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Spirulina (*Arthrospira platensis*) protein-rich extract as a natural emulsifier for oil-in-water emulsions: Optimization through a sequential experimental design strategy

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HIGHLIGHTS

- Spirulina protein-rich extracts valorization can impact algal biorefinery.
- Spirulina protein-rich extracts are effective natural emulsifiers.
- Sequential experimental design is an effective tool to optimize emulsion production.
- Stable emulsions against creaming for up to 30 days of storage were obtained.
- The best conditions were achieved using 4 wt% of Spirulina protein extract.

GRAPHICAL ABSTRACT

ARTICLE INFO

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ABSTRACT

Spirulina (*Arthrospira platensis*) proteins have been proven to present emulsifying properties. In this work, a Spirulina protein-rich extract obtained by ultrasound extraction (SpE) was tested to stabilize oil-in-water (O/W) emulsions. For this purpose, a sequential experimental design strategy (Fractional Factorial Design (FFD) 2^{4-1} followed by a Central Composite Rotatable Design (CCRD) 2^2)) was applied. The effect of four variables, SpE concentration, O/W weight ratio, pH and storage time, on emulsions' zeta potential and number-mean droplet diameter was considered for the FFD 2^{4-1}, indicating SpE concentration and storage time as the relevant variables for the CCRD 2^2. According to zeta potential and number-mean droplet diameter evaluation, for the studied SpE concentration range (2–5 wt%), quite stable emulsions were obtained along the tested 30-days period. Even so, for 5%, visual inspection revealed extract segregation after 20-days. The optimal solution comprised 4 wt% of SpE, for an O/W weight ratio of 30/70 and a pH of 7.0 (number-mean droplet diameter of 55.66 nm and zeta potential of −43.83 mV). Overall, SpE has proven to be an excellent emulsifier, offering the potential to

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substitute animal-based proteins and synthetic emulsifiers. In addition, no signs of contamination by microorganisms were observed, suggesting that the SpE may also act as an antimicrobial agent.

1. Introduction

Proteins are amphiphilic macromolecules consisting of one or more polypeptide chains, being obtained from a wide variety of sources such as animal, plant, fungal and algae [1]. Due to the amphiphilic nature imparted by the acid-base characteristics of the amino acids, proteins are surface-active molecules able to stabilize emulsions and foams. These macromolecules can be adsorbed at the oil/water or air/water interfaces, forming strong viscoelastic films, increasing emulsion thermodynamic and kinetic stability. They can also generate repulsive interactions between the oil droplets avoiding emulsion destabilization (e.g., flocculation and coalescence) [2,3].

Nowadays, the demand for natural and sustainable ingredients is becoming a priority driven by consumer's awareness regarding health and environmental concerns. For this reason, research and industry efforts are aligned to find sustainable and natural alternatives [4]. In this context, proteins of plant-based (e.g., pea protein, soy protein, lupin protein) [5,6] and cell-based (e.g., microalgae, yeast, bacteria, fungi) [7,8] origin are being proposed, not only to replace synthetic (e.g., Spans and Tweens) but also animal-based (e.g., gelatine, egg proteins, whey protein) counterparts. Likewise, these solutions are at high demand to fulfill the needs of some dietary restrictions, namely of vegan/vegetarian diets [9]. In this context, researchers have been exploring microalgae protein-rich extracts to form and stabilize emulsions and foams, being reported similar adsorption kinetics and surface tension reduction to benchmark proteins like whey protein isolate [2,10].

Microalgae are considered viable sources of proteins presenting several benefits over conventional plant crops. They can proliferate and generate higher protein yields per unit of cultivation area (4–15 tons/Ha/year), comparatively with traditional crops, including soybean and wheat (0.6–1.2 tons/Ha/year, and 1.1 tons/Ha/year, respectively). Moreover, they do not require arable land for cultivation and do not compete with food chains [11]. Among them, *Arthrospira platensis* (well-known as Spirulina), which is phylogenetically characterized as a cyanobacterium, presents a high amount of protein (50–70%), vitamins (A, K, B12), minerals (Fe, Ca, Se, Mg) and pigments (e.g., chlorophylls, carotenoids and phycobiliproteins). Spirulina is generally recognized as safe for human ingesting (GRAS) and consumed for centuries. It presents high commercial relevance as a food supplement and as a source of the natural blue pigment C-phycocyanin (C-PC), which is currently used as a food colorant [12–15].

Many studies have demonstrated the ability of Spirulina proteins to form and stabilize emulsions and foams [16–20]. Böcker et al. [7] showed that extracts from *A. platensis* (Spirulina) stabilize emulsions independently of the achieved degree of purity. However, the higher the protein purity is, the higher is its functionality, resulting in a faster adsorption at the oil/water interface, producing a finer emulsion with smaller droplet diameters. Therefore, and comparatively with the use of the whole biomass, the use of protein-rich extracts resulted in an improved emulsifying performance. This fact demands an integrated valorization of the protein-rich extracts, which can be combined with other co-products in the same productive chain (e.g., carbohydrates, lipids) to reduce production costs, in-line with the biorefinery concept [19–21].

Most of the available studies dealing with the use of algal protein-rich extracts address the characterization of the emulsifying properties [16,18–20,22,23], lacking of studies dealing with emulsion formulation optimization, which is crucial to achieve viable technological applications. In this context, this work reports the obtainment of a Spirulina protein-rich extract (SpE) and subsequent testing as an oil-in-water (O/W) emulsifier. The extraction was conducted, after a defatting step, using ultrasound technology under an alkaline medium followed by isoelectric precipitation, and the obtained extract characterized concerning protein and lipid contents and color parameters. A sequential experimental design strategy was applied, firstly using a Fractional Factorial Design 2^{4-1} to evaluate the effects of four variables (O/W weight ratio, SpE concentration, pH, and storage time) on the number-mean droplet diameter and zeta potential, and then a Central Composite Rotatable Design 2^2 addressing the most relevant variables to achieve the best conditions to obtain stable O/W emulsions.

2. Materials and methods

2.1. Samples and reagents

The dried *Arthrospira platensis* (Spirulina) biomass was kindly donated by the Portuguese company A_4F – Algae for Future. Samples were kept stored at − 24 ºC and protected from light. The Pierce™ Modified Lowry Protein Assay Kit, purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) was used to determine the soluble protein content. For pH adjustments, NaOH and HCl 37% purchased from Sigma-Aldrich (Saint Louis, MO, USA), were used. n-Hexane, which was used to extract lipids from Spirulina biomass, was purchased from Carlo Erba Reagents (Barcelona, Spain). The phosphate buffer solution was produced using sodium di-hydrogen phosphate 1-hydrate and di-sodium hydrogen phosphate anhydrous purchased from Panreac AppliChem (ITW Reagents) (Barcelona, Spain). The olive oil used for emulsions preparation, a neutral oil characterized by having neutral flavor and color, was purchased from Vencilab (Vila Nova de Gaia, Portugal). All other chemicals and solvents were of analytical grade and purchased from common suppliers.

2.2. Protein-rich extract production

The protein extraction methodology was adopted from the one reported by Lupatini et al. [21], but using an ultrasound bath of lower frequency (25 Hz instead of 37 Hz). Previously to the extraction step, the Spirulina biomass (SpB) was subjected to a fat extraction with hexane using a Soxhlet apparatus and dried in an oven at 50 ºC for 24 h to obtain the Spirulina-defatted biomass (SpDB). For the extraction, the SpDB was dispersed in ultra-purified water (30 g/L), the pH adjusted to 9 using a 2 mol/L NaOH solution, and the resulting mixture sonicated in the ultrasound bath (45 min, 30 ºC) (J.P. SELECTA s.a, Barcelona, Spain), followed by stirring at 30 ºC (50 min, 80 rpm), then centrifuged (15 min, 7500 g, 20 ºC) (Eppendorf, 5810R, Hamburg, Germany). The resulting supernatant was adjusted to pH 3 (Spirulina protein isoelectric point (pI)) [18,24], and the suspension placed in a water bath without stirring (10 ºC, 30 min) for protein precipitation. Afterwards, the suspension was centrifuged (15 min, 7500 g, 20 ºC) (Eppendorf, 5810R, Hamburg, Germany), and the precipitate collected and washed three times with ultra-purified water, adjusted to pH 7 using 2 mol/L NaOH, and freeze-dried (48 h) (Labogene, ScanVac CoolSafe, Lillerod, Denmark) to obtain the Spirulina protein extract (SpE). The extraction process from SPDB was characterized concerning extract and protein yields using Eq. 1 and Eq. 2, respectively.

$$\text{Extract yield (wt\%)} = \frac{\text{m-SpE}}{\text{m-SpDB}} \times 100 \quad (1)$$

where m-SpE is the obtained mass (g) of the Spirulina protein extract (SpE) and m-SpDB is the used mass (g) of Spirulina-defatted biomass (SpDB).

Table 1
Variables range applied in the Fractional Factorial Design 2^{4-1} and in the Central Composite Rotatable Design 2^2.

Fractional factorial design 2^{4-1}						
Variable	Symbol	Coded variable level				
		-1	0	1		
O/W weight ratio	x_1	10/90	20/80	30/70		
SpE concentration (wt%)	x_2	1	2.5	4		
pH	x_3	4	5.5	7		
Storage time (Days)	x_4	0	7	14		
Central Composite Rotatable Design 2^2						
		-1.41	**-1**	**0**	**1**	**1.41**
SpE concentration (wt%)	x_1	2	2.4	3.5	4.6	5
Storage time (Days)	x_2	10	13	20	27	30

$$\text{Protein yield (wt\%)} = \frac{(\text{m-SpE} \times \text{p-SpE})}{(\text{m-SpDB} \times \text{p-SpDB})} \times 100 \quad (2)$$

where m-SpE is the obtained mass (g) of the Spirulina protein extract (SpE), p-SpE is the protein content (wt%) of SpE, m-SpDB is the used mass (g) of Spirulina-defatted biomass (SpDB), and p-SpDB is the protein content (wt%) of SpDB. p-SpE and p-SpDB were determined by the macro-Kjeldahl method as described next in Section 2.3.

2.3. Protein-rich extract characterization

The protein-rich extract was characterized concerning protein and lipid contents, and color parameters. For comparison purposes, SpB and SpDB were also characterized. Moreover, the protein rich extract was characterized concerning protein solubility.

The protein and lipid content and color parameters were determined according to well established methodologies of the group [25]. Briefly, the protein content was determined by the macro-Kjeldahl method using an N-factor of 5.95 according to López et al. [26]. The lipids determination was performed using a Soxhlet apparatus and petroleum ether as the solvent. The color parameters (L^*, a^* and b^*) (CIELAB) were obtained using the equipment Konica Minolta CR-400 (Chiyoda, Tokyo, Japan).

Protein solubility (PS) was determined according to the methodology described by Menegotto et al. [19]. Briefly, 0.1 g of SpE were dispersed in 20 mL of distilled water and stirred for 10 min. The pH was adjusted from 2 to 9 using HCl 2.0 mol/L or NaOH 2.0 mol/L as needed, and the respective dispersions centrifuged (4100 g, 25 ºC, 15 min). The supernatants were collected to determine the soluble protein concentration. A control sample was prepared under the same conditions but not subjected to pH adjustment and centrifugation. The soluble protein concentration (g/L) of the supernatants (SSP) and the control sample (CSP) was determined using the Pierce™ Modified Lowry Protein Assay. The calibration curve was constructed using bovine serum albumin (BSA) (1.5 g/L), and fitted to a polynomial equation ($y = 298.05\ x^4 - 236.75\ x^3 + 57.111x^2 - 2.4694x + 0.0204$, where y is the protein concentration (g/L) and x is the absorbance, $R^2 = 0.9923$). The absorbance of the supernatants was measured at 750 nm using a spectrophotometer (Jasco Inc., V-730, Tokyo, Japan). For each pH, PS was expressed in wt% according to Eq.3.

$$\text{PS(wt\%)} = \frac{\text{SSP}}{\text{CSP}} \times 100 \quad (3)$$

2.4. Evaluation of the protein-rich extract as an O/W emulsifier

2.4.1. Experimental design strategy

To evaluate the ability of the protein-rich extract as an O/W emulsifier, a sequential experimental design strategy was applied, firstly using a Fractional Factorial Design (FFD) 2^{4-1} to define the most relevant variables influencing the number-mean droplet diameter (nm) and zeta potential (mV), followed by a refined study where a Central Composite Rotatable Design (CCRD) 2^2 was applied to the identified variables. Briefly, to determine the statistically significant variables ($p \leq 0.10$), the FFD comprised 2^{4-1} trials plus 3 central points (11 runs). The effect of four independent variables (O/W weight ratio, SpE concentration (wt%), pH, and storage time (days)) on the number-mean droplet diameter, and zeta potential of the emulsions was considered. This experimental design was chosen since it provides reliable information about the main effects of the studied variables on the desired responses while requiring a lower number of experiments than a full design, reducing time, energy and costs [27].

Thereafter, the CCRD comprised 3 replicates at the central point and 4 axial points (2^2 plus star configuration, 11 runs) to obtain the second-order models for emulsion's number-mean droplet diameter and zeta potential (dependent variables) as a function of the identified most relevant independent variables (SpE concentration (wt%) and storage time (days)). CCRD design is usually applied for process optimization since it enables evaluating the synergistic and antagonistic effects among variables and optimizing several responses at a time [27]. The ranges of the studied variables are shown in Table 1 for both experimental designs. For both experimental designs only one single emulsion was prepared per trial, and the repeatability of the process was checked by doing 3 replicates at the central point.

2.4.2. Emulsion preparation procedure

Emulsions were prepared according to the methodology described and optimized by Schreiner et al. [28]. Briefly, the SpE was dispersed in the aqueous phase (phosphate buffer solution 0.1 M, at pH defined by the FFD 2^{4-1}) and stirred for 5 min to prepare the aqueous phase. Thereafter, the oil phase (olive oil) was added to the flask and dispersed using an Ultraturrax (11,000 rpm, 3 min) (CAT, Unidrive X 1000, Ballrechten-Dottingen, Germany). These pre-emulsions were, subsequently, submitted to a high-pressure homogenization (HPH) (Avestin, Emulsiflex C3, Ottawa, Ontario, Canada) for six cycles at 100 MPa. A heat exchanger was coupled to the system to avoid temperature increase. The obtained emulsions were stored in the dark at 4 ºC until analysis.

For the FFD 2^{4-1}, the emulsions were prepared using different O/W weight ratios, SpE concentrations, pHs, and storage times, according to the design matrix proposed in Table S1 (Supplementary material). For the CCRD 2^2, the O/W weight ratio was set at 30/70 and the pH at 7.0, being the SpE concentration and storage time defined by the CCRD 2^2 matrix Table S3 (Supplementary material).

2.4.3. Emulsion characterization

The obtained emulsions were characterized concerning the number-mean droplet diameter, relative span factor, zeta potential, and morphology. The number-mean droplet diameter and relative span factor were determined by the Laser Diffraction technique using a Malvern Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) equipped with a Hydro MV dispersion unit using water as the dispersing medium. The retrieved data were treated using the Malvern Software version 3.10. Zeta potential was evaluated using an electrophoresis instrument (Zetasizer Nano-ZS90, Malvern Instruments, Worcestershire, UK). The refractive index for the dispersed phase (olive oil) and continuous phase (water) were 1.47 and 1.45, respectively. The obtained emulsions were dispersed in water (1:100–1:300) to avoid multiple scattering effects. For each individual emulsion sample, the measurements for both droplet diameter and zeta potential, were performed in quintuplicate. Morphological analysis of the emulsions was performed by optical microscopy. The used microscope was a Ni-U Nikon (Nikon Instruments Europe, Amsterdam, The Netherlands) outfitted with a digital camera. To determine the type of emulsion (O/W or W/O) the drop test was used. For that, two or three drops of the emulsion were added into the water or oil phases followed by gentle stirring. If the emulsion droplets disperse in water and agglomerate in

Table 2
Protein and lipid contents, color parameters (a^*, b^*, and L^*), and RGB color for SpB, SpDB, and SpE.

	SpB	SpDB	SpE
Protein (wt%)	$58.7^{a} \pm 1.71$	$62.2^{b} \pm 0.06$	$66.6^{c} \pm 0.25$
Lipid (wt%)	$3.50^{a} \pm 0.42$	$0.15^{b} \pm 0.04$	$0.44^{b} \pm 0.07$
Colour			
L^*	$10.1^{a} \pm 0.38$	$12.8^{b} \pm 0.99$	$21.3^{c} \pm 0.92$
a^*	$-3.31^{a} \pm 0.09$	$-5.09^{b} \pm 0.15$	$-5.06^{b} \pm 0.21$
b^*	$6.02^{a} \pm 0.12$	$3.96^{b} \pm 0.04$	$-5.28^{c} \pm 0.37$
RGB colour			

Each value is expressed as mean ± standard deviation (n = 3). Different letters in the same line represent significant differences ($p \leq 0.05$).

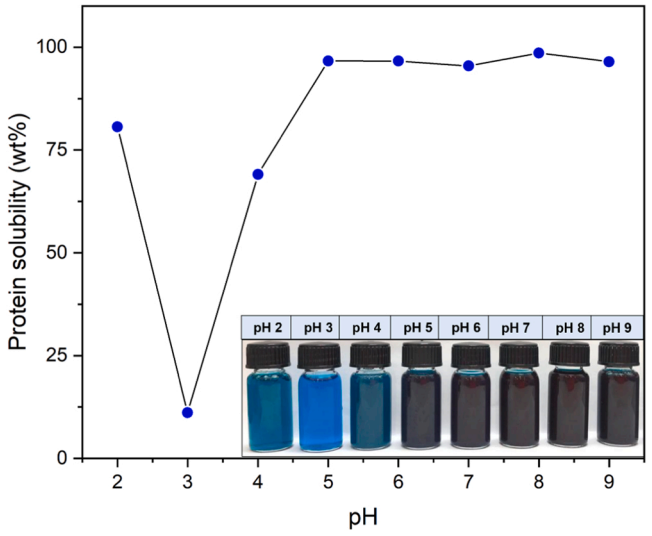

Fig. 1. : Protein solubility and visual appearance of the Spirulina protein extract (SpE) in solution at different pHs after centrifugation.

oil, the emulsion type is O/W, whereas if the inverse occurs the emulsion is of W/O type.

2.5. Statistical analysis

The experimental designs were analysed, and the response surface plots generated by TIBCO Statistica 14.0 software (Statsoft Inc, USA). The model adequacy was evaluated by the coefficient of determination (R^2) and the ANOVA (F-test) (CCRD 2^2 5% significant level (p-value ≤ 0.05). All the experimental designs (FFD and CCRD) were performed randomly.

The Tukey's test was carried out to compare the differences among the mean values of SpB, SpDB and SpE for protein and lipid contents, and color parameters (L^*, a^*, and b^*). The t Student's test was performed to evaluate the difference between means during the model validation. These statistical tests were carried out using IBM SPSS Statistics 28.0 software (IBM Corp., USA) at a 95% of confidence interval (p-value ≤ 0.05).

3. Results and discussion

3.1. Protein-rich extract

3.1.1. Extraction process

The used extraction process to obtain SpE from SpDB, which was an ultrasound-assisted process followed by isoelectric precipitation, was characterized by an extraction yield of 40.0 ± 1.91 wt%, and a protein yield of 43.6 ± 0.93 wt%. The obtained protein yield was 1.4 times higher than the one found by Yucetepe et al. [29] (30.1 wt%) using the same technology but applying different extraction conditions (ultrasound bath (4 °C, 60 min), followed by stirring in a water bath (45 °C, pH 7.46, time 120 min). A similar extraction yield (40.6 wt%) was obtained by applying high-shear homogenization (Ultraturrax, 9000 rpm) followed by isoelectric precipitation, as reported by Pereira et al. [30] in a study using Spirulina biomass. Nevertheless, comparatively with the present work, they disclosed a higher protein yield (63.3 wt%). Considering the work of Lupatini et al. [21], a higher extraction yield, but a lower protein yield was achieved in this work, which might be related to the used lower frequency (25 Hz). Higher ultrasonic frequencies can generate stronger cavitation, which can be more effective in disrupting the Spirulina cell walls, facilitating the protein released into the medium, resulting in higher protein yields [31]. The reference work that studied the optimization process by a sequential experimental design for protein and carbohydrate extraction from Spirulina (30 g/L; pH 9.0) by sonication (ultrasound bath; 35 min; 37 Hz) and mechanical stirring (50 min, 80 rpm), reported an extraction yield of 38.0 wt% and a protein yield of 75.76 wt%.

Many studies have found higher protein yields after an optimization procedure, being ultrasound cell-disruption technique followed by isoelectric precipitation the most widely used extraction process. Sánchez-Zurano et al. [24] have performed a sequential experimental design to optimize the protein solubilization and protein recovery by ultrasound-assisted technology (ultrasonic probe; 400 W; 24 kHz; 2 min) followed by isoelectric precipitation from *Arthrospira platensis*. The optimized precipitation conditions (pH 3.89; 45 min of stirring) generated a protein yield of 75.2 wt%.

3.1.2. Characterization

The results obtained for SpE characterizations concerning protein and lipid contents, and color parameters, are summarized in Table 2. For comparison purposes, namely to check the effect of the defatted step on lipids reduction and of the extraction process on protein concentration, the results obtained for SpB and SpDB were also included. Spirulina biomass is well-known for its high protein content, in the present work

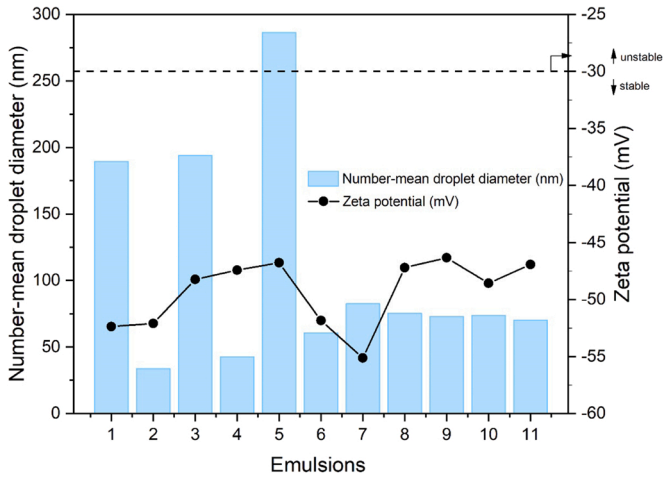

Fig. 2. : Results of the 11 trials (Emulsions) obtained by the Central Composite Rotatable Design 2^2 for number-mean droplet diameter (nm) and zeta potential (mV).

58.7 wt%, being possible with the applied methodology to concentrate it up to 66.6 wt%. After lipid extraction, an intermediate protein value of 62.2 wt% was obtained for SpDB. The obtained protein values are found to be significantly different for SpB, SpDB, and SpE, corroborating the positive effect of the performed treatment in the protein concentration. The amount of lipids decreased from 3.50 (SpB) to 0.15 wt% (SpDB), then increased to 0.44 wt% (SpE), showing the expected effect of the defatted process in decreasing lipids (significant differences were found), and a slight increase due to the extraction process (but not significantly different from SpDB).

Regarding color parameters, SpB showed a dark green color with a higher a^* value (−3.31) than SpDB (−5.09) and SpE (−5.06). Moreover, SpE showed a blue color (negative b value) attributed to the presence of the characteristic blue protein-pigment C-phycocyanin. This color modification might be an attractive attribute for industrial applications (e.g., food, cosmetic and pharmaceutical industries), as the dark green color of the Spirulina biomass was minimized. Compared to SpDB, SpE presented significant differences regarding L^* and b^* value.

Protein solubility is an important parameter affecting its techno-functional properties such as emulsification, foaming and gelation, as it measures the balance between protein-protein and protein-solvent interactions. The SpE solubility as a function of pH is shown in Fig. 1. The graphic presents a U-shaped trend with the lowest solubility at pH 3.0 (11.2 wt%), corresponding to the pI of the protein extract. The pI corresponds to the pH at which a protein has a neutral charge, which means that positive and negative charges are balanced, and thus no repulsion interactions exist. In contrast, at pHs far from pI (higher or lower), the protein carries a net charge that generates repulsive forces, and thus its solubilization [22,24]. The highest solubility was found at pH 8.0 (98.6 wt%), even from pH 5–9 the solubility remained basically constant. Fig. 1 also shows the visual appearance of the SpE solutions at different pHs after centrifugation. As the pH increases, the color of the solution turns to a deep blue, indicating that the extract presented higher solubility far from the pI (pH 3.0), enabling their application in aqueous systems.

Menegotto et al. [19] found the same behavior, but with the lowest solubility at pH 3.0 (3.23%) and the highest value (98.07%) achieved at pH 9 instead of 8. Benelhadj et a. [18] reported a lower maximum solubility (59.6%; pH 10). According to Jambrak et al. [32], ultrasonic radiation can improve solubility due to the partial unfolding of the protein structure, making the hydrophilic amino acids located inside the structure more exposed. In addition, cavitation can break non-covalent interactions, namely the hydrophobic and hydrogen bonding, improving protein-water interactions.

3.2. Evaluation of the protein-rich extract as an O/W emulsifier

The oil-in-water (O/W) emulsions were prepared using the SpE and by applying high-pressure homogenization (100 MPa; 6 cycles). The used preparation procedure has been reported to produce stable emulsions when using natural emulsifiers such as saponins [28]. Moreover, the combination of SpE with high-pressure homogenization might synergistically improve stability, a fact associated with the partial unfolding of the proteins and consequent exposition of some hydrophobic groups, contributing to better emulsifying performance [33]. The O/W type of the emulsion was confirmed by the drop test, as they become dispersed in water while remaining intact in oil.

Previous works have shown that pH and protein concentration are the main parameters affecting Spirulina-based extract's emulsifying properties [18,19]. However, from a technological point of view, to prepare O/W emulsions the O/W ratio and storage time are relevant parameters to consider since they impact emulsion formation and

Table 4
Analysis of Variance (ANOVA) (Percentage of the explained variance (R^2), $F_{calculated}$ and $Ft_{abulated}$ values) for the zeta potential (mV) and number-mean droplet diameter (nm) responses.

Sources of Variation	SS	df	QM	F_{cal}	F_{tab}	p-value
Zeta potential (mV) $R^2 = 75.08\%$						
Regression	66.1	2	33.0	12.0	4.5	0.0039 *
Residues	21.9	8	2.74			
Lack of fit	19.2	6	3.20	2.4	19.3	0.3258
Pure error	2.7	2	1.35			
Total	88.0	10				
Number-mean droplet diameter (nm) $R^2 = 99.41\%$						
Regression	63,002.0	2	31,501.0	669.3	4.5	0.0000 *
Residues	376.51	8	47.1			
Lack of fit	369.4	6	61.6	17.43	19.3	0.0553
Pure error	7.07	2	3.5			
Total	63,378.51	10				

* p-value ≤ 0.05. SS: Sum of squares; df: degrees of freedom; QM: quadratic means.

Table 3
Regression coefficients estimates of the models for the zeta potential (mV) and number-mean droplet diameter (nm) from the CCRD 2^2 results.

Factor	Zeta potential (mV)				Number-mean droplet diameter (nm)			
	Regression coefficient	Standard error	t (5)	p-value	Regression coefficient	Standard error	t (5)	p-value
Mean	-47.28	0.90	-52.73	0.0000 *	72.31	4.96	14.57	0.0000 *
x_1 (L)	-0.76	0.55	-1.38	0.2273	-78.37	3.04	-25.75	0.0000 *
x_1 (Q)	-0.97	0.66	-1.47	0.2010	47.97	3.63	13.21	0.0000 *
x_2 (L)	2.51	0.55	4.56	0.0061 *	0.36	3.04	0.12	0.9108
x_2 (Q)	-1.90	0.66	-2.89	0.0342 *	0.41	3.63	0.11	0.9154
$x_1 \cdot x_2$	0.14	0.78	0.17	0.8688	1.13	4.30	0.26	0.8026

(L): Linear terms; (Q): quadratic terms; Zeta potential: R^2: 86.29%; adjusted R^2: 72.58%; Number-mean droplet diameter: R^2: 99.42%; adjusted R^2: 98.83%; * Significant factors ($p \leq 0.05$). x1: SpE concentration (wt%); x2: storage time (Days).

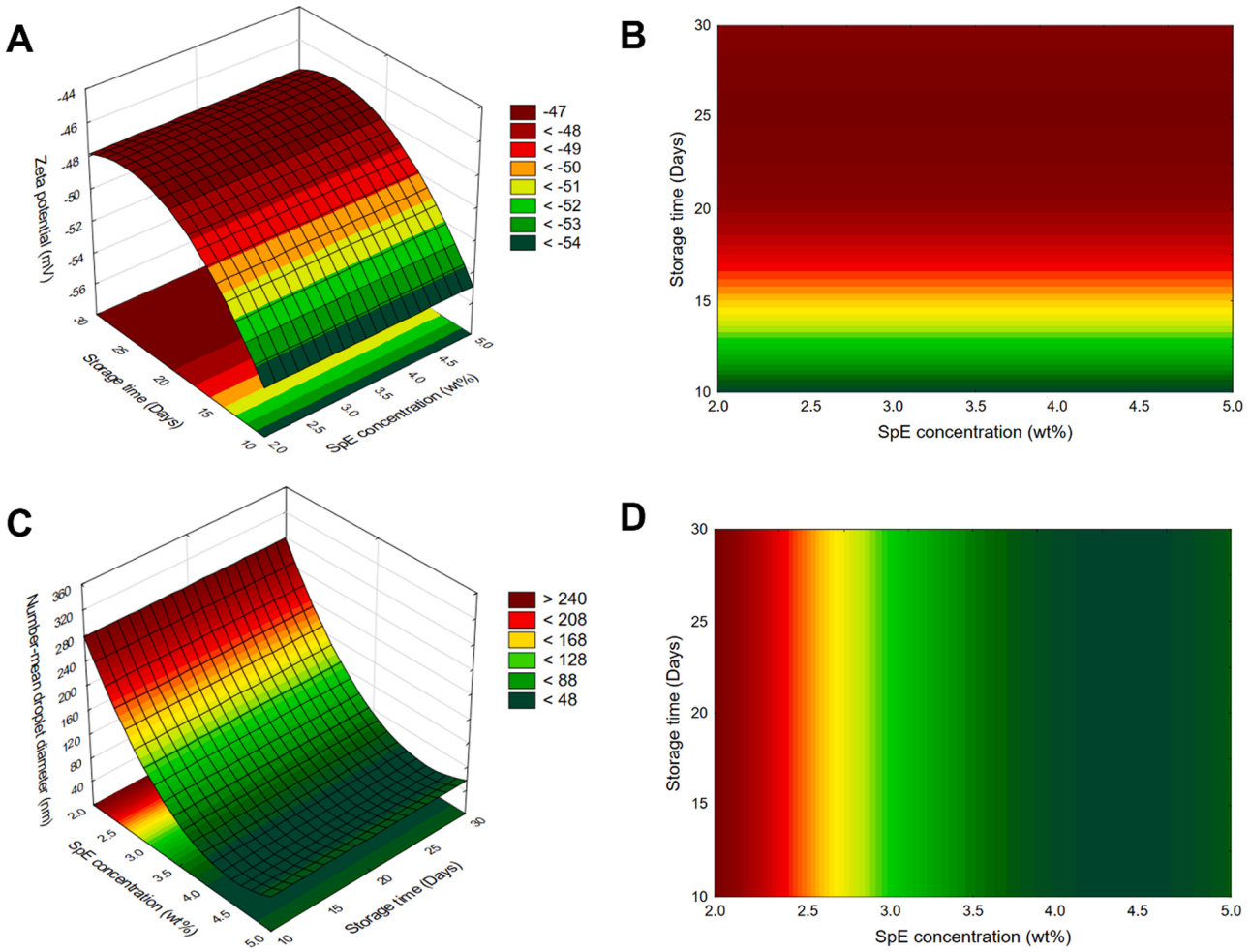

Fig. 3. : Response surfaces (A, C) and contour plots (B, D) for Zeta potential (mV) (Y_1), Number-mean droplet diameter (nm) (Y_2) as a function of SpE concentration (wt%) (x_1) and Storage time (Days) (x_2) of the emulsions, respectively.

stability [9]. Thus, in this work a first screening design was carried out through a Fractional Factorial Design 2^{4-1} to get insights into the effects of these four variables (O/W weight ratio, SpE concentration, pH, and storage time) on the emulsion's number-mean droplet diameter and zeta potential.

Zeta potential (mV) and droplet diameter are the most common used characterizations for colloidal systems, which can be related with emulsion stability. Zeta potential provides the net charge of the particle surface, which can be positive or negative. In general, particles with more negative or positive zeta potential (< -30 mV or $> +30$ mV) have enough repulsive forces to present high colloidal stability. Otherwise, less negative or positive zeta potential values (> -30 mV or $< +30$ mV) can generate particle's aggregation and flocculation [34–36]. Droplet diameter can be determined by Laser Diffraction (LD), a well-established technique for size distribution determinations in the range 0.01–3500 μm. Generally, the smaller is the droplet diameter, the higher is the emulsion stability [37,38].

The variable's range for the FFD 2^{4-1} was chosen according to previous studies and preliminary experiments. They showed that at pH 3.0 (SpE pI) no emulsion was formed, letting to fix the pH in the range 4–7. The O/W weight ratio was considered in the range 10/90–30/70, allowing to inspect the ability of the SpE to stabilize emulsions for different oil contents. The storage time was considered from 0 to 14 days to analyse the effect of time on the defined responses, and thus stability. Menegotto et al. [19] earlier reported that the studied Spirulina protein concentrate showed good emulsifying capacity in the range 0.9–4%. Accordingly, in this work, the SpE concentration was ranged from 1% to 4% to verify its effect on emulsions formation.

The results and the effects estimated by the analysis of the FFD 2^{4-1} are shown in Table S1 and Table S2, respectively (Supplementary material). All the obtained emulsions presented negative zeta potential values (−55.06 to −34.68 mV), being within the stability range, and the number-mean droplet diameters were in the range 18.28–518.2 nm. Among the determined effects for the O/W weight ratio, SpE concentration, pH and storage time on the zeta potential and number-mean droplet diameter, only the storage time was found to have a significant effect ($p \leq 0.10$) on zeta potential. The other studied variables (O/W weight ratio, SpE concentration and pH) had no statistically significant effects for both studied responses in the defined ranges. Although SpE concentration was not statistically significant ($p > 0.10$), it presented a p-value near 0.1 (p-value = 0.1175), being thus also considered as a relevant variable to include in the CCRD 2^2 design.

Since pH and O/W weight ratio were not significant, their values were fixed in the CCRD 2^2 experimental design, namely a pH of 7.0 (neutral pH) was chosen to evaluate the emulsion in accordance with drinking-water pH, facilitating future applications development. The O/W weight ratio was set to 30/70, the higher analysed O/W weight ratio. The SpE concentration (wt%) and storage time (days) were established as the most relevant variables to evaluate the effect on number-mean droplet diameter and zeta potential. The SpE concentration range was increased (2–5 wt%) since, according to the previous screening analysis, higher SpE concentrations decrease zeta potential and the number-mean droplet diameter and thus increase the potential stability of the emulsion. Regarding the storage time, the period was increased up to 30 days

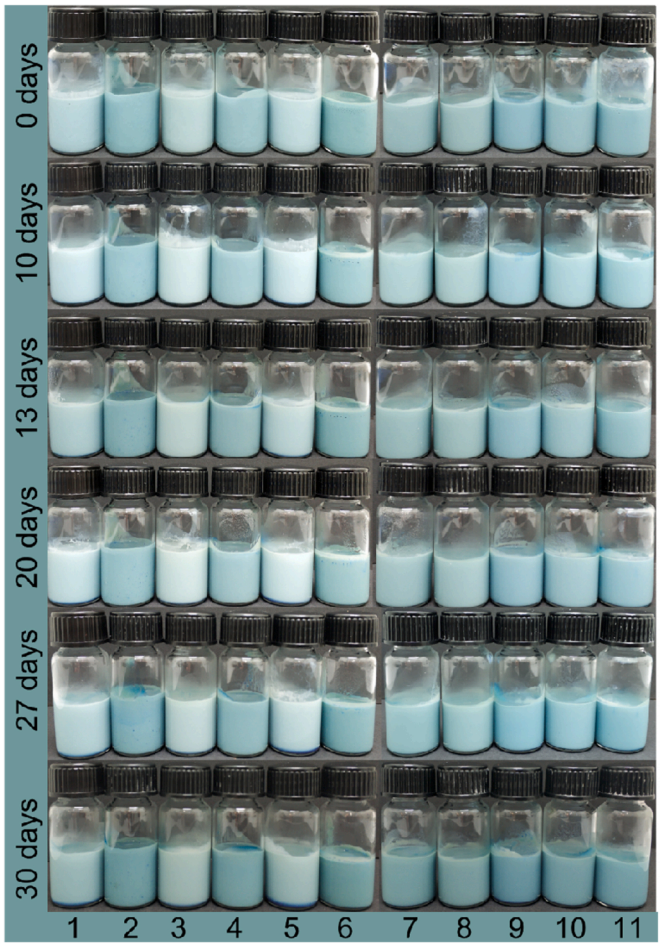

Fig. 4. : Visual appearance of the obtained emulsions from the CCRD 2^2.

since the emulsions remained stable up to 14 days. Thus, the objective was to evaluate SpE potential to stabilize emulsions for a long-term storage time (1 month).

The results of the CCRD 2^2 are depicted in Fig. 2. The detailed predicted and experimental values, as well as the relative errors are summarized in Table S3 (Supplementary material). The central points (trials 9–11) showed negligible variations, which indicates a good reproducibility of the process. The produced emulsions presented negative zeta potential values ranging from − 55.12 mV to − 46.34 mV, within the stability range (< −30 mV).

The relative span factor of the emulsions obtained for the FFD 2^{4-1} and DCCR 2^2 designs are displayed in Tables S1 and S3, respectively. It can be noticed that higher relative span factors were obtained using higher SpE concentrations, indicating that wider droplet diameter distributions were achieved. From the regression analysis, it was possible to obtain the regression coefficients of the studied variables (SpE concentration and storage time), which are highlighted in Table 3. The CCRD 2^2 showed that only the storage time had a significant positive effect on the zeta potential response ($p \leq 0.05$). These findings indicate that the zeta potential increased over time (from 10 to 30 days), which means that zeta potential values become less negative and, therefore, the emulsions less stable. However, it remained within the stability range (≤ -30 mV).

For the produced samples, the lowest number-mean droplet diameter was 33.68 nm (4.6 wt% SpE;13 days), and the highest was 286.3 nm (2 wt% SpE; 20 days). Results showed that the higher the SpE concentration is, the smaller is the number-mean droplet diameter, while the higher the storage time is, the higher is the number-mean droplet diameter. From the CCRD 2^2, only SpE concentration had a significant and negative effect on number-mean droplet diameter, indicating that it can be decreased using the higher SpE concentration within the studied range (2–5 wt%). These findings clearly show that SpE act as an effective emulsifier for O/W emulsions, similarly to other soluble protein extracts and protein isolates that have shown to present interfacial activity and stabilize O/W emulsions [7].

Thereafter, ANOVA was applied to identify the adequacy of the fitted model (Table 4). The coefficient of determination (R^2) and calculated F values ($F_{calc} > F_{tab}$) were adequate to obtain the second-order model. Moreover, the relative errors between the experimental and predicted model values showed their fitting to the experimental data (Table S3, Supplementary material).

Concluding, the mathematical models for zeta potential and number-mean droplet diameter as a function of the coded independent statistically significant ($p \leq 0.05$) variables (x1: SpE concentration (wt%); x2: storage time (days)) were constructed within the studied ranges. The non-statistically significant parameters were incorporated into the residues, and the mathematical models re-parameterized (Eqs. 4 and 5):

$$Y_1 \quad (\text{zeta potential, mV}) = -48.19 + 2.50x_2 - 1.61x_2^2 \tag{7}$$

$$Y_2 \quad (\text{number-mean droplet diameter, nm}) = 72.69 - 78.37x_1 + 47.85x_1^2 \tag{8}$$

The predictive models were used to generate the response surface and contour plots. In Fig. 3 A and B, it can be noticed that the higher the storage time is (10–30 days), the higher the zeta potential becomes, but remaining within the stability range (≤ -30 mV). On the other hand, from Fig. 3 C and D, it was possible to see that the higher the SpE concentration is (2–5 wt%), the lower is the number-mean droplet diameter, showing an explicit dependency of this response from protein concentration.

These statements were corroborated by the visual inspection of the emulsions (Fig. 4). All the emulsions presented a light blue-green color, and most of them remained stable along the storage period of 30 days. Emulsions with the highest SpE concentrations (3.5, 4.6, and 5 wt%) presented higher stability over time. This fact can be explained due to their smaller number-mean droplet diameter, which is less prone to gravitational forces, preventing instability phenomena such as creaming and coalescence [39]. Otherwise, emulsions with lower SpE concentrations (2 and 2.4 wt%) were less stable, showing a blue sediment from 20 days. Additionally, no signs of contamination by microorganisms (e.g.,

Table 5
Results of the validation experiments regarding experimental and predicted values of the chosen conditions (4 and 5 wt% of SpE; 20 and 30 days of storage) for zeta potential (mV) and number-mean droplet diameter (nm).

Trial x_1 (x_2)	Zeta potential (mV)				Number-mean droplet diameter (nm)			
	Experimental	Predicted	Re	p-value	Experimental	Predicted	Re	p-value
4 (20)	-45.97 ± 1.13	-48.19	-4.82	0.3023	55.81 ± 8.79	47.16	15.5	0.1150
5 (20)	-44.79 ± 1.30	-48.19	-7.58		34.20 ± 16.4	57.46	-68.0	
4 (30)	-43.83 ± 1.46	-47.87	-9.22	0.4239	55.66 ± 8.18	47.16	15.3	0.0782
5 (30)	-42.46 ± 2.23	-47.87	-12.73		33.67 ± 14.0	57.46	-70.6	

Mean ± standard deviation of triplicates. p-value ≥ 0.05 indicates that there is no difference between trials by Student's *t*-test. Re: [((Experimental – Predicted)/Experimental)* 100)] relative error (%). x_1: SpE concentration (wt%); x_2: storage time (days).

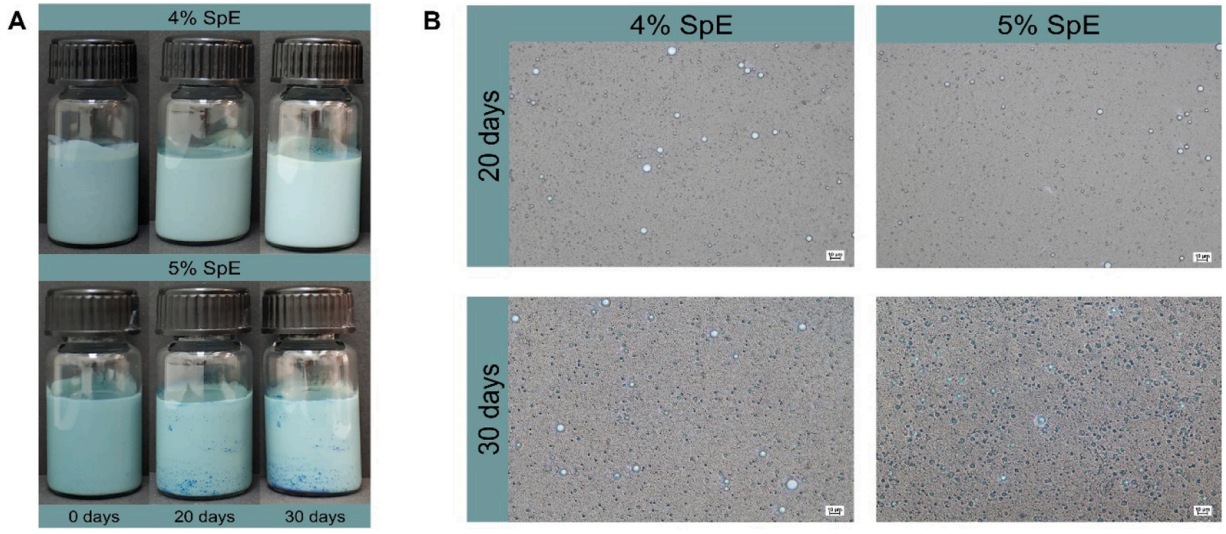

Fig. 5. : Visual appearance of the emulsions obtained from the validation experiments (A). Microscopic visualization (200 X) of the emulsions obtained from the validation experiments (B).

mold (fungi)) were observed in the emulsions over time, suggesting that the SpE may also acts as an antimicrobial agent. However, to have more conclusive statements, further studies on the emulsion microbiological stability are needed to be carried out.

As shown in Fig. 2, the emulsions (7–11) where 3.5 wt% of SpE was applied, no significant changes on the number-mean droplet diameter were observed (10, 20 and 30 days). From this analysis, it can be noticed that intermediate SpE concentrations (3.5 wt%) give rise to a suitable behavior along time. All the other sample trials remained stable against creaming along the studied storage time, indicating that SpE can be an effective emulsifier when concentrations higher 3.5 wt% are used.

After analysing the response surfaces, a validation experiment was performed to authenticate the observed results. As the smaller number-mean droplet diameters were found for the higher SpE concentrations, the chosen conditions considered 4 wt% and 5 wt% of SpE, being analysed after 20 and 30 days of storage to validate that the emulsions remained stable. The results of the three runs, the predicted values and relative errors are displayed in Table 5.

Only the relative error for number-mean droplet diameter (5 wt%, 20 and 30 days) presented values higher than 30%. This fact happened because the experimental number-mean droplet diameter (34.20 nm (20 days); 33.67 nm (30 days)) were lower than the predicted values (57.46 nm (20 and 30 days)), indicating that emulsions with lower number-mean droplet diameters than the predicted ones could be obtained. The differences between the number-mean droplet diameter of the two conditions were analysed by the Student's *t*-test ($p \leq 0.05$). Regarding zeta potential and number-mean droplet diameter, no differences were found between the two conditions on the 20th and 30th day of storage, suggesting that applying 4 wt% or 5 wt% of SpE can give rise to stable emulsions. Microscopic visualization also corroborated this behavior (Fig. 5B) since both emulsions presented a morphology of spheric and tiny droplets (number-mean droplet diameter $\leq$ 55.66 nm) after 30 days of storage (4ºC). Nevertheless, according to the visual appearance (Fig. 5A), the emulsion with 4 wt% of SpE presented a homogeneous appearance. In comparison, the emulsion with 5 wt% showed some blue points after the 20th day, indicating an excess of emulsifier.

These findings show that higher SpE concentrations (5 wt%) can generate emulsions with smaller number-mean droplet diameters; however, in this specific case (O/W weight ratio (30/70); pH (7.0)), the emulsifier concentration might be in excess, which means that there is more emulsifier than the one needed to be adsorbed and cover the complete oil/water interfacial area. In this situation, the number-mean droplet diameter and emulsions' stability mainly depend on the energy input of the homogenization technology [3].

According to Rowe [40], to produce a physically stable emulsion a minimum emulsifier concentration is needed. Increasing the emulsifier concentration over this threshold will result in a decreased number-mean droplet diameter but the gain in stability can become not significant. Likewise, the use of high amounts of natural emulsifiers in emulsions is a critical point since it leads to process cost increase, compromising the economic viability of the process.

Therefore, this work demonstrates that, apart from applying two or more characterization techniques, the visual inspection is also an important tool to better establish the proper conditions to obtain and validate stable emulsions. In this context, the best formulation and production conditions to produce a highly stable O/W emulsion up to 30 days under refrigerated storage conditions (4 ºC), correspond to 30/70 O/W weight ratio, pH 7.0 and 4 wt% of SpE, applying high-shear followed by high-pressure homogenization (100 MPa; 6 cycles). Results also show that 3.5 wt% of SpE can be a satisfactory condition; however, emulsions with 4 wt% of SpE showed smaller number-mean droplet diameters.

4. Conclusions

Through this work, the ability of a Spirulina protein-rich extract to form stable emulsions for a long-term storage time (30 days) was demonstrated. The optimal conditions were determined using a sequential experimental design strategy. Concerning the effect of the most influential variables, SpE concentration increase imply lower number-mean droplet diameters, and storage time increase gives rise to higher zeta potential, but within the stability range (<-30 mV). The best conditions correspond to the formulation using 4 wt% of protein-rich extract, a O/W weight ratio of 30/70 and a pH of 7.0 (number-mean droplet diameter of 55.66 nm and zeta potential of −43.83 mV). For higher SpE concentrations (5 wt%), and after 20 days, the emulsion showed signs of emulsifier segregation due to a potentially excessive extract concentration.

Overall, the obtained results pointed out the promising use of the Spirulina protein-rich extract as a natural O/W emulsifier, which can be proposed to replace animal-based proteins and synthetic counterparts. Nevertheless, purification processes and low protein yield can constitute a bottleneck to the economic competitiveness of the productive process, demanding an integrated valorization of the whole productive chain. Through this integrated approach, other compounds can be obtained

and valorized, such as the lipid fractions, which can be targeted for biodiesel or essential fatty acids production, reducing the costs of the protein extraction process, promoting sustainability and enhancing Spirulina technological use, within a biorefinery concept. Moreover, although Spirulina is not generally considered as a source of allergens, the allergenicity risk assessment should be considered in future studies for the consolidation of microalgal protein applications development. Other needed stages are final product development, acceptability evaluation, and stability studies against external environmental stresses to achieve marketable products with impact in several industrial areas.

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CRediT authorship contribution statement

Samara Silva: Methodology, Investigation, Validation, Data curation, Writing – original draft**. Thayná Almeida:** Methodology, Investigation, Data curation. **Giovana Colucci**: Methodology, Investigation. **Arantzazu Santamaria-Echart**: Methodology, Investigation; **Yaidelin A. Manrique**: Methodology, Investigation. **Madalena M. Dias**: Supervision, Writing – review&editing. **Lillian Barros**: Methodology, Resources. **Ângela Fernandes**: Methodology, Supervision. **Eliane Colla**: Conceptualization, Methodology, Supervision, Writing – review & editing; **M. Filomena Barreiro:** Conceptualization, Methodology, Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2022.129264.

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