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Functional properties and antioxidant activity of protein fractions of spirulina (*Arthrospira maxima*)

Propiedades funcionales y actividad antioxidante de las fracciones proteicas de la espirulina (*Arthrospira maxima*)

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Abstract
In this research, spirulina of the species *Arthrospira maxima*, produced and donated by the producer association called "NanoMex Espirulina" located in the city of Tlaxcala, Tlax., Mexico, was used. The proximal chemical composition of spirulina was determined. Sequential protein extraction was performed from frozen and macerated spirulina. Fractions were obtained based on protein solubility: albumins (water), globulins (0.4 mM Na_2SO_4) and glutelins (pH 11), but not prolamins (70% ethanol). Some functional properties, antioxidant activity and electrophoretic profile of the protein fractions obtained were determined. The albumins fraction presented the highest yield, high water retention capacity and high solubility. The highest antioxidant activity was observed in the albumins and glutelins fractions. Most of the proteins in spirulina are soluble in water (albumins) and of low molecular weight, these proteins have potential use in the food industry in the design and production of protein-added beverages.
Keywords: albumins, antioxidant activity, functional properties, spirulina, proteins.

Resumen
En esta investigación se utilizó espirulina de la especie *Arthrospira maxima*, producida y donada por la asociación productora llamada "NanoMex Espirulina" ubicada en la ciudad de Tlaxcala, Tlax., México. Se determinó la composición química proximal de la espirulina. Se realizó una extracción proteica secuencial a partir de espirulina congelada y macerada. Se obtuvieron fracciones con base en la solubilidad las proteinas: albúminas (agua), globulinas (0.4 mM Na_2SO_4) y glutelinas (pH 11), no así de prolaminas (etanol 70%). Se determinaron algunas propiedades funcionales, la actividad antioxidante y el perfil electroforético de las fracciones proteicas obtenidas. La fracción de albúmina presentó el mayor rendimiento, elevada capacidad de retención de agua y elevada solubilidad. La mayor actividad antioxidante se observó en las fracciones de albúmina y glutelina. La mayoría de las proteínas de la espirulina son solubles en agua (albúminas) y de bajo peso molecular, estas proteínas tienen potencial uso en la industria alimentaria en el diseño y elaboración de bebidas adicionadas de proteinas.
Palabras clave: albúminas, actividad antioxidante, propiedades funcionales, espirulina, proteínas.

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1 Introduction

The term spirulina is used to refer to the species *Arthrospira platensis* and *Arthrospira maxima* (Tomaselli, 1997; Vonshak and Tomaselli, 2000; Sánchez *et al*., 2003; Ramírez-Moreno and Olvera-Ramírez, 2006). Spirulina is a cyanobacteria, but because it contains chlorophyll it is also considered a microalga (Ramírez-Moreno and Olvera-Ramírez, 2006). *Arthrospira maxima* and *Arthrospira platensis* form massive populations in tropical and subtropical water bodies characterized by high carbonate and bicarbonate levels and high pH (up to 11) (Sánchez *et al*., 2003). *Arthrospira platensis* appears to be a more widely distributed species, found mostly in Africa, however also in South America and Asia, while *Arthrospira maxima* appears to be essentially confined to Central America. This last species represented the main component of the phytoplankton of Lake Texcoco, Mexico, which could have been the original habitat of this species (Sánchez *et al*., 2003). Similarly, the alkaline saline lakes of the semi-desert zone of the Sudan-Sahel, with an epicenter in Lake Chad, and those of the Rift Valley, dominated by water blooms of *Arthrospira platensis*, can be considered the starting points of this species (Tomaselli, 1997). Currently, spirulina is produced in many parts of the world, it is worth mentioning that the main producers in Latin America are Cuba, Chile, Peru, Ecuador, Brazil and Mexico. It is worth mentioning that Mexico presents a great biodiversity due to its territorial extension and the different climates and geographic surfaces, for which it includes a great diversity of algae, many of which have been studied for their production and human and animal consumption, within these algae are spirulina (Sosa-Hernández *et al*., 2019). In Mexico there are companies, organizations and artisan producers of spirulina, highlighting the production in the states of San Luis Potosí, State of Mexico, Monterrey, Jalisco, Puebla, Hidalgo, Sonora and recently Tlaxcala. The industrial production of spirulina has been carried out in open raceway ponds or in greenhouses, seeking to establish the conditions that favor the increase in its production, among which is the type and concentration of the carbon source, which are mainly $NaHCO_3$, Na_2CO_3 and CO_2, together with the alkaline pH that favors the solubility and availability of CO_2 and nutrients, phosphorus is an essential micronutrient. Temperature, relative humidity, and light-dark cycles are also important during its production (Costa *et al*., 2002; Rodríguez-Mata *et al*., 2019; Zhu *et al*., 2020; Solis-Méndez *et al*., 2020). Spirulina has bioactive compounds and has been considered an important source of nutrition for humans and animals (Costa *et al*., 2002; Gershwin and Belay, 2007). There are many studies on the bioactive properties of spirulina, among which can mention: anti-nephrotoxicity, anti-genotoxicity, antiviral activity, anti-inflammatory property, anti-obesity and weight loss, hypoglycemic and hypolipidemic property, neuroprotective property, anti-cancer effects, anti-anemic activity, probiotic property, spirulina for eyesight, immunological applications (Anvar and Nowruzi, 2021). In addition, spirulina biomass is called unicellular protein, which can be used to solve malnutrition problems, and currently plays an important role in a wide range of industries; to mention some products added to spirulina are cookies, ice cream, fresh cheeses, biscuits, etc., where it is possible to increase the content of proteins (including phycobiliproteins), minerals, vitamins, fatty acids and antioxidant activity, among others (Gershwin and Belay, 2007; Ducat *et al*., 2011; Taton *et al*., 2012; Anvar and Nowruzi, 2021). Although proteins are among the most specific substances obtained from algae, serious gaps remain to be filled in terms of protein characterization. Spirulina seems to be among the best sources of protein with values of 60-70% dry weight (Paoletti *et al*., 1980). Spirulina protein contains a large amount of phycobiliprotein (approximately 20% of the total protein content) among which are the phycocyanins known for their attractive blue color and their great effect on health in the human body (Hirata *et al*., 2000; Bhat and Madyastha, 2001). Phycocyanins include C-phycocyanin, R-phycocyanin, and allophycocyanin. The hepatoprotective effect (Deng and Chow, 2010), antioxidant and anti-inflammatory effect of C-phycocyanin (Ferreira-Hermosillo *et al*., 2010), as well as its behavior as a free radical scavenger have been observed (Gantar *et al*., 2012). On the other hand, the classification of proteins according to Osborn classification based on their solubility mentions four, albumins (soluble in water), globulins (soluble in diluted saline solutions), glutelins (soluble in acidic or alkaline media) and prolamins (soluble in 50-80% ethanol) (Chen *et al*., 2018; Yang and Sagis, 2021). The solubility of proteins depends largely on the amount, type and distribution of the amino acids that they contain, as well as on the state of their structure, that is, the polar and non-polar amino acids that are exposed and available for interaction with the solvent will determine the solubility of the protein. Functional properties are defined as any physical-chemical property of proteins that modifies the behavior and characteristics of the foods in which they are added or already found and that affect the final quality of the product, so it is important to study their behavior for use in the design and preparation of food. The functional properties of proteins are classified into three groups based on the interaction of proteins with each other or proteins with water, 1) protein-water interactions (such as solubility, water retention, viscosity, etc.); 2) protein-protein interactions (precipitation, gelation, among others) and 3) protein-interface interaction (foaming, emulsification, etc.). The functional properties of proteins may depend mainly on intrinsic factors of the protein (structure, type and number of amino acids as well as their arrangement, ratio of hydrophilic and hydrophobic amino acids, electrical charge, shape and molecular weight), and also on extrinsic factors, that is, of the medium in which they are found and that is variable (temperature, pH, water activity, ionic strength and dielectric constant) (Badui,

2019; Flores-Silva *et al*., 2022). It is important to mention that these properties can vary depending on the degree of protein denaturation. There are few scientific publications available on the functional properties of spirulina proteins, being studies carried out on all the proteins and mainly on those obtained from *Arthrospira platensis*. Among the functional properties of spirulina proteins that have been reported are mainly water solubility, water and oil retention capacity, as well as foaming and emulsification capacity and stability (Bleakley and Hayes 2021; Benelhadj *et al*., 2016; Mahajan and Ahluwalia, 2010; Nirmala *et al*., 1992). In this sense, *Arthrospira maxima* proteins were fractionated based on their solubility and some functional properties, their antioxidant capacity, as well as their electrophoretic profile were determined.

2 Materials and methods

2.1 Spirulina and proximal chemical analysis

Spirulina of the species *Arthrospira maxima* was used, which was donated by the producer association called "NanoMex Espirulina" located in the city of Tlaxcala, Tlax., Mexico, which is produced in ponds inside a greenhouse with control of temperature, relative humidity, light and nutritional composition, after harvesting it is filtered until reaching a concentration of 20% solids and is frozen at -20ºC. Light microphotographs were taken of the spirulina, using a microscope Nikon Optiphot-2 with TOUPCAM camera and ToupView (x64, 3.7.1460) software. Proximal chemical analysis (moisture content, ash, ether extract, crude protein, dietary fiber and nitrogen-free extract by difference) was performed at the spirulina and crude protein content was determined for all protein fractions obtained. All analyzes were performed using the AOAC methodology (AOAC, 2019).

2.2 Obtaining protein fractions

The fractionation process based on the Osborne classification dependent on the solubility of proteins (Yang and Sagis, 2021), was carried out from dehydrated spirulina (dried in an oven at 45 ºC until constant weight, the resulting paste was pulverized in a mortar) and also from the maceration of frozen spirulina, looking for the method that favors a higher yield of protein fractions. With both samples, a suspension of spirulina at 0.9% (dry weight of spirulina/water) was prepared to start with the extraction of albumins, later with the globulins, glutelins and finally the prolamins, where in each case the solvent was added until reaching the dry weight concentration of 0.9% w/v. The extractions were performed sequentially, at each stage, the mixture was kept under stirring for 3 h, at refrigeration temperature to solubilize the proteins, subsequently, they were centrifuged at 5000 rpm for 15 min, the sediment was used to obtain the following fraction; solubilized proteins were precipitated with 5% trichloroacetic acid, proteins were recovered by centrifugation at 5000 rpm for 5 min, then dried and pulverized. To obtain the extraction yield of the protein fractions, the amount of spirulina in dry weight used at the beginning of the extraction (0.9% w/v) was taken as reference. The yield of crude protein in each fraction was determined based on the amount of protein in the spirulina used at the beginning of the process. The albumins were obtained through an aqueous extraction at pH 7.0, the globulins with 0.4 M Na_2SO_4 (pH 7.0), the glutelins in water and the pH was adjusted to 11 with 0.1 M NaOH, the prolamins in 70% alcoholic solution (pH 7.0). Given that the extraction yield of the protein fractions was higher with frozen and macerated spirulina (see results section), all other activities were carried out with these fractions.

2.3 Evaluation of functional properties

2.3.1 Solubility of protein fractions

A 1% (w/v) suspension of each protein fraction was prepared in the solvent of each extraction (water for albumins and saline solution for globulins, both at pH 7.0, for glutelins the pH was adjusted to 11, prolamins were not obtained). The suspension was kept under stirring at room temperature for 30 min. The suspensions were centrifuged (5000 rpm for 15 min), the non-solubilized and sedimented residue was weighed to calculate the amount of solubilized fraction (g/100g) (Wang and Kinsella, 1976).

2.3.2 Water or oil retention

0.5 g of each protein fraction was homogenized at maximum speed in a vortex (Genie-2, Scientific Industries) for 2 min in the presence of water or oil. Subsequently, they were left to stand for 30 min and centrifuged at 3500 rpm for 25 min. Finally, the amount of water or oil not absorbed by the material was measured and by difference with the original volume added, the amount absorbed was calculated (Wang and Kinsella, 1976).

2.3.3 Emulsification and emulsification stability

Water and oil (in equal volumes) were mixed in the presence of the protein fraction (1% w/v), after stirring in a vortex at maximum speed for 1 min, then centrifuged at 1500 rpm for 5 min, the amount of oil and water, comparing with the initial values the amount emulsified was calculated (Wang and Kinsella, 1976). Stability was determined in the presence of heat; the emulsion was kept in a water bath at 80 ºC for 30 min. After cooled in water to 15°C, the emulsion was centrifuged at 1500 rpm for 5 min. For the evaluation of stability, the height of the emulsion was measured before the

thermal treatment and the remaining height of the emulsion after centrifugation (Wang and Kinsella, 1976).

2.3.4 *Foaming and foaming stability*

For each protein fraction, a 1% (w/v) suspension in distilled water was prepared. For the formation of the foam, each suspension was subjected to homogenization at a speed of 4000 rpm for 90 s and the volume of the foam obtained was measured, after 1 h, at rest the stability of the foam was determined (Kabirullah and Wills, 1982).

2.4 *Antioxidant activity evaluation*

Antioxidant activity was evaluated with the ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) methods (modified of Gonzalez-Palma *et al*., 2016). To determine the IC_{50} (sample concentration at which 50% of $ABTS^{+}$ or DPPH free radicals are inhibited), were used 0.2, 0.5, 1.0, 2.0 and 4.0 mg/mL for the albumins fraction and 0.1, 0.25, 0.5, 1.0 and 2.0 mg/mL for globulins and glutelins, adjusting the final volume to 200 μL with water and adding 800 μL of the radical, either $ABTS^{+}$ or DPPH. The percentage of inhibition of both radicals was obtained by the relationship between the initial and final absorbance in the test, and the IC_{50} values were calculated from the equation of the straight line resulting from the graph representing the sample concentration required to scavenge 50% of the ABTS or DPPH free radicals. ABTS (2mM) was reacted with potassium persulfate (2.45 mM final concentration) in the dark for 12-16 h at room temperature to obtain the radical. The absorbance (734 nm) of the $ABTS^{+}$ radical was adjusted to 0.700 (±0.2) with water. The mixtures were incubated in the dark at room temperature for 6 min and their absorbance was read. For the test with the radical DPPH (0.5 mM in methanol), the mixtures were incubated in the dark for 45 min at room temperature, recording the decrease in absorbance (517 nm).

2.5 *Electrophoretic profile*

The electrophoretic profile was obtained from the spirulina proteins and from each protein fraction in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli (1970), using the MiniProtean III equipment (BioRad) at 150 V, staining was done with Coomassie Brilliant Blue R-250. Molecular weights were determined using molecular weight markers (Low 14.4-97.4 kDa, Catalog Number 1610304. BIO-RAD). 5 μL of the molecular weight marker and 20 μL of each sample containing 60 μg in the case of spirulina and albumins and 20 μg of either globulins or glutelins were placed.

2.6 *Statistical analysis*

All evaluations were performed in triplicate and the mean ± standard deviation (SD) is reported. Analysis of variance and Tukey's test ($p<0.05$) were performed using the StatView 5.0 program.

3 Results and discussion

3.1 *Proximal chemical composition*

Figure 1 shows a light microphotograph of the spirulina used, where the typical morphology of *Arthrospira maxima* can be observed, such as the arrangement of multicellular cylindrical trichomes in a helix throughout the entire body (Lee *et al*., 2017). The proximal chemical composition of spirulina is reported in Table 1, it should be mentioned that the spirulina provided by the producer contains 20% dry matter. It was observed that spirulina had a high crude protein content (61.6 g/100 g dry weight), and low values of ethereal extract and dietary fiber.

Table 1. Proximal chemical composition of spirulina.

Component	+Wet basis (g/100g)	Dry basis
Moisture	7.5±1.2	–
Crude protein	57.0±2.0	61.6
Ash	10.0±0.5	10.8
Ethereal extract (Lipids)	6.0±0.3	6.5
Dietary fiber	4.5±0.2	4.9
*Nitrogen free extract (Carbohydrates)	15	16.2

+Mean ± SD. *Obtained by difference.

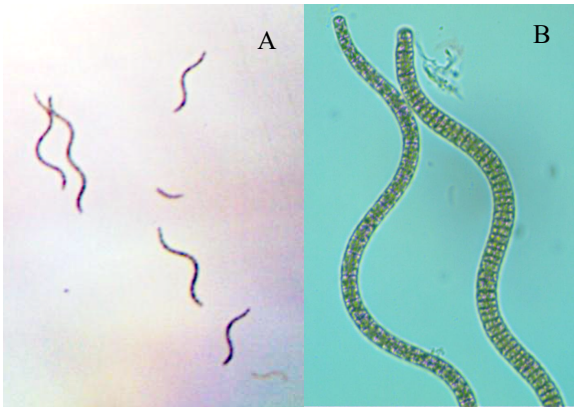

Figure 1. Light microphotographs of spirulina used in this study. 4X (A), 40X (B).

Table 2. Yield of protein fractions in dry weight.

Fractions	Dried spirulina (g/100 g)	Frozen spirulina (g/100 g)
Albumins	8.9 ± 1.0^{b}	43.1 ± 1.5^{a}
Globulins	3.23 ± 0.2^{b}	4.15 ± 0.1^{a}
Glutelins	1.02 ± 0.1^{b}	1.76 ± 0.05^{a}

Values are mean ± SD. In the same row, different letters indicate that there are significant differences, $p<0.05$.

In our study, a higher protein content was observed than that reported in other studies; to mention a few, Affan *et al*., (2015a) obtained 56.59% for the same species, and in *Arthrospira platensis* it has been reported 58.20% (Alvarenga *et al*., 2011), 50.24 and 58.61% (Ngakou *et al*., 2012), 56.79% (El-Moataaz *et al*., 2019), 61.57% in Arthrospira sp. (Sharoba *et al*., 2014), however, Seghiri *et al*. (2019), indicated a value of 76.65% in *Arthrospira platensis*. In the same way, the other chemical components of spirulina in our work presented variations in their values with respect to other reports. Few studies have evaluated the effect of the culture medium of *Arthrospira maxima* compared to *Arthrospira platensis*, however, it has been observed that in general the protein content of the first species is higher than that of the latter. It is also known that the proximal chemical composition of spirulina depends on the species and strain in question, as well as the culture conditions, whether in artificial or natural ponds, which implies, among other factors, the pH, temperature and type and concentration of nutrients, although it should also be considered that the analytical techniques used could report differences in the values (Affan *et al*., 2015b).

3.2 Yield and crude protein content of the obtained protein fractions

When using dehydrated or frozen spirulina, only three protein fractions were obtained, the prolamins fraction was not obtained, which suggests that spirulina proteins contain low proline content, however, physical and/or chemical treatments can be performed on the spirulina proteins seeking to partially or totally denature them to expose amino acids that could modify the yields of each protein fraction. Table 2 shows the yield of the protein fractions, it is worth mentioning that, when using frozen spirulina for the extraction of the protein fractions, an increase in the yield of 4.8 times was observed in the albumins compared to that obtained when dehydrated spirulina was used. The other fractions showed very low yields, suggesting that most of the protein in spirulina is solubilized in water, based on these results, it is considered that the albumins fraction is the one with potential uses in the food industry, mainly in the design and production of added protein drinks. Table 3 shows the content of crude protein, being around 90 g/100g of crude protein in each fraction, considering

Table 3. Crude protein content in the fractions (dry basis).

Fractions	Protein content in fraction	Amount of total protein in each fraction (g/100 g)
Spirulina	-	61.6
Albumins	90.97 ± 4.1^{a}	39.21 ± 1.1^{a}
Globulins	89.63 ± 3.1^{a}	3.72 ± 0.8^{b}
Glutelins	88.63 ± 5.1^{a}	1.56 ± 1.1^{c}

Values are mean ± SD. Different letters in the same column indicate significant differences, $p<0.05$.

that the extraction also resulted in a process of protein concentration; the remaining 10% of their composition is represented by soluble compounds in each solvent that could include pigments, carbohydrates and salts, which are cellular components. The protein content in the albumins fraction represents almost 64% of the total in spirulina, while the content in the globulins and glutelins fractions was only 8.6% of the total protein of the spirulina, so the efficiency extraction was approximately 73%.

There are no reports on the solubility fractionation of spirulina proteins, the reported studies are on the extraction of the total proteins and are more abundant on *Arthrospira platensis* proteins. Recently, a microalgae suspension of *Arthrospira platensis* powder in phosphate buffer (pH 7, 0.1 M) was homogenized and insoluble components were separated by centrifugation; the proteins were precipitated at their isoelectric point (pH 3.5) and dialyzed, reporting 58.0 ± 2.5% protein on a dry basis (Böcker *et al*., 2021). Martínez-Palma *et al*. (2015), performed the aqueous extraction of proteins from *Arthrospira maxima* using a biomass-water ratio of 0.08 mg/mL, at 25 °C for 24 h. The obtained fraction was considered phycobiliprotein extract; the crude extract was centrifuged and the supernatant precipitated with 50% ammonium sulfate, re-centrifuged and the blue precipitate resuspended in distilled water. The powder obtained had a blue color, characteristic of phycobiliproteins, and the protein content was 72.3 g/100 g, representing 26% more than the spirulina used in our study. Recently, an aqueous extract was obtained from *Arthrospira platensis*, this microalgae was mixed with Milli-Q ultrapure water at a concentration of 2% (w/v) and sonication was carried out. The protein content was 85.50%, while the reported protein values for this species range from 55-77% (Bleakley and Hayes, 2021). In another study, a protein concentrate from *Arthrospira platensis* was obtained using spirulina powder in 0.1 N NaOH solution, then the proteins were precipitated at pH 3 and finally resuspended at pH 6.8-7.0 to finally lyophilize them. A protein content in the extract of 74.01% was obtained with a yield of 52.6% (Bashir *et al*., 2016). The protein content in the fractions of our study was higher than the values reported in other studies, this could be due to the cell lysis conditions used in spirulina before carrying out the protein extractions, as well as the extraction conditions that involve volumes of the solvent used as well as techniques and times in the solubilization of proteins.

In this study, two methods were used to carry out the extraction of the protein fractions, with frozen spirulina being the one that had the highest yield in the extraction of the fractions, where the effect was greater in the albumins fraction, this could be due to the fact that during the freezing process ice crystals form and grow, which by themselves are capable of breaking cell and organelle membranes, and combined with the maceration process, a more efficient cell lysis is carried out, causing the release of membrane proteins and intracellular content, allowing protein solubilization, while the conditions to dry the spirulina caused a loss of protein solubilization capacity possibly caused by chemical reactions that could include Maillard's generating insoluble molecules for having high molecular weight involving proteins and carbohydrates. It is worth mentioning that there are other methods that include ultrasonification (Yucetepe *et al*., 2018) or ultrafiltration (Nisticò *et al*., 2022), seeking to make protein extraction more efficient.

3.3 Functional properties of the protein fractions

The functional properties of proteins depend mainly on their interaction with three components of food systems including oil, water and gas, and are based on the amount and proportion of hydrophobic and hydrophilic amino acids present in the protein, as well as its size and structure (Bashir *et al*., 2016). Figure 2 shows the amount (g/100 g) of each solubilized protein fraction, it should be mentioned that the fractions were solubilized in the solvent used for their extraction. The three protein fractions obtained from spirulina showed a high solubilization capacity, being 98 g/100 g for the albumins fraction and close to 90 g/100 g for the other two fractions with no significant difference ($p<0.05$) between them.

Figure 3 shows the values of the water retention capacity of the protein fractions. In general, the three fractions showed high values, without significant differences ($p<0.05$) between them, the values were around 83-89 g/100 g.

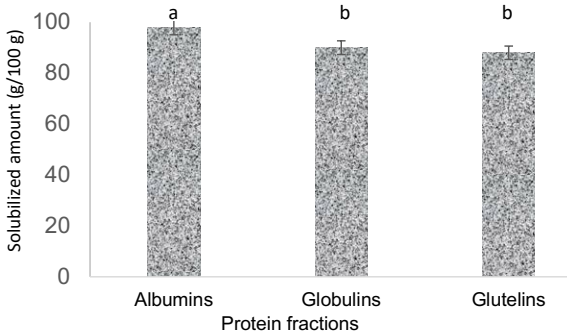

Figure 2. Solubilization capacity of the protein fractions of spirulina in the extraction solvent of each fraction. Different letters indicate significant differences, $p<0.05$.

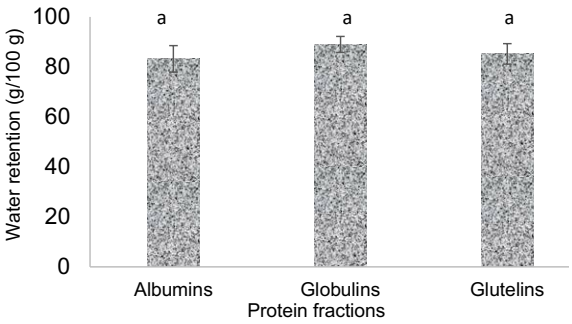

Figure 3. Water retention capacity of the protein fractions of spirulina. Different letters indicate significant differences, $p<0.05$.

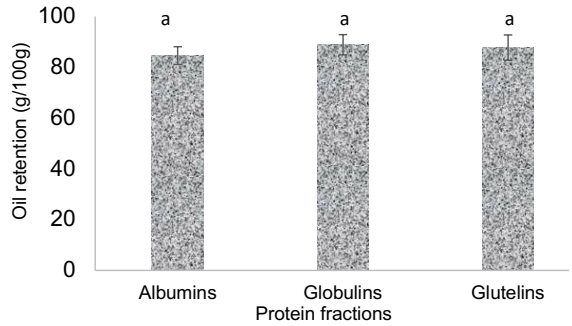

Figure 4. Oil retention capacity of the protein fractions of spirulina. Different letters indicate significant differences, $p<0.05$.

Figure 4 shows the oil retention capacity of the three protein fractions, being approximately 84-87 g/100 g, with no significant difference ($p<0.05$) between them.

The solubility, as well as the water and oil retention capacities, have not been reported in protein fractions obtained by the methods used in this study, and there are minimal reports of these properties evaluated in spirulina proteins. In a study, the solubility, as well as the oil retention capacity and the water retention capacity of a protein extract of *Spirulina platensis* (obtained in water at a concentration of 2% (w/v) and sonicated) were determined. The solubility of the protein extract was evaluated at different pH values (from 2 to 12) in concentrations of 1% w/v in water. The minimum solubility was observed at pH 4 (4.99%), rising to 32.44% at pH 10, with the maximum solubility at pH 12 (62.99%). The water retention capacity was 2.25 g of water/g of protein extract, and the oil retention capacity was 5.80 g of oil/g of protein extract, which are higher values compared to the observed in this study, which could be due to the extraction method of the fractions (Bleakley and Hayes, 2021).

The emulsification capacity of each fraction was evaluated as a function of pH (Table 4). The albumins fraction showed values between 47-69%; being the highest

Table 4. Capacity and stability of emulsion as a function of the pH of the protein fractions from spirulina.

pH	Capacity (%)			Stability (%)		
	Albumins	Globulins	Glutelins	Albumins	Globulins	Glutelins
2	54.1±2.1^b	42.8±0.9^c	69.5±3.9^a	90.0±2.0^a	78.8±12.5^a	64.1±4.6^c
4	58.3±3.9^b	61.1±0.5^a	59.9±1.6^c	66.8±4.5^c	72.7±3.1^a	76.4±2.1^b
6	47.6±0.9^c	61.2±1.1^a	55.5±4.9^c	80.1±3.8^b	72.9±2.6^a	70.0±1.5^c
8	65.0±5.4^a	58.3±3.9^a	61.8±1.0^b	86.4±5.2^a	76.4±5.14^a	85.9±5.8^a
10	68.8±0.8^a	51.3±1.9^b	57.2±2.3^c	72.3±6.4^b	80.0±4.1^a	90.1±2.8^a

Values are mean ± SD. Different letters in the same column indicate significant differences, p<0.05.

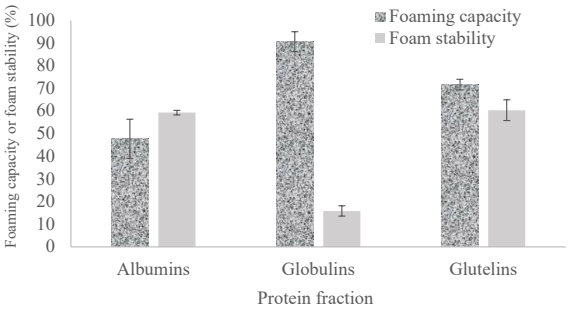

Figure 5. Foaming capacity and foam stability of the protein fractions of spirulina. Different letters indicate significant differences, p<0.05.

values at alkaline pH (pH 8 and 10, without significant difference (p<0.05) between them), while at pH below 7 the lowest values were obtained, being the lowest at pH 6. The emulsification capacity of the globulins fraction showed that at pH values of 4, 6 and 8 the highest values (around 60%) were obtained without significant difference (p<0.05) between them. At pH 2 and 10 the lowest values were observed (approximately 42 and 51%, respectively). Regarding the emulsification capacity of the glutelins fraction, it was observed that at pH 2 the highest value was obtained (approximately 70%) and at pH 8 the value was reduced by 13%, while at pH's of 4, 6 and 10 values (with no significant difference (p<0.05) between them) were approximately 20% lower than that observed at pH 2. The emulsions formed with the albumins fraction showed the highest values of stability at pH 2 and 8 (90.0 and 86.4% respectively) without significant difference (p<0.05) between them, while at pH's of 6 and 10 values close to at 80% were observed, and at pH 4 the lowest value (67%) was reported. The stability of the emulsions prepared with the globulins fraction in all pH evaluated (Table 4), reported values of approximately 73-80%, with no significant difference (p<0.05) between them. The emulsions that were developed with the glutelins fraction showed the highest stability values (approximately 90%) at alkaline pH (8 and 10), while at pH 4 the value decreased by 15.5%; at pH's 2 and 6, values of approximately 25% lower than that observed at alkaline pH's were observed (Table 4). In another study, a protein extract from *Spirulina platensis* was mixed with olive oil and showed the highest emulsifying activity (22.41%) while with canola oil showed the lowest (21.00%), such values were very low compared to those obtained in this investigation. *Spirulina platensis* protein extract showed emulsion stability values (85.91%) when evaluated in olive oil, being similar to those observed in this study. Emulsion stability can be affected by several factors, including pH, droplet size, net charge, interfacial tension, viscosity, and protein conformation (Bleakley and Hayes, 2021).

Figure 5 shows the foaming capacity and foam stability of the protein fractions. Regarding the foaming capacity of the fractions a very low value was observed for the albumins fraction (48%) with respect to the value shown by the globulins fraction, which reached 91%, while the glutelins showed a value of approximately 71%. The stability of the foam formed with the three protein fractions showed very low values, being approximately 60% for albumins and glutelins (with no significant difference (p<0.05) between them) and only 16% for globulins. Bleakley and Hayes (2021), evaluated the foaming capacity at different pH's (2, 4, 6, 8 and 10) of a *Spirulina platensis* protein extract at a concentration of 1.5% (w/v) in ultrapure water. The volumes of the protein suspensions before homogenization and the foam generated after homogenization were measured using a Vernier. Foaming stability was expressed as a percentage of the initial foam volume and was determined by measuring the foam volume at 15, 30, 60, 90 and 120 min after homogenization. Low foaming capacity values were observed, being approximately 60% at pH 2, 4 and 6, while at pH 8 and 10, the values were approximately 50 and 40%. The stability of the foams after 2 h was 70% at pH 2 and 4, dropping to 45% at pH 6, 8 and 10; these results are similar to those observed in this research. Bashir *et al*. (2016) evaluated the foaming capacity and stability of a *Spirulina platensis* protein isolate. The isolate was obtained from spirulina powder using 2M NaOH. For the evaluation of the foaming capacity, they used 1 g of the protein isolate in 50 mL of distilled water, after vigorous stirring the foaming capacity was determined and 60 min later the stability of the foam was measured. Values of 10.74 and 19.26% were obtained for foaming capacity and stability, respectively, which also show low values for this property.

It is important to mention that the functional properties of proteins are not necessarily related to or conditioned by

their ability to be extracted, since the processes to obtain protein fractions by solubilization cause minimal changes in the native structure of proteins, while in the determination of its functional properties, the process conditions such as mechanical agitation, air injection, change in pH or heating, among others, denature proteins causing the exposure of amino acids that would not be exposed in their native form, favoring a certain functional property. It should also be noted that chemical and enzymatic modifications of proteins modify their functional properties, which in most cases are improved.

3.4 Antioxidant activity of the protein fractions

The antioxidant activity evaluated with the ABTS and DPPH radicals can be reported as the percentage of radical inhibition under the test conditions, however, these results can be subjective since the amount of sample used can change, so the calculation of the IC_{50} considers the amount of sample necessary to inhibit 50% of the free radical. Table 5 shows the antioxidant activity of the three protein fractions, where it can be seen that all showed antioxidant activity with both free radicals used. With the ABTS radical, the glutelins fraction was the one that showed the best antioxidant activity with the lowest IC_{50} value, followed by the albumins fraction and the globulins fraction showed the highest value. The reduction of the DPPH radical was carried out with low values of the three protein fractions, being the albumins fraction the one that showed the highest antioxidant activity with the lowest value of IC_{50}, followed by the glutelins and the highest IC_{50} value was that of globulins.

The globulins fraction had the lowest antioxidant activity, being approximately less than half the amount of the albumins and glutelins fractions to obtain the value of IC_{50}. It is worth mentioning that there are few studies where the antioxidant capacity of spirulina protein extracts has been evaluated, and there are a few others where the antioxidant activity has been carried out on extracts with solvents from spirulina biomass. Martínez-Palma *et al*. (2015), evaluated the antioxidant capacity (with ABTS and DPPH radicals) of a protein isolate obtained from *Arthrospira maxima*, reporting the percentage of radical inhibition, with very low values, however, they applied enzymatic hydrolysis to the protein extract and observed an increase in antioxidant activity with the two free radicals dependent on hydrolysis time, which suggests that low molecular weight peptides have greater antioxidant activity than larger peptides. Piñero-Estrada *et al*. (2001), evaluated the antioxidant activity by means of the DPPH method of an aqueous extract of *Spirulina platensis* proteins, as well as of the fractions obtained after the separation of the proteins by molecular weight. The percentage of inhibition of the DPPH radical by the protein extract was 38.12%, while the lower molecular weight proteins had 50.90%, which also supports the suggestion that the antioxidant activity is greater in the presence of low molecular weight peptides.

Table 5. Antioxidant capacity of the protein fractions of spirulina.

Fractions	ABTS	DPPH
	IC_{50} (mg/mL)	
Albumins	2.15 ± 0.12^{b}	1.17 ± 0.01^{c}
Globulins	4.97 ± 0.31^{a}	1.88 ± 0.02^{a}
Glutelins	1.47 ± 0.01^{c}	1.60 ± 0.01^{b}

Values are mean ±SD. Different letters in the same column indicate significant differences, $p<0.05$.

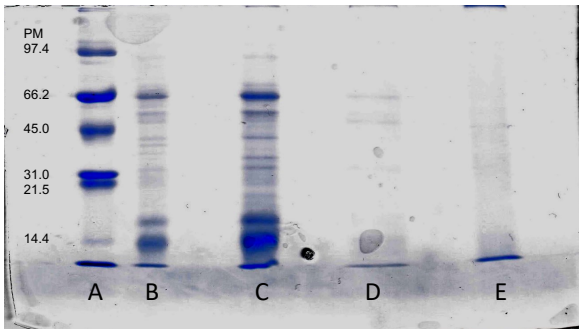

Figure 6. Electrophoretic profile of the protein fractions. Molecular weight marker (A), spirulina (B), albumins (C), globulins (D), glutelins (E).

3.5 Electrophoretic profile of protein fractions

The electrophoretic profile of spirulina and protein fractions is shown in Figure 6. It is worth mentioning that the proteins observed in spirulina had a molecular size of less than 100 kDa, and the largest number of them was concentrated between 66 and 14 kDa. Only 4 bands between 66 and 97.4 kDa can be seen. The highest number of spirulina proteins can be observed in the albumins fraction, in the globulins and glutelins fractions very few bands were observed. Martínez-Palma *et al*. (2015), reported the electrophoretic profile of the proteins of spirulina (*Arthrospira maxima*), showing that most proteins were found in the range of 4.5-63 kDa and few proteins in 63-95 kDa. In another study, an SDS-PAGE with a high molecular weight marker was shown, observing that the proteins of spirulina (*Spirulina platensis*) were concentrated in the lower part of the gel, which indicates that the proteins are of low molecular weight (Piñero-Estrada *et al*., 2001). Bleakley and Hayes (2021), showed an electrophoretic profile of *Spirulina platensis* proteins, where it was observed that the proteins were found in the range of 10 to 100 kDa. Nisticò *et al*. (2022), also reported an electrophoresis gel with *Arthrospira maxima* proteins, showing that the proteins had molecular weights between 15 and 50 kDa.

Conclusions

- Fractions of albumins, globulins and glutelins were obtained from spirulina, prolamins were not obtained, suggesting that spirulina proteins have a low proline content.
- The albumins fraction was the one that presented the highest yield, the amount of the other two fractions being minimal. Therefore, the albumins fraction is the one that has potential use in the design and preparation of foods where its high solubilization in water and its high-water retention capacity are used.
- The highest antioxidant activity in the albumins and glutelins fractions was observed.
- Spirulina proteins are generally of low molecular weight and most are grouped in the albumins fraction.

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Nomenclature

ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid
AOAC	Association of Official Analytical Chemists
DPPH	2,2-diphenyl-1-picrylhydrazyl
SDS-PAGE	Polyacrylamide gels in the presence of sodium dodecyl sulfate

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