Suarez S, Ibáñez E, Señorans FJ (2010) LWT-Food Sci Technol 43:105-112-REP].

Among the four methods tested, the higher extraction yields (calculated as dry weight/initial weight expressed in %) were obtained by treating the sample under freezing-thawing or cryogenic grinding, as shown **Table 2**. Regarding the amount of carotenoids extracted, a preliminary quantification of carotenoids (using β -carotene equivalent to quantify and expressing the results as mg β -carotene/g extract) was carried out considering all those chromatographic peaks whose UV spectra could be assigned to a carotenoid [Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoids Handbook. Birkhäuser, Basel (Switzerland)]. As it can be observed in **Table 2**, the higher amount of carotenoids was

obtained when a treatment of cryogenic grinding was used. Based on these results, this method was selected as treatment previous to PLE extraction.

In order to test the interest of optimizing PLE as a useful alternative to conventional extraction procedures, a conventional solvent extraction with acetone (under conditions previously published by Cha *et al.* [Cha KH, Koo SY, Lee D-U (2008) J Agric Food Chem 56:10521-10526-REP] and Sarada *et al.* [Sarada R, Vidhyavathi R, Usha D, Ravishankar GA (2006) J Agric Food Chem 54:7585-7588-REP]) was also performed. Cryogenic grinding was also selected as pretreatment of the sample. Both, extraction yield and amount of carotenoids were lower than those obtained using PLE (see **Table 2**), thus demonstrating the interest of this environmental friendly extraction technology to produce extracts enriched in carotenoids from microalgae.

3.2. Optimization of PLE conditions and chemical characterization by LC-MS.

Once selected the pretreatment previous to PLE extraction, a three-level factorial design was performed to optimize the extraction temperature and the solvent composition using as responses variables the extraction yield and the total amount of carotenoids in the extract. Regarding solvent composition, different percentages of limonene in the mixture were tested; limonene is a green biodegradable solvent that has been suggested as a good alternative to hexane for lipid extraction since it possess a dielectric constant very close to this toxic organic solvent [Virot M, Tomao V, Ginies C, Visinoni F, Chemat F (2008) J Chromatogr A, 1196-1197:147-152]. In fact, limonene has been previously used to extract non-polar substances such as oils from matrices as rice brand [Mamidipally PK, Liu SX (2004) European J Lipid Sci Technol 106:122-125***Liu SX, Mamidipally PK (2005) Cereal Chemistry 82:209-215] olive residues [Virot M, Tomao V, Ginies C, Chemat F

(2008) *Chromatographia* 68:311-313] and microalgae [Tanzi CD, Vian MA, Ginies C, Elmaataoui M, Chemat F (2012) *Molecules* 17:8196-8205], or carotenoids from tomatoes [Chemat-Djenni Z, Ferhat MA, Tomao V, Chemat F (2010) *J Essential Oil-Bearing Plants* 13:139-147]. On the other hand, in previous works we demonstrate the ability of pressurized ethanol to extract carotenoids from different microalgae [Herrero M, Jaime L, Martín-Álvarez PJ, Cifuentes A, Ibáñez E (2006) *J Agric Food Chem* 54:5597-5603**Jaime L, Rodríguez-Meizoso I, Cifuentes A, Santoyo S, Suarez S, Ibáñez E, Señorans FJ (2010) *LWT-Food Sci Technol* 43:105-112***Jaime L, Mendiola JA; Herrero M, Soler-Rivas C, Santoyo S, Señorans FJ, Cifuentes A, Ibáñez E (2005) *J Sep Sci* 28:2111-2119*** Plaza M, Santoyo S, Jaime L, Avalo B, Cifuentes A, Reglero G, García-Blairsy Reina G, Señorans FJ, Ibáñez E (2012) *LWT-Food Sci Technol* 46:245-256 (YA ESTA TODAS)]. Therefore, it is expected that a combination of both green solvents with their particular properties would favor the extraction of carotenoids from *N. oleabundans*. **Table 3** shows the experimental matrix design with the levels of the experimental factors along with the results obtained for the two responses analyzed.

Figure 1 depicts two chromatograms of the carotenoids profile obtained at 100 °C using 100 % ethanol (Figure 1.A) and 100 % limonene (Figure 1.B), corresponding to experiments 3 and 8 of **Table 3**. As can be seen, important differences were observed in the chromatographic profile of pigments extracted with the two different solvents that basically depend on their distinct polarity. The main differences are observed in the first part of the chromatogram (see **Figure 1**), where a higher proportion of polar compounds is obtained when ethanol is used as extraction solvent, demonstrating the different selectivity of both solvents.

In order to obtain a more in depth information about the extract composition to quantify the carotenoids and to use this data as response variable for the experimental design, *N. Oleoabundans* extracts were analyzed by LC(APCI)MS. Thus, a tentative identification of different carotenoids was carried out combining the information provided by the two detectors (i.e. DAD and MS) with the use of commercial standard and the data found in the literature. Information about characteristic UV maxima, $[M+H]^+$ and the main fragments obtained by MS for the different detected pigments is shown in **Table 4**. As it can be observed, from the twenty peaks whose UV spectra pointed out to pigment compounds, two chlorophylls and eleven (free or diester) carotenoids could be identified by MS. Since LC(APCI)MS was performed in positive ion mode, free pigments were detected as quasimolecular ion at $[M+H]^+$, except lutein (peak 5) whose $[MH-H_2O]^+$ ion was obtained as main fragment. It is important to highlight that, as it has been mentioned in the introduction, the studies described in the literature about *N. Oleoabundans* has been mainly focused on the extraction of oil and the analysis of lipid [Gouveia L, Oliveira AC (2009) J Ind Microbiol Biotechnol 36:256-274***Beal CM, Webber ME, Ruoff RS, Hebner RE (2010) Biotechnol and Bioeng 106:573-583***Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008) Appl Microbiol Biotechnol 81:629-639*** Gatenby CM, Orcutt DM, Kreeger DA, Parker BC, Jones VA, Neves RJ (2003) J Appl Phycol 15:1-11 (YA ESTAN TODAS)] so that, to the best of our knowledge, this is the first study in which the carotenoids of *N. oleabundans* extracts has been tentatively identify.

Among the carotenoids of *N. oleabundans* extracts, it was possible to the identify β -carotene (peak 17), lutein (peak 5), violaxanthin (peak 2), chlorophyll a (peak 9) and chlorophyll b (peak 4), that have been described as the major carotenoids in chlorophycean

algae, as well as other minor carotenoids as zeaxanthin (peak 7) [Takaichi S (2011) Mar Drugs 9:1101-1118***Plaza M, Herrero M, Cifuentes A, Ibáñez E (2009) J Agric Food Chem 57:7159-7170-REP]. Between them, lutein was shown in higher proportion as occurs in other green microalgae as *Chlorella vulgaris* [Cha KH, Koo SY, Lee D-U (2008) J Agric Food Chem 56:10521-10526-REP]. Thus, lutein was the main carotenoid accumulated from *N. oleabundans*.

Along with these primary carotenoids, others secondary as canthaxanthin, echinenone and esterified forms of astaxanthin could be also identified in the extracts (see Table 4). Their presence could be related to the ability of some microalgae (among them, *Neochloris Wimmeri*, one specie from the same family of *N. Oleoabundans*) to synthesize, under unfavourable culture conditions, certain amount of a complex mixture of secondary carotenoids [Orosa M, Torres E, Fidalgo, Abalde J (2000) J. Appl Phycol 12:553-556*** Orosa M, Valero JF, Herrero C, J. Abalde (2001) Biotechnol Letters 23:1079-1085]. Regarding peak 10, it was assigned as “related to canthaxanthin” taking into the account their UV and MS characteristics as well as the information obtained from the literature in which is described the presence of canthaxanthin and *cis*-canthaxanthin in green microalgae [Yuan J-P, Chen F, Liu X, Li X-Z (2002) Food Chem 76:319-325].

The accumulation of astaxanthin in the form of di- or monoesters has been described in other green microalgae such as *Neochloris Wimmeri* [Orosa M, Torres E, Fidalgo, Abalde J (2000) J. Appl Phycol 12:553-556*** Orosa M, Valero JF, Herrero C, J. Abalde (2001) Biotechnol Letters 23:1079-1085-YA ESTAN] or *Haematococcus pluvialis* [Miao F, Lu D, Li Y, Zeng M (2006) Anal Biochem 352:176-181***Frassanito R, Cantonati M, Flaim G, Mancini I, Guella G (2008) Rapid Communications in Mass Spectrometry 22:3531-3539***Holtin k, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K (2009)

395:1613-1622***Jaime L, Rodríguez-Meizoso I, Cifuentes A, Santoyo S, Suarez S, Ibáñez E, Señorans FJ (2010) LWT-Food Sci Technol 43:105-112-YA ESTAN] but only in the second they have been characterized. In the *N. Oleabundans* extracts studied in this work, a typical fragmentation pattern of carotenoids fatty acids monoesters was obtained for peaks 13-16. In the MS and MS² spectrums of these compounds, it was possible to observe not only the quasimolecular ion ([M+H]⁺) but also the fragment corresponding to the loss of fatty acid ([MH-FA+H₂O]⁺). Thus, peaks 14 and 16 were tentatively assigned to astaxanthin monoesters C18:4 and C18:3 respectively. Both monoester has been described previously in *Haematococcus pluvialis* [Miao F, Lu D, Li Y, Zeng M (2006) Anal Biochem 352:176-181-REP]. Unfortunately, the monoester corresponding to peaks 13 and 15 could not be assigned to a specific carotenoid what helps to understand the great difficulties related to the carotenoid identification. Regarding astaxanthin diesters, two different compounds were also identified in the extracts, astaxanthin diester (C16:0, C18:1) and astaxanthin diester (C16:0, C16:0) whose [M+H]⁺ are 1099 *m/z* and 1073 *m/z*, respectively. The tentative identification of both diester was carried out taking into account both their quasimolecular ions and the fragments obtained from their fragmentation pattern, which are shown in **Table 4**.

After chemical characterization, quantification of the identified carotenoids was carried out. To overcome the limitation imposed by the lack of commercial standards for some carotenoids, their quantification was done using as standards those with the closest chemical structure. For instance, violaxanthin (peak 2), lutein (peak 5), zeaxanthin (peak 7), canthaxanthin (peaks 8 and 10), and echinenone (peak 12) were quantified using the calibration curve of lutein, while β-carotene (peak 17), monoesters (peaks 13-16) and

diesters (peaks 18-20) were quantified using, respectively, β -carotene, astaxanthin monopalmitate and astaxanthin dipalmitate calibration curves. This more accurate quantification enabled to obtain the values corresponding to the total amount of carotenoids in the extracts. Considering these results along with those obtained for the extraction yields (see **Table 3**), the statistical treatment of the experimental design was performed.

Figure 2 shows the standardized Pareto charts for the two response variables evaluated, illustrating the importance and the statistical significance of each term in the model. Different bars shadings indicate positive and negative effects of the factors in the response variables and the vertical line tests the significance of the effects at the 95 % confidence level. From this figure, it can be deduced that the term that mostly influence the extraction yield is the interaction T x solvent composition whereas the quadratic effect of the solvent composition was the most important term in the total amount of carotenoids extracted. Those terms in the equation not significantly different from zero ($P > 0.05$) were excluded from the model and the mathematical model was refitted by MLR. Results obtained are listed in Table 5, which also includes statistics values for goodness of fit of the model. From these results, the following conclusions can be drawn: (i) the determination coefficient (R^2), which indicates the variability of the response variable explained by the model, was 0.944 for the yield and 0.968 for the amount of carotenoids extracted, (ii) the RSD of the fit for both response variables was below 1.0, (iii) the RRSD values (that provide a measure of the relative error of the fit and are expressed as percentage of the mean value of the response ($RRSD (\%) = RSD/\bar{Y} \times 100$)) were below 2 %. Therefore, the estimated model was found to be adequate enough to describe the data (P -value of lack-of-fit test higher than 0.05).

Figure 2 also shows the surface plots obtained for both response variables as a function of temperature and solvent composition (as % of limonene in the mixture). By analyzing the plot for the extraction yield, it can be seen that the optimum values for temperature and solvent composition that enable to obtain the higher extraction yield, can be found around the intermediate values of both factors in the experimental study. In fact, the statistical programme define a temperature of 109 °C and a percentage of limonene of 51 % to maximize the yield (optimum calculated yield = 35.9 %). On the other hand, the analysis of the surface plots for the amount of carotenoids shows an increase in the response by decreasing not only the temperature but also the limonene content in the solvent mixture. Thus, the lower experimental levels of the factors (40 °C and 0 % limonene) are predicted by the statistical program as the optimum values to obtain the maximum amount of carotenoids (optimum calculated carotenoids = 63.8 mg carotenoids/g extract). This result can be correlated with the above-mentioned fact that a higher fraction of polar carotenoids was obtained employing ethanol as solvent.

From the obtained results it seems quite difficult to optimize both response variables at the same time. To reach a compromise between them, a multiple response optimization was performed in order to find the values of temperature and solvent composition which enabled to obtain simultaneously the maximum yield and the maximum amount of carotenoids. To do that, both response variables were considered equally important (weight factor and impact were set at 1.0 and 3.0, respectively). Applying this methodology, the optimum level of the factors was obtained: 116 °C was the optimum extraction temperature while 0 % limonene (thus 100% ethanol) was the optimum solvent composition. Under these conditions, the values predicted by the model were around 32 % of extraction yield and 53.4 mg carotenoids/g extract with overall desirability value of 0.6682. Comparing

these results with those obtained under the experimental conditions closer to the optimum (run 3, 100 °C and 0% limonene, see **Table 3**), it can be seen that the values predicted by the model and the experimental values were very close.

4. Concluding remarks

In this work, PLE with GRAS solvents has shown their potential to extract carotenoids from *N. oleoabundands*. The optimization of the extraction process was carried out by means of an experimental design in which the effect of experimental factor, such as extraction temperature and solvent composition were investigated. According to the mathematical model, maximum values of extraction yield and total amount of carotenoids in the extract could be simultaneously obtained using as extracting solvent 100 % ethanol at 116 °C as extracting temperature.

Combination the data obtained from the analysis of the extracts by LC-DAD and LC-MS was possible to carry out a tentative identification of different carotenoids present in the *N. oleoabundands* extracts, so that under cetartain cultivation conditions, the main carotenoids accumulated in this microalga were lutein, cantaxanthin, zeaxanthin, and mono and diester of astaxanthin, among others.

The results obtained in this work have demonstrated for the first time that the microalga *N. oleoabundands* can be considered as a novel potential source of natural carotenoids. Due to the carotenoids content and composition are influences by culture conditions, more in depth investigations about the capacity of this microalga to accumulate carotenoids under different environmental parameters are being currently carry out.

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Figure captions

Figure 1 LC-DAD chromatogram (456 nm) of a 100% ethanol extract (A) and of a 100 % limonene extract (B). Both were obtained at 100 °C. For peak identification see Table 4.

Figure 2 Standarized Pareto charts with the effect of each term in the model and response surface plots of the two response variables depending on the extraction temperature and solvent composition (as % of limonene in the mixture).

Table 1. Linear regression equations of the carotenoids and chlorophylls standards.

Compound	Linear regression equation	Correlation coefficient (r^2)
Lutein	$Y = 154641x - 59.765$	0.9999
Chlorophyll b	$Y = 13.780x - 14.834$	0.9999
Chlorophyll a	$Y = 45477x - 64.107$	0.999
β -carotene	$Y = 4286.9x - 82.227$	0.9985
Astaxathin monopalmitate	$Y = 92274x + 15.379$	1
Astaxathin dipalmitate	$Y = 28658x + 2.894$	1

Table 2. Extraction yields and amount of carotenoids obtained after different pretreatments previous to extraction.

Pretreatment/Extraction method	Extraction yield (%)	mg β -carotenoids/g extract
Freezing-thawing/PLE	39.4	162.7
Cryogenic grinding/PLE	32.6	176.1
Acid hydrolysis/PLE	26.6	142.6
Basic hydrolysis/PLE	12.6	62.0
Cryogenic grinding/conventional extraction	28.3	110.2

Table 3. Experimental matrix design and result obtained for each response variable studied.

Exp.	Temperature (°C)	Solvent composition (% limonene in the mixture)	Extraction yield (%)	mg carotenoids/g extract
1	160	0	33.7	49.0
2	40	100	31.0	44.8
3	100	0	30.1	52.2
4	40	50	33.4	40.9
5	40	0	23.4	65.7
6	100	50	36.1	36.5
7	100	50	35.3	36.8
8	100	100	34.1	44.6
9	100	50	35.6	38.0
10	160	100	26.5	37.8
11	160	50	33.3	30.1

Table 4. Detected pigments in *N. Oleoabundans* PLE extracts.

ID	Tr (min)	Identification	UV-Vis max (nm)	[M+H] ⁺	Main fragments
1	9.3	Not identified	398,422,448	-	-
2	10.5	Violaxanthin	412,435,464	601	-
3	13.7	Not identified	448, 470	565	-
4	14.3	Chlorophyll b ^a	466, 650	908	-
5	15.1	Lutein ^a	420, 444, 472	551 ^b	-
6	15.9	Not identified	456, 470	565	-
7	16.9	Zeaxanthin	424, 450, 476	569	-
8	18.0	Canthaxanthin	475	565	-
9	18.2	Chlorophyll a ^a	432, 664	894	-
10	20.4	Related to canthaxanthin	466	565	-
11	21.1	Not identified	455, 468	551	-
12	23.8	Echinenone	461	551	-
13	27.3	Monoester	455, 469	883	599
14	27.8	Astaxanthin monoester C18:4	474	855	597 [M+H-FA+H ₂ O] ⁺
15	29.3	Monoester	456, 468	857	599
16	29.8	Astaxanthin monoester C18:3	474	857	597 [M+H-FA+H ₂ O] ⁺
17	30.2	β-carotene ^a	424, 451, 476	537	-
18	39.1	Astaxanthin diester C16:0/C18:1	478	1099	861 [M+H-FA(C16:0) +H ₂ O] ⁺ 817 [M+H-FA(C18:1)] ⁺ 579[M+H-2FA+H ₂ O] ⁺
19	40.6	Astaxanthin diester C16:0/C16:0 ^a	478	1073	817 [M+H-FA] ⁺ 579 [M+H-2FA+H ₂ O] ⁺
20	42.3	Diester	478	1101	-

FA: fatty acid, ^aidentification corroborated using commercial standards; ^b [M+H-H₂O]⁺,

Table 5. Regression coefficients (values of variables are specified in their original units) and statistics for the fit obtained from MLR.

Terms of the model	Extraction yield (%)	mg carotenoids/g extract
Constant	15.16*	69.2583
T	0.2393*	-0.1362*
solvent comp	0.3045*	-0.7154*
T*T	-0.0008*	-
T*solvent comp	-0.0012*	0.0008*
solvent comp*solvent comp	-0.0016*	0.0050*
<i>Statistics for goodness of fit of the model</i>		
R ²	0.944	0.968
RSD	0.404	0.794
P	0.054	0.083
RRSD (%)	1.26	1.83

R², determination coefficient ; RSD, residual standard deviation ; P, P-value of the lack of fit test for the model ; RRSD, the residual standard deviation expressed as a percentage of the mean value of the response. *Regression coefficient significantly different from zero (P < 0.05)

570 **Figure 1**

Figure 2