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Enzymatic hydrolysis allows an integral valorization of *Nannochloropsis oceanica* resulting in the production of bioactive peptide extracts and an eicosapentaenoic acid enriched fraction

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Abbreviations

ALA - α-linolenic acid

ARA – arachidonic acid

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DHA - Docosahexaenoic acid

DOE – Design of experiments

EPA - Eicosapentaenoic acid

GI - Gastrointestinal

iACE – Angiotensin-I-converting enzyme inhibition

LA – Linoleic acid

MW – Molecular weight

ORAC – Oxygen radical absorbance capacity

PUFAs – Polyunsaturated fatty acids

scCO2 – supercritical CO2

Abstract

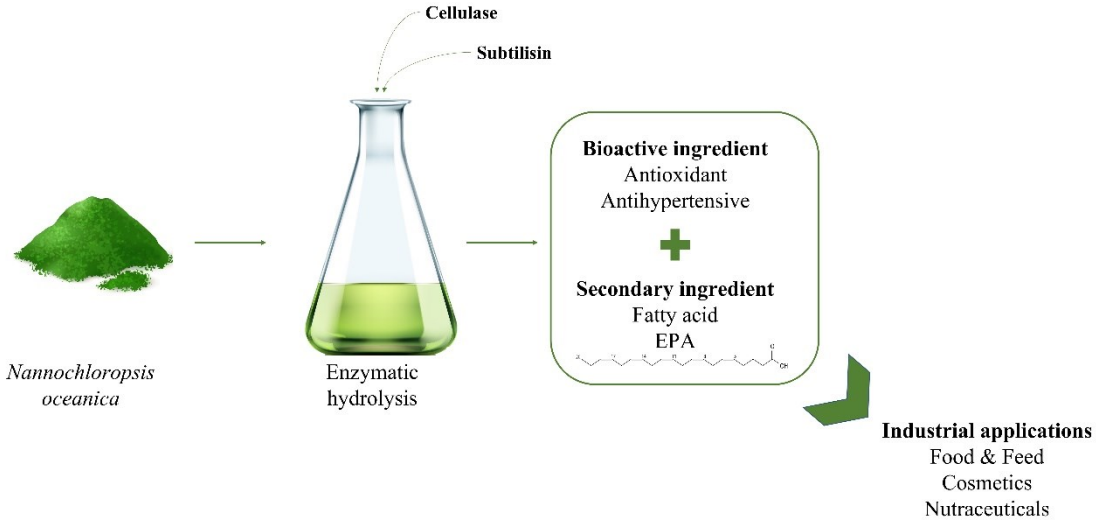
Nannochloropsis oceanica is a microalga with relevant protein content, making it a potential source of bioactive peptides. Furthermore, it is also rich in fatty acids, with a special focus on eicosapentaenoic acid, an omega-3 fatty acid mainly obtained from marine animal sources, with high importance for human health.

N. oceanica has a rigid cell wall constraining protein extraction, thus hydrolyzing it may help increase its components' extractability. Therefore, a Box-Behnken experimental design was carried out to optimize the hydrolysis.

The hydrolysate A showed 67±0.7% of protein, antioxidant activity of 1166±63.7 μmol TE/g of protein and an ACE inhibition with an IC50 of 379 μg protein/mL. The hydrolysate B showed 60±1.8% of protein, antioxidant activity of 775±13.0 μmol TE/g of protein and an ACE inhibition with an IC50 of 239 μg protein/mL. The by-product showed higher yields of total fatty acids when compared to "raw" microalgae, being 5.22 and 1%, respectively.

The sustainable developed methodology led to the production of one fraction rich in bioactive peptides and another with interesting EPA content, both with value-added properties with potential to be commercialized as ingredients for different industrial applications, such as functional food, supplements or cosmetic formulations.

Keywords: bioactive peptides; eicosapentaenoic acid; functional foods; microalgae; marine hydrolysates; sustainability.



Graphical abstract

1. Introduction

Microalgae production may offer several advantages for the environment and from an economic perspective, since they do not need cultivable land or intensive production factors to have a high growth rate, but also their production can be allied with industries that generate CO_2, thus decreasing the gas release to the environment [1].

N. oceanica is a good protein source, with values ranging from 28.7 – 47.7 %. However, it has a recalcitrant cell wall, decreasing protein bioavailability when ingested as a whole and limiting its extraction [2]. So, it is important to break or weaken the cellular wall to help with protein extraction. Bead milling and high-pressure homogenization are examples of highly described

mechanical processes. However, these are high-energy consuming techniques that imply the acquisition of specific equipment. Another alternative is enzymatic hydrolysis, considered more sustainable, cheaper and simpler [3], with different enzymes being studied for *Nannochloropsis* species [4]. In addition, enzymatic hydrolysis is one of the main methods for hydrolyzing proteins and releasing bioactive peptides (with several properties already identified, such as anticancer, antimicrobial, antioxidant, and antihypertensive, among others [5].) from several sources, increasing their bioavailability. Microalgae-derived bioactive hydrolysates may be used as natural industrial ingredients with several applications, such as the development of functional foods, nutraceuticals with promising health-enhancing properties, or even as bioactive cosmetic ingredients [5].

Beyond being a promising peptide source, *N. oceanica* has a high fatty acid content (ca. 120 mg per g of dry matter (DM)) [2]. Furthermore, this microalga is rich in eicosapentaenoic acid (EPA) (29 – 39% of the total fatty acid content) [2], an omega-3 fatty acid essential to the human diet. Human EPA consumption is mainly made from marine animal sources since it is rarer in non-animal supplies. Polyunsaturated fatty acids (PUFAs), such as DHA (22:6 n-3), EPA (20:5 n-3), linoleic acid (LA) (18:2 n-6) and α-linolenic acid (ALA) (18:3 n-3) are associated with proved benefits for human health and wellbeing [6]. They may help with several health conditions, and have benefits for neurodegenerative and neurological disorders [7]; but are also associated with skin benefits [8]. In western countries, omega-3 intake is lower than recommended [9]. Thus, *N. oceanica* besides being a protein source, could also be used as a non-animal source of these important fatty acids with increased interest for food, cosmetic and cosmeceutical vegetarian and vegan products.

Despite the increasing interest in microalgae, few species are approved for human consumption, namely *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Chlorella luteoviridis*,

Odontella aurita and *Tetraselmis chui*. However, as shown by Zanella & Vianello [10], *Nannochloropsis* sp. presents all the specifications required to be approved for human consumption. One of the most known and used microalgae is *Chlorella vulgaris*, which has great interest due to its high protein content that can reach more than 60% [11,12]. *Nannochloropsis oceanica* usually shows a lower protein value than *Chlorella vulgaris*, however, it shows a great commercial advantage when compared to *Chlorella* and *Tetraselmis* species, which is the great content of the essential fatty acids already mentioned. Thus, approving *Nannochloropsis* species for human consumption may allow the creation of novel functional foods rich in EPA and DHA, that can be consumed on a vegan diet.

These microalgae are suitable for intensive culture, rich in valuable compounds, do not have known toxins and have been used in aquaculture feed with no toxicological effects associated. Furthermore, several studies have been performed in mammals, namely rats and mice, showing no toxicity after *Nannochloropsis* or derivatives ingestion [13–16].

According to the literature, there are not many studies regarding *N. oceanica* bioactive hydrolysates production and, at the same time, the valorization of the produced by-product. Thus, in this study, it is intended to valorize *N. oceanica* as a sustainable source of natural ingredients with commercial potential. For that, a sustainable methodology was developed using a design of experiments (DOE) to optimize sequential enzymatic hydrolysis. Furthermore, the resulting water-insoluble fraction was explored as a source of important fatty acids. Thus, the optimized hydrolysis produced two novel ingredients, whose biological and bioactive potential was studied.

2. Materials and methods

2.1. Materials

Nannochloropsis oceanica was provided by Allmicroalgae - Natural Products, S.A (Pataias, Portugal) (Table S1). Cellulase (New Cell Supreme 4000L) (activity - 40000 ECU/g) and subtilisin (New Pro 16L) (activity - 16 KNPU-S/g) were supplied by NewEnzymes. Boric acid and hydrochloric acid (32%) were purchased from Merck (Damdstadt, Germany); sodium hydroxide from LabChem (USA); sulphuric acid and Kjeldahl tablets (Catalyst with 0.3% CuSO4.5H2O) from VWR Scientific (VWR chemicals, Karlsruhe, Germany).

Fluorescein, 2,2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (≥97%), 6-hydroxy-2,5,7,8-tetramethylbroman-2-carboxylic acid (Trolox) (≥97%), angiotensin-I converting enzyme (EC 3.4.15.1, 5.1 U mg-1) and α-glucosidase (EC 3.2.1.20) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Abz-Gly-Phe(NO2)-Pro from Bachem Feinchemikalien (Switzerland). The proteins standard (Thyroglobulin, Ferritin, Aldolase, Conalbumin, Ovalbumin, Carbonic anhydrase, Ribonuclease A and Aprotinin) were obtained from GE Healthcare (USA) and the peptide KGYGGVSLPEW from GenScript (China).

2.2. Design of experiments (DOE)

2.2.1. Experimental design

A Box-Behnken DOE was performed to produce the most protein/peptide-rich and bioactive hydrolysates. The DOE evaluated the combined effect of four factors which were evaluated with three established levels coded as −1 (low), 0 (central), and +1 (high). The factors studied were hydrolysis temperature in °C (X_A) (40, 50 and 60 ºC), cellulase percentage (X_B) (1.5, 3.0 and 5.0 %), protease percentage (X_C) (1.5, 3.0 and 5.0 %) and protease hydrolysis time in hours

(X_D) (2, 4 and 6 h),. The effect of the factors was evaluated on the selected response variables (protein content, antioxidant and antihypertensive potential). A DOE experimental matrix was generated, resulting in an arrangement of 27 treatments performed in duplicate. Each hydrolysis was performed as described in 2.3.

After the optimization process, two scaled-up hydrolysis (200 times the initial amount) were performed using the optimized conditions, for validating the DOE. The scaled-up hydrolysates were freeze-dried before proceeding with further analysis.

2.2.2. Statistical analysis

The DOE matrix generation and analysis were performed using the software Statgraphic Centurion 19®. A significance level of 5% ($p < 0.05$) was considered.

2.2.3. Statistical model

For the optimization, the responses (Y) were adjusted to the model expressed in equation 1, considering the linear, quadratic and linear-linear interaction between the factors.

$$Y = \beta_0 + \beta_A X_A + \beta_B X_B + \beta_C X_C + \beta_D X_D + \beta_{A,B} X_A X_B + \beta_{A,C} X_A X_C + \beta_{A,D} X_A X_D + \beta_{B,C} X_B X_C + \beta_{B,D} X_B X_D + \beta_{C,D} X_C X_D + \beta_{A,A} X_A^2 + \beta_{B,B} X_B^2 + \beta_{C,C} X_C^2 + \beta_{D,D} X_D^2 + \varepsilon \quad (1)$$

where β0 is the constant; β_A, β_B, β_C and β_D are the regression coefficients associated with linear effects of the variables X_A, X_B, X_C and X_D; $\beta_A^2 - \beta_D^2$ corresponds to the quadratic effect; $\beta_{A,B} - \beta_{C,D}$ represents the coefficient for the interaction effects; and ε is the residual error. On each response's final model, the effects that maximized the adjusted R^2 were considered ($p < 0.05$). For multiple response optimization, Derringer's desirability function was applied (Suich & Derringer, 1980), since it describes the degree of desirability for the optimal conditions specified in the model, considering all the responses and using a scale ranging from 0 to 1 [17].

2.3. Enzymatic hydrolysis

N. oceanica, used in powder format (spray-dried), was mixed with deionized water (10% w / v) and the pH was adjusted to 7.5. The enzymatic hydrolysis was performed in two sequential steps, first samples were incubated with cellulase, and then with the protease. So, first, the cellulase was added and incubated at 50 ºC for 2 h. Next, assuring pH 7.5, the protease was added in the test percentage and incubated using the temperature and time test. Cellulase and protease percentages were added according to the DOE generated matrix . The protein breakdown leads to a pH decrease, thus, pH was verified and adjusted to 7.5 along with the protease hydrolysis. The samples were incubated at 90 ºC for 10 min to stop the enzymatic reaction. Finally, samples were centrifuged at 5000 x g for 20 min and both the supernatant (the water-soluble fraction) and the pellet (the water-insoluble fraction) were recovered and henceforth called hydrolysate and secondary ingredient, respectively. All the hydrolysis steps were performed in an orbital shaker (Thermo Scientific™ MaxQ™ 6000) with an agitation of 125 rpm.

The hydrolysates resulting from the experimental design were tested directly, dry weight was determined and the results were expressed by dry weight.

2.4. Hydrolysate's characterization

2.4.1. Protein quantification

The total nitrogen content of each hydrolysate was determined by the Micro-Kjeldahl method, as described before [18]. For the hydrolysates resulting from the DOE, 1.0 mL of sample was used, and the dry weight was used to achieve the protein content (%) per 100 g of dry hydrolysate. For the scaled-up samples, 0.2 g of the freeze-dried hydrolysate was digested. A nitrogen to protein conversion factor of 6.25 was used [19,20].

2.4.2. Antioxidant potential determination

The antioxidant potential of the hydrolysates was evaluated, in triplicate, by the oxygen radical absorbance capacity (ORAC), performed in a black 96-well micro-plate (Nunc, Denmark) according to a method described before [21]. For the DOE samples, the results were based on volume and expressed in µmol TE (Trolox equivalent) / mL, while for scaled-up hydrolysates the results were based on dry weight (DW) and expressed in µmol TE / g hydrolysate (eq. 4) and µmol TE / g protein (eq. 4).

$$\mu M/g\ \text{hydrolysate} = \frac{\text{TE concentration}}{\text{Sample concentration}} \times 1000 \quad (2)$$

$$\mu M/g\ \text{protein} = \frac{\text{TE concentration}}{\text{Total protein content in the weighted sample}} \times 1000 \quad (3)$$

Where, TE concentration is expressed in µmol TE / L, sample concentration in mg / mL and total protein content in the weighted sample is expressed in mg.

2.4.3. Antihypertensive potential determination

To understand if the hydrolysates had antihypertensive potential, their ability to inhibit the Angiotensin-I converting enzyme (iACE) was determined. The assay was performed in a black 96-well microplate (Nunc, Denmark) according to a previously described method [22].

For the DOE, iACE results were expressed as percentage of inhibition at 0.5 mg / mL. For the scaled-up hydrolysates, results were expressed as the concentration able to inhibit 50% of the ACE activity (IC50).

2.4.4. Analysis by size exclusion chromatography

The molecular weight (MW) profile of the *N. oceanica* optimized hydrolysate was determined as previously described [3] .

2.5. Fatty acids profile analysis

The water-insoluble fraction (secondary ingredient) resulting from the scaled-up hydrolysis and the "raw" microalgae (without any treatment) were submitted to further extraction and characterization regarding the fatty acid profile.

The two samples were subjected to n-hexane solid-liquid extraction, using Soxhlet in a ratio of 1:50 (solid:solvent) for 6 h at 68.7 ºC, and to a supercritical CO_2 extraction (scCO2) process.

The scCO2 experiments were carried out in a supercritical fluid extraction system (Thar Technology, Pittsburgh, PA, U.S.A., model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell and two different separators, each of them with 500 mL of capacity, with independent control of temperature and pressure. In this process, 5 g of microalgae were submitted to an extraction process using carbon dioxide at a pressure and temperatures of 300 bar and 50 ºC, respectively, for 4 h preceded by a period of 20 min of static contact, using a flow rate of 5 g / min of CO_2. The samples were collected and stored at -20 ºC until derivatization and subsequent analysis with a flame ionization detector (GC-FID).

Fatty acid methyl esters for gas chromatography analysis with GC-FID were prepared by a saponification step (methanolic NaOH) followed by acid esterification with BF3 in methanol, according to ISO 5509:2000 [23], and heptadecanoic acid was used as internal standard. Analysis was performed using a Thermo Scientific TRACE GC Ultra (Thermo Scientific, Milano, Italy) GC-FID. The sample components were separated using a J&W DB-23 capillary column (Agilent Technologies, Inc., Santa Clara, CA, USA), 60 m, 0.25

mm internal diameter and 0.25 µm phase thickness. GC-FID analysis conditions and fatty acids identification were described previously [24].

3. Results and discussion

3.1. Enzymatic hydrolysis optimization

N. oceanica has about 33% of protein (Table S1). Thus, extracting and hydrolyzing these proteins may allow the production of bioactive hydrolysates. Microalgae have a protective cellular wall to guarantee the integrity of their internal components. So, to achieve protein and peptide release, the cell wall must be weakened or broken. For that, a methodology was studied and optimized based on two successive enzymatic hydrolyses with cellulase and subtilisin (protease). Cellulase degrades cellulose, one of the most abundant polysaccharides of this microalga cell wall, helping the protein release. Subtilisin acts by hydrolyzing proteins, leading to peptides release. Several factors may influence not only protein release but also the bioactive potential of the hydrolysates. So, it is essential to evaluate and analyze the combination of different conditions that can interfere. For that, a Box-Behnken DOE was performed to evaluate the effect of different combinations of temperature, enzymes percentage and hydrolysis time on the hydrolysate's protein content, ORAC and iACE (Table 1).

The DOE's results were expressed graphically for a more straightforward analysis (Fig. 1 and 2). Fig. 1 represents the recalculated Pareto charts obtained for the three evaluated responses, allowing immediate identification of the estimated significant coefficients.

Table 1. Box-Behnken factorial design matrix for four factors and three responses.

Run	Factors	Response[1]

	Hydrolysis temperature (ºC) (X_A)	% Cellulase (X_B)	% Protease (X_C)	Hydrolysis time (h) (X_D)	Protein content (%)	ORAC (µmol TE / ml)	iACE (%)[2]
1	50	1.5	3.0	2	37.73 ± 0.09	23.77 ± 4.56	56.38 ± 0.39
2	40	3.0	1.5	4	30.78 ± 0.34	17.23 ± 3.83	47.08 ± 15.66
3	50	3.0	3.0	4	32.85 ± 0.09	23.38 ± 0.26	62.77 ± 0.33
4	50	5.0	3.0	2	34.28 ± 0.35	23.09 ± 0.41	54.15 ± 0.07
5	40	5.0	3.0	4	28.87 ± 0.36	25.49 ± 3.69	59.40 ± 5.91
6	50	3.0	1.5	6	29.58 ± 0.04	30.88 ± 0.13	61.20 ± 0.27
7	50	5.0	3.0	6	33.39 ± 1.61	27.23 ± 0.00	58.19 ± 0.29
8	60	3.0	1.5	4	39.98 ± 6.74	9.86 ± 0.88	59.32 ± 0.04
9	40	3.0	5.0	4	34.53 ± 0.06	22.50 ± 6.36	54.36 ± 0.36
10	60	1.5	3.0	4	39.68 ± 0.16	9.70 ± 0.00	80.24 ± 0.13
11	50	3.0	3.0	4	42.16 ± 0.06	26.76 ± 0.00	67.04 ± 0.07
12	60	5.0	3.0	4	42.82 ± 0.94	8.61 ± 0.00	69.99 ± 0.02
13	50	3.0	1.5	2	32.95 ± 0.14	22.82 ± 0.00	58.22 ± 0.14
14	50	5.0	1.5	4	49.53 ± 0.71	21.57 ± 0.00	65.60 ± 0.00
15	40	3.0	3.0	6	39.01 ± 5.49	20.15 ± 3.60	55.12 ± 0.05
16	50	3.0	3.0	4	32.23 ± 0.12	19.88 ± 5.56	66.09 ± 0.05
17	40	3.0	3.0	2	35.08 ± 0.30	24.89 ± 0.01	47.07 ± 0.18
18	50	3.0	5.0	6	29.90 ± 0.30	19.66 ± 0.01	50.49 ± 0.39
19	40	1.5	3.0	4	28.24 ± 0.16	21.61 ± 2.68	48.24 ± 0.17
20	50	3.0	5.0	2	31.52 ± 0.16	27.08 ± 0.01	58.90 ± 0.00
21	60	3.0	5.0	4	41.20 ± 0.62	13.92 ± 2.68	67.22 ± 0.18
22	50	1.5	3.0	6	22.84 ± 0.72	30.82 ± 0.00	52.55 ± 0.50
23	60	3.0	3.0	2	44.92 ± 0.51	9.60 ± 0.00	62.73 ± 0.21
24	50	1.5	5.0	4	28.18 ± 0.04	25.44 ± 0.01	53.40 ± 0.17
25	60	3.0	3.0	6	39.62 ± 1.65	10.76 ± 0.00	57.10 ± 0.06
26	50	5.0	5.0	4	37.24 ± 0.06	17.08 ± 0.01	57.25 ± 0.00
27	50	1.5	1.5	4	25.21 ± 0.07	22.40 ± 4.52	54.06 ± 0.02

[1] Values expressed as mean ± SD of two replicates
[2] ACE inhibitory activity measured in 0.5 mg hydrolysate / mL

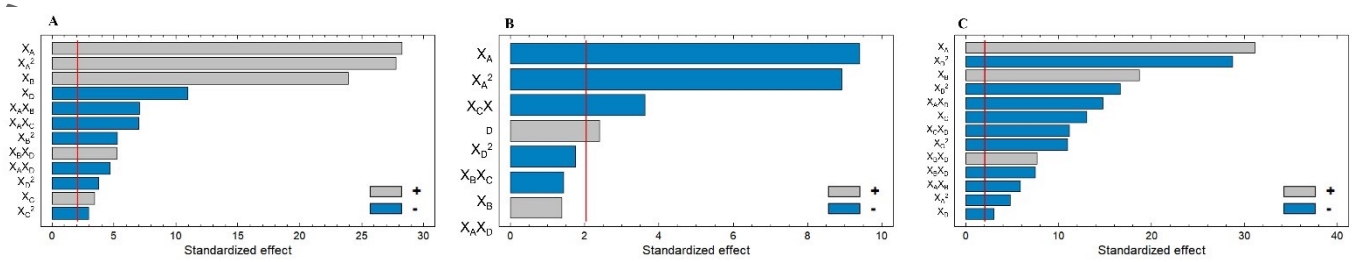


Fig. 1. Pareto charts recalculated models with the effect of four experimental factors, in decreasing order, obtained for protein (A), antioxidant (B) and antihypertensive (C) properties in the experimental design, showing the most influent factors. The vertical lines represent the level of significance (p=0.05). X_A – Temperature; X_B - Cellulase; X_C - Protease; X_D – Hydrolysis time.

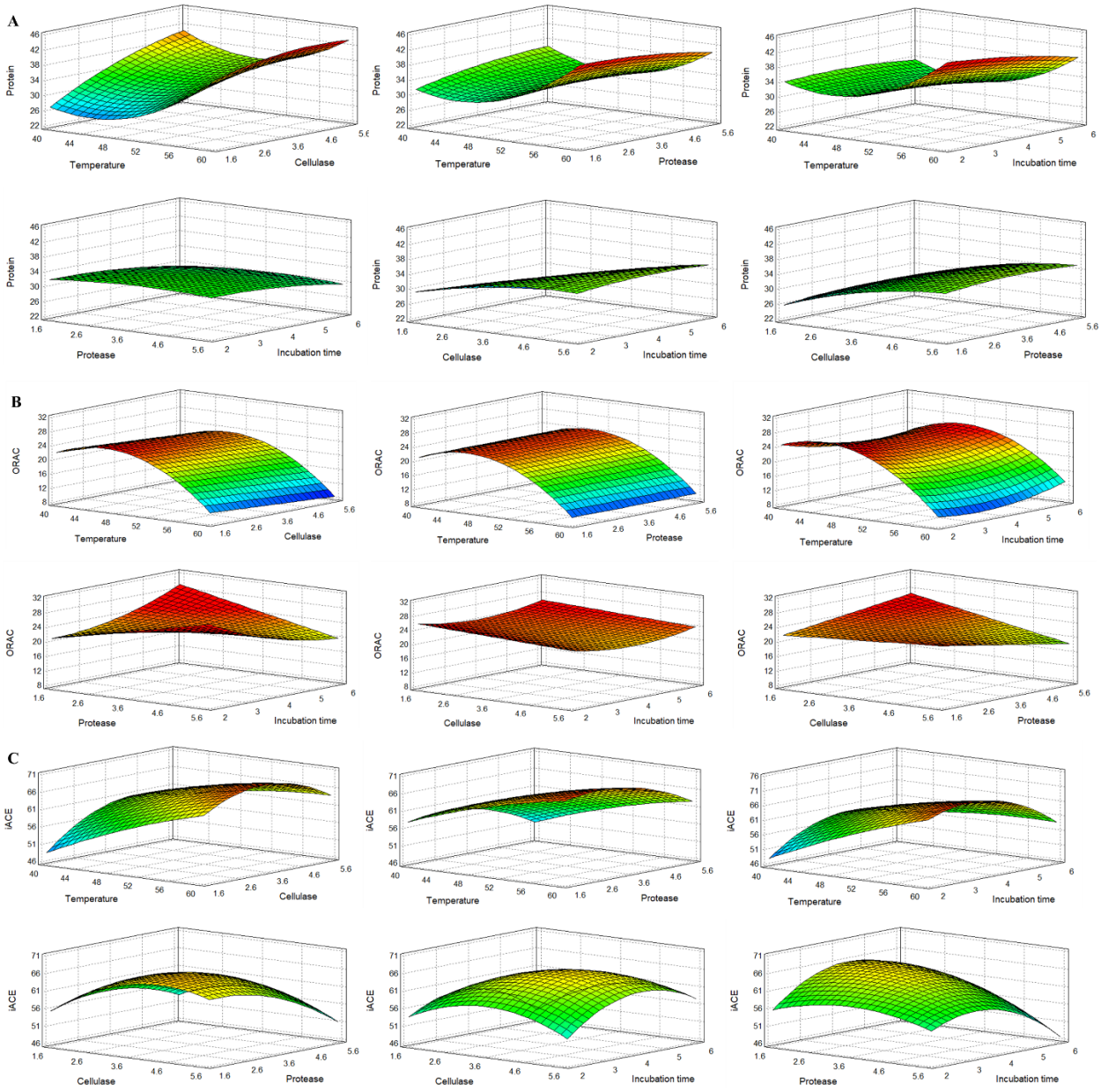


Fig. 2. Response surface models obtained for the protein content (A), ORAC (B) and iACE (C). Each surface corresponds to the combined effect of the variations of two experimental factors, keeping the other two factors at their central level.

The protein content of the 54 runs was measured by Kjeldahl and expressed as protein percentage (dry basis) of the two replicates (Table 1). The highest protein percentage obtained values were 49.53%, 44.92% and 42.82% (runs 14, 23 and 12). The highest values were observed in runs using the higher or central cellulase levels. On the other hand, the lower protein values (22.84%, 25.21% and 28.24%) were obtained for runs held with the lower cellulase percentage (runs 22, 27 and 19).

The recalculated Pareto chart (Fig. 1A) shows that temperature linear and quadratic effects, as well as cellulase and time linear effects, are the main influencing coefficients on protein percentage. Protein is directly influenced by the temperature linear effect, meaning its increase leads to a high protein percentage. Its quadratic coefficient corroborates that the central values are not so favorable. Protein is also directly influenced by the cellulase linear coefficient. Despite the information from its quadratic effect, indicating higher protein percentage when using cellulase central levels, it was observed higher protein percentage with cellulase higher levels (Table 1).

Time is also one of the main influencing factors, with both linear and quadratic coefficients having an inverse influence, so longer protease hydrolysis leads to lower protein content, with the highest results being obtained in the central values. The protease linear coefficient has a directly proportional effect, which, allied with the information from the quadratic coefficient, reveals that the highest protein values are obtained with protease central levels.

The multiple regression analysis (Table S2) showed that the predicted response for the protein content could be obtained by the model in equation 4, which explained 95.38% of protein variability.

$$\text{Protein (\%)} = 109.225 - 4.87052X_A + 9.51055X_B + 6.85798X_C + 2.23306\,X_D + 0.062409\,X_A^2 - 0.119219X_AX_B - 0.0999176X_AX_C - 0.0554677X_AX_D - 0.437173X_B^2 + 0.435588X_BX_D - 0.236002X_C^2 - 0.211535\,X_D^2 \quad (4)$$

The antioxidant potential was measured by ORAC, and the highest values (30.88. 30.82 and 27.08 µmol TE / ml - runs 6, 22 and 20) were obtained at 50 ºC. The lowest ORAC values (8.61, 9.60 and 9.70 µmol TE / ml - runs 12, 23 and 10) were obtained at 60 ºC and with protease at the central level. The recalculated Pareto charts (Fig. 1B) reveal that ORAC was only influenced by four coefficients, with temperature (linear and quadratic coefficient) being the most important. ORAC values were inversely affected by temperature linear coefficient (X_A), and together with its quadratic effect (X_A^2), it was observed that the highest antioxidant

properties are obtained at 50 ºC (central temperature value). The time quadratic effect (X_D^2) reveals that hydrolyzing for 4h (central values) is less effective for ORAC than incubating for 2 or 6h (extreme values). Furthermore, the interaction between protease percentage and time (X_CX_D) had a meaningful contribution to the model's predictive capacity.

The model expressed in equation 5 can be used to predict the ORAC response, and it is able to explain 74.14% of ORAC variability (Table S3).

$$ORAC = -134.474 + 6.65019X_A + 1.72551X_B + 6.90302X_C - 3.8827X_D - 0.0752427X_A^2 + 0.073875X_AX_D - 0.677703X_BX_C - 1.161X_CX_D + 0.50737X_D^2 \quad (5)$$

To evaluate the antihypertensive potential of the hydrolysates, their capacity to inhibit ACE was determined. The higher inhibition percentage was obtained for runs 10, 12 and 21, with values of 80.24%, 69.99% and 67.22%, respectively. All runs were held at 60 ºC, with protease central or high levels and with protease hydrolysis for 4h (central value). The lower iACE percentage were 47.08%, 47.07% and 48.24%, in runs 2, 17 and 19, respectively. Those runs were held at 40 ºC, with cellulase and protease lower or central values.

The iACE percentage was significantly influenced by several coefficients, with X_A, X_D^2, X_B and X_B^2 being the four most important. Temperature linear coefficient (X_A) had a directly proportional effect, and its quadratic effect (X_A^2) corroborates the associated increase. However, a higher value is achieved at the temperature central value. The iACE is inversely influenced by time (X_D), with the maximum result being obtained at central temperature levels. Cellulase linear coefficient (X_B) had a directly proportional influence, which, together with the quadratic coefficient (X_B^2), reveals that a higher iACE percentage is obtained with cellulase central values. Regarding protease, it showed an inversely proportional influence, suggesting

that higher iACE is produced with the enzyme central percentage. Furthermore, the interactions X_AX_D and X_CX_D also represent meaningful influence.

The iACE can be predicted by the multiple regression model expressed in equation 6, which explains 86.42% of the variability (Table S4).

$$\text{iACE (\%)} = -126.352 + 3.1485X_A + 18.4315X_B + 10.8687X_C + 24.7449X_D - 0.0129183X_A^2 - 0.13406X_AX_B - 0.205564X_AX_D - 1.49189X_B^2 - 0.691201X_BX_C + 0.590251X_BX_D - 0.963094X_C^2 - 0.854627X_CX_D - 1.72719\ X_D^2 \quad (6)$$

To predict the optimal conditions for multiple responses, a Derringer desirability analysis was performed [25] (Table 2). For an optimal response for protein and ORAC, the desirability was 0.75, and the model predicts hydrolyzates with 38% of protein and an ORAC of 26.26 µmol TE/mL. For the optimization of a multiple response for protein, ORAC and iACE, a desirability of 0.71 was obtained. Using the optimal conditions, the model predicts hydrolyzates with 35% of protein, ORAC of 25.5 µmol TE / mL and 66% of iACE.

3.2. Optimal hydrolysate scale-up

3.2.1. Bioactive properties

The predicted optimal conditions on the DOE were combined (Table 2) to produce hydrolysates in 200 times scaled-up experiments. The microalgae:water ratio was maintained, and each scale-up was performed in triplicate. So, for the scale-up aiming at producing hydrolysates rich in proteins and antioxidant peptides (Hydrolysate A), the microalga was hydrolyzed with 4.7% cellulase for 2 h at 50 ºC. Secondly, 5.0% of subtilisin was used to continue the hydrolysis at 40 ºC for 2 h. Regarding the scale-up aiming the optimization of the three responses tested

(Hydrolysate B), the microalga was firstly hydrolyzed with 5.0% cellulase for 2h at 50 ºC, and then with 1.7% of subtilisin at 50 ºC for 5 h (temperature was adjusted to 50 ºC for simple application).

The predicted optimal conditions on the DOE were combined to produce two final hydrolysates, one with conditions A and the other with conditions B (Table 2). Both hydrolysates were prepared due to the different extraction conditions. Since method B requires longer incubations which may result in higher energy costs, it is important to understand the differences between both methods, so the hydrolysate may be produced according to the intended needs. Condition A resulted in a yield of 67% (soluble fraction). The hydrolysate showed 31% protein, corresponding to a release of 63 ± 0.7% of the total protein present in the microalga biomass used. The bioactive characterization revealed 361 ± 16.5 µmol TE / g hydrolysate or 1166 ± 63.7 µmol TE / g protein on ORAC and an IC_{50} for iACE of 379 ± 66.8% µg of protein / mL. Conditions B resulted in a hydrolysate production yield of 60%, with 29% protein, 224 ± 0.16 µmol TE / g hydrolysate, or 775 ± 13.0 µmol TE / g protein on ORAC and an IC_{50} for iACE of 239 ± 17.4% µg of protein / mL (Table 2).

3.2.2. Protein and peptide profile

The protein/peptide profile of the hydrolysates produced under optimized conditions A and B were analyzed by size exclusion chromatography (SEC) at 280 nm, and 12 peaks were identified for each hydrolysate (Fig. 3) (Table S5 and S6). The chromatograms obtained showed a similar profile. At 280 nm, for hydrolysate A, the highest MW with a relevant area percentage was found to have 163.76 kDa, corresponding to 16% of the total area. However, the most abundant absorbance was observed for peptides with MW equal to or lower than 1.2 kDa, representing about 70% of the total chromatogram area. The peak at 35.02 mL, around 21% of the area, corresponds to 1.2 kDa, while the other 49% of the area corresponds to peptide

mixtures with MW lower than that. The lowest MW corresponds to a free amino acid, with a MW of around 204 Da. The main differences observed in the hydrolysate B are the existence of a more prominent peak at 15.6 mL and the absence of a peak at 55.6 mL.

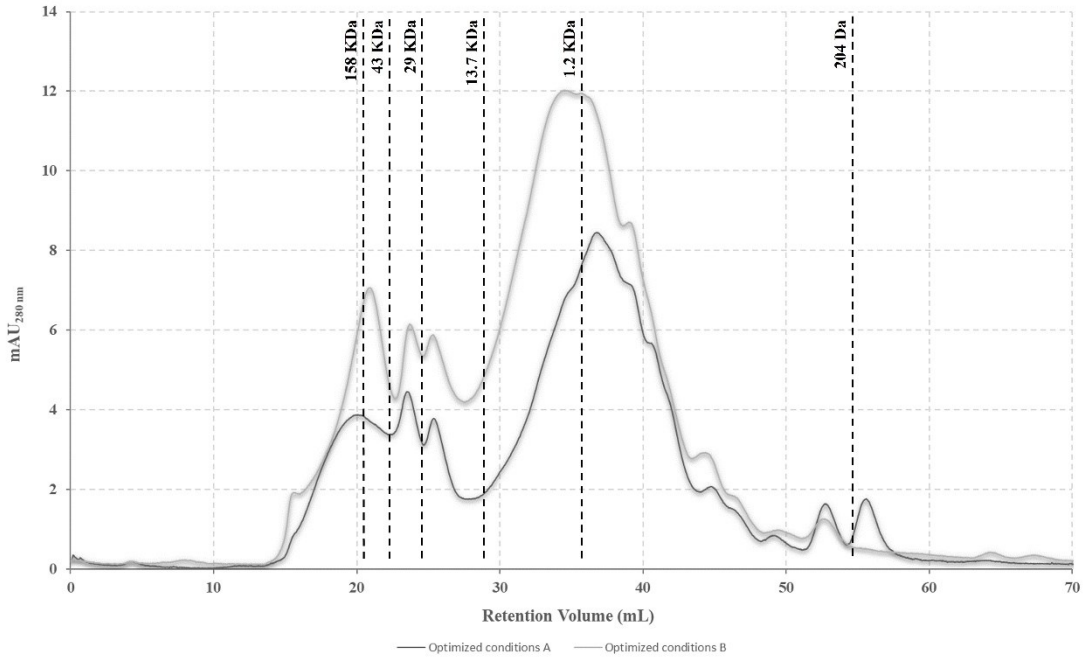


Fig. 3. Protein and peptide profile by the size of molecular weight of the *N. oceanica* hydrolysates, with some MW identified.

3.3. Fatty acids profile

N. oceanica is described as a source of relevant fatty acids. However, when consumed they may not have excellent bioavailability, due to the cells rigid wall. With the developed methodology, microalga fatty acids remain in the insoluble fraction. Thus, to understand if our method affects the fatty acid release, an extraction was performed, by Soxhlet and scCO2, in the "raw" microalgae biomass and in the insoluble fraction resultant from the enzymatic hydrolysis.

The lipidic extract obtained from the "raw" sample showed a lower mass yield than the hydrolyzed sample, either after Soxhlet or after scCO2 (Table S7). For Soxhlet, the extraction mass yield obtained was 1.48% and 4.47% for "raw" and hydrolyzed microalgae, respectively. Regarding scCO2, the increase in yield was more noticeable for the hydrolyzed sample, which presented a mass yield of 5.22% against a yield of less than 1% for the "raw" sample.

Regarding fatty acid composition, the same compounds were identified in both samples (Supplementary materials). However, the total fatty acid content was higher in the hydrolyzed sample, with the main difference being observed in the scCO2 extraction . Those that are in greater quantity in both samples are C16 (Palmitic acid) and C16:1 cis9 (Palmitoleic acid), representing 65% of the total fatty acids for the hydrolyzed scCO2 extract and 70% for the "raw" sample. For Soxhlet extractions, these two fatty acids represent 60% of the total.

The main differences between the hydrolyzed and "raw" samples are in the amount of EPA extracted, from the omega-3 family, and arachidonic acid (ARA), of the omega-6 family. For the hydrolyzed sample EPA content corresponds to 11% of the total, while in the "raw" sample only 4%, which demonstrates an increase of almost 3 times in the extraction of this fatty acid after hydrolysis. ARA is also 4 times more concentrated in the hydrolyzed sample than in the "raw" one. For the extracts obtained by Soxhlet this difference was not so noticeable. However, the hydrolyzed microalgae extract also has higher EPA and ARA values than the "raw" extract. Figure 4 shows the differences between the profiles and amount of each fatty acid in terms of extract obtained for the two microalgae samples extracted by scCO2. Figure 4 only shows the comparison regarding the major fatty acids found in both samples, although the complete profile may be found in supplementary data (Table S8 and S9).

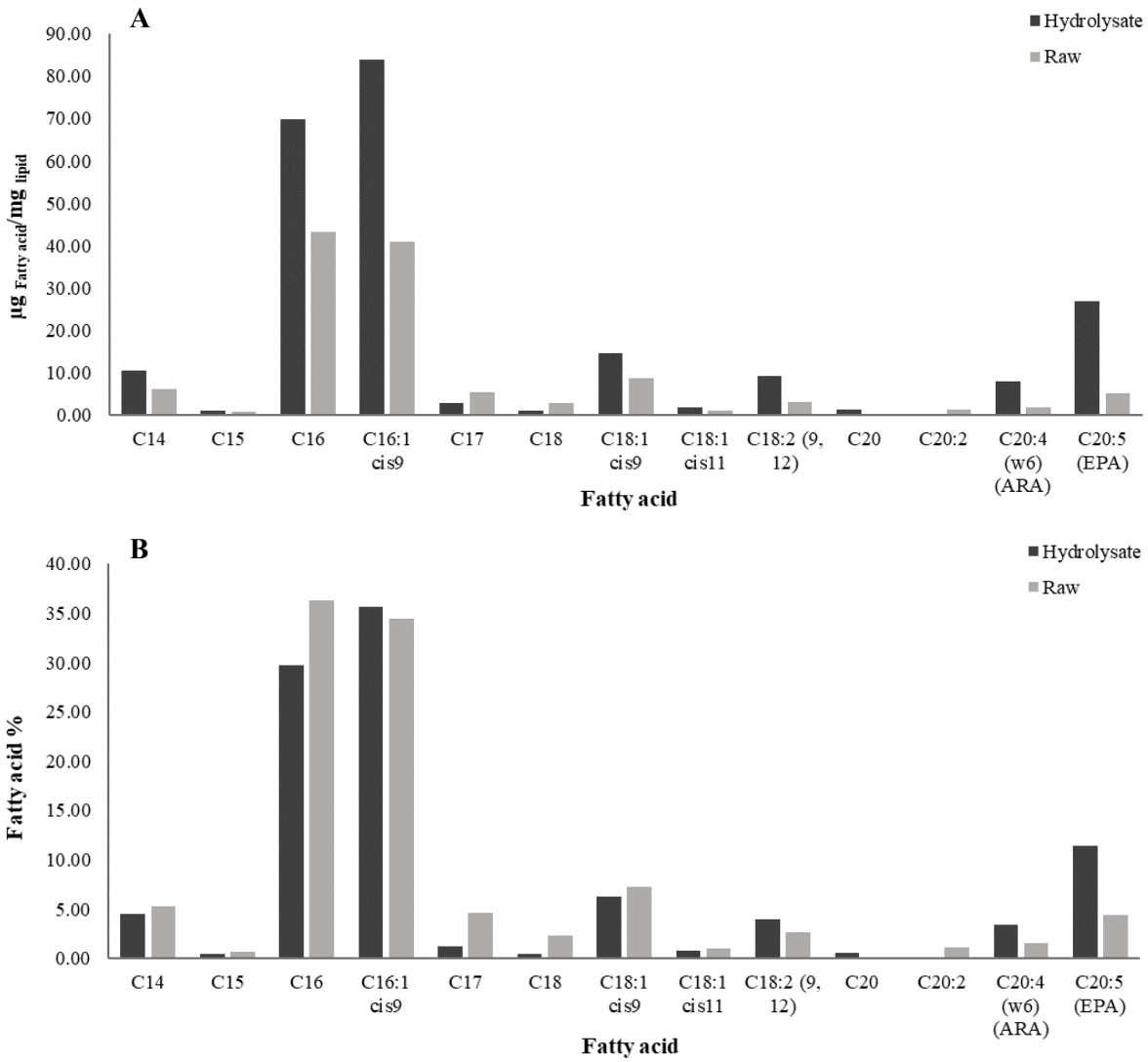


Fig. 4. *N. oceanica* hydrolysate and "raw" biomass major fatty acid composition (>0.5%), obtained by scCO2. A – Content of each Fatty acid in µg / mg lipid. B – Percentage of each fatty acid concerning the total lipids content in the sample.

4. Discussion

N. oceanica is an undervalued microalga species, especially regarding bioactive peptides with limited information available. The main focus of the studies on this species is related to its lipid

content, for biofuels, or its profile, due to its richness in EPA. EPA is an omega-3 fatty acid essential for human nutrition, however, most sources are marine animal-derived. Thus, *N. oceanica* has been studied as a vegetal EPA source. Nevertheless, this microalga has an interesting protein content, making it promising to produce bioactive peptides.

N. oceanica cell wall has a high cellulose content [26], thus an enzymatic hydrolysis with a cellulase was performed in order to weaken the cellular wall and release proteins that could be further hydrolyzed by the protease. Secondly, the protease was used to break proteins down, resulting in peptides production, expected to have bioactive properties.

The DOE allowed the optimization of a methodology to produce hydrolysates rich in proteins/peptides and bioactive properties. For that, a Box-Behnken design was used to evaluate the effect of the combination of four factors on three responses. The temperature was the most crucial factor for all the evaluated responses, being directly proportional for protein and iACE, and inversely for ORAC. Regarding the enzymes percentage, cellulase was relevant for protein and iACE, showing to be directly proportional, with higher values being predicted when using the enzyme central value. Furthermore, the higher iACE percentage was obtained in the runs that originated low ORAC values, the reason why two multiple optimizations were performed, one for producing hydrolysates rich in proteins and antioxidant properties and the other for the three evaluated responses. The predicted conditions to optimize protein and ORAC or the three responses were very different, with the triple optimal point needing higher temperatures, longer hydrolysis, and less protease. Thus, regarding the differences, it was expected that we could not obtain the higher response values for both properties when using the same conditions. So, we present both optimizations, so that, conditions should be chosen according to the intended goal. Furthermore, when protein and antioxidant properties are the most relevant for the final application, the conditions obtained for the protein and ORAC should be used since they are less time and energy-consuming.

Using the conditions obtained for the multiple optimization of the three responses, a scaled-up hydrolysis with an increase of 200 times was performed. The final hydrolysate, after being freeze-dried, showed a greenish powder appearance. The *N. oceanica* used has a high content of chlorophyll and carotenoids, which seems to have been divided between the soluble and insoluble fractions since both demonstrated a green color. Thus, the chloroplast wall must have been broken, so few pigments were released from the thylakoids to the soluble extract, conferring the greenish color.

The protein/peptide profile of the final hydrolysate revealed that most of the peaks as well as the higher absorbance abundance at 280 nm, represent peptide mixtures with MW lower than 1.2 kDa. This may be explained by the hydrolysis process, which led to protein breakdown, resulting in smaller peptides. The peak with MW lower than 204 Da may be tyrosine (MW of 181.2), another aromatic amino acid that can absorb light at 280 nm, despite absorbing less than tryptophan.

Both hydrolysates showed interesting protein content as well as a high bioactive potential. On the one hand, the high ORAC value highlights the potential as an antioxidant ingredient, with hydrolysate A showing higher antioxidant values. Our method revealed to produce an extract with higher antioxidant potential than other microalgae such as *Chlorella vulgaris* obtained by ultrasound-assisted extraction using water/ethanol (31.21 µmol TE / g DW) [27], *Phormidium autumnale* aqueous extract (46.95 µmol TE/ g DW) or *Scenedesmus obliquus* aqueous extract (33.22 µmol TE/ g DW) [28]. Considering the antihypertensive potential, both hydrolysates have a high ACE inhibitory capacity, with an IC_{50} lower than the reference value of 500 µg protein/mL [18], with hydrolysate B being more efficient in inhibiting ACE. Natural alternatives for hypertension control may be studied and developed from these microalga hydrolysates. Also, the development of functional foods with hypertension protective effects should be studied, as well as supplements. Finally, a hydrolysate combining these two bioactive

properties opens a wide range of possible commercial applications for at least three different industries, food, cosmetics and pharmaceutics.

The presented methodology resulted in the production of a main ingredient as a water-soluble fraction, and a water-insoluble fraction, as the secondary ingredient. Despite the first one having the more significant potential to be used in the development of high-value products, the secondary ingredient (lipids) has remarkable features for other commercial applications. First, the hydrolysate is only composed of soluble compounds or insoluble but able to form micelles, and such may happen with the insoluble pigments responsible for the green color. Thus, the secondary ingredient retains the remaining noteworthy compounds of this microalga, such as lipids, polysaccharides and also the remaining proteins that were not released by the hydrolysis.

The secondary ingredient showed interesting results regarding the lipid extraction yield and profile when compared to the "raw" microalgae. The "raw" samples showed a lower mass yield than the hydrolyzed sample, either after Soxhlet or after scCO2. The fact that the hydrolyzed samples had their cell walls weakened due to the enzymatic treatment, clearly promoted the extraction of fatty acids from their interior. This explains why the Soxhlet and scCO2 yield for this sample was almost 3 and 5 times greater than for the "raw" sample, respectively. The C16 and C16:1 cis9 fatty acids are the most abundant, being 65% and 70% of the total fatty acids of the hydrolyzed and "raw" samples, respectively. Palmitic acid is present not only in the diet but is also synthesized endogenously and palmitoleic acid is an omega-7 monounsaturated fatty acid often found in vegetal and marine biomass. Even though they have been associated with adverse effects on chronic diseases in adults, palmitic acid is an essential component of cell membranes, secretory and transport lipids [29] and palmitoleic acid has been related to beneficial effects on insulin sensitivity, cholesterol metabolism, and hemostasis [30].

The increased fatty acids in the hydrolyzed microalgae are EPA (of the omega-3 family) and ARA (of the omega-6 family), being 3 and 4 times more concentrated than in the "raw" sample.

In fact, one of the most relevant compounds present in this microalga is EPA. The *N. oceanica* used has 14.3 g of lipids/100 g, with 27.7% being EPA. The results showed that after submitting the microalga to this methodology, the EPA bioavailability was increased compared to the "raw" biomass. This finding adds an enormous potential for secondary ingredient commercialization since EPA is not frequently found in non-animal sources. Some fatty acids may benefit the skin, such as palmitoleic acid (C16:1 cis-9) and squalene, which can act against lipid peroxidation and, consequently, reduce skin cell damage [31]. So, the secondary ingredient richness in fatty acids may also be interesting to the cosmetic industry in reducing skin damage and promoting healthier skin.

This work reveals the enormous potential of *N. oceanica* as a source of bioactive hydrolysates rich in proteins and antioxidant and antihypertensive properties. However, a deeper characterization needs to be performed to fully understand all the compounds found in it. Additionally, further studies are needed before incorporating these hydrolysates in the final matrixes. On the one hand, a gastrointestinal digestion simulation should be performed since not all the nutrients ingested can reach the bloodstream, nor the bioactivities are guaranteed to be maintained [32]. On the other hand, skin permeation studies should be performed to evaluate the ability of the hydrolysates to reach their target and retain the active compounds [33]. If bioactive properties are lost within the GI tract, or if the peptide mixture does not show the capacity to reach the dermis, encapsulation strategies must be developed so that this hydrolysate may be commercialized with its total capacity.

5. Conclusion

Our study describes a methodology for valorizing the microalga *N. oceanica* using an integral approach where the most relevant ingredients are sequentially extracted and quality preserved. The sequential enzymatic hydrolysis led to the production of a hydrolysate with relevant

protein content, but especially with high antioxidant and antihypertensive potential. Furthermore, the resulting water-insoluble fraction showed a different fatty acid profile, compared to the "raw" microalga, with an emphasis on improving omega-3 EPA levels. So, it is presented a sustainable and food-compatible methodology that can produce two ingredients with commercial potential, leading to almost zero waste production.

Future studies may be performed to confirm peptide bioactive properties and resistance to gastrointestinal tract conditions, as well as peptide skin permeation capacity for cosmetic application.

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Declarations

Conflict of Interest: The authors declare no competing interests.

Data Availability Statement:

The data that supports the findings of this study are available in the supplementary material of this article.

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