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Evaluation of cushuro (*Nostoc sphaericum)* as an alternative source of minerals, functional protein and bioactive peptides

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ABSTRACT

Cushuro (*Nostoc sphaericum*) is an underexplored blue-green alga currently used as a food ingredient, natural medicine, and organic fertilizer in Peru. Our paper aims to address the nutritional and biological values of cushuro protein. Dried cushuro had a high amount of minerals as calcium (567 ± 64 mg/100 g to 1357 ± 153 mg/100 g), iron (13.5 ± 1.5 mg/100 g to 26.2 ± 2.9 mg/100 g, magnesium (95 ± 11 mg/100 g to 126 ± 14 mg/100 g) and protein content (32.4 ± 0.5 g/100 g to 36.9 ± 0.2 g/100 g. Protein solubility was assessed to optimize the extraction of cushuro protein by ultrasound-microwave assisted extraction, after that, the protein contents in cushuro fractions were over 70%. All detected cushuro protein fractions had Mw < 24 kDa and a better digestibility than legume products. In addition, cushuro protein products tend to have umami taste, and does not bring a bitter flavor. Moreover, cushuro flour and protein fractions contain all essential amino acids, which are responsible for over 40% of total amino acids. Meanwhile, hydrophobic amino acids content of around 40 g/100 g also indicated their excellent antioxidant potential. Thus, the data obtained supports cushuro as a promising alternative source for minerals, protein and bioactive peptides.

1. Introduction

Nowadays, global population is growing dramatically, it is projected that in 2050, the world's population will reach 10 billion people (Adam, 2021; Van Dijk et al., 2021), necessitating a 35–56 g/100 g increase in food production, which is closely correlated to an increasing demand for foods of high nutritional value and protein content. Likewise, due to shifts in dietary preferences toward protein-rich food consumption and as an alternative to combat anemia and child malnutrition the demand for dietary protein continues to grow. Considering the land resources and environmental issues, while improving global nutrition, as outlined by the United Nations' Sustainable Development Goals (Clark H, 2016) traditional protein sources (e.g., milk, egg and meat) are labelled as low conversion efficiency sources with high environmental footprint (Geada et al., 2021; Kumar et al., 2023). The exploration of alternative and complementary protein sources that are sustainable, nutritious, and affordable becomes crucial (Gephart et al., 2016; Willett et al., 2019). The concept of 'sustainability' has been highlighted when seeking new alternative protein sources, rather than only considering the safety and well-balanced nutrients. Therefore, alternative protein sources, including insects, quinoa, oilseeds, algae, and seaweed have been recommended as potential options for replacing traditional animal proteins.

Microalgae are rich in nutrients and biologically active substances, such as proteins and bioactive peptides, polysaccharides, lipids, polyunsaturated fatty acids, vitamins, pigments. These compounds are capable of exhibiting antioxidant, antibacterial, antiviral, antitumor, regenerative, antihypertensive, antidiabetic, neuroprotective and immunostimulant effects (Gürlek et al., 2020). They are in demand in pharmacology, medicine, cosmetology, chemical industry, fish farming, energy industry, agriculture, and in the production of feed and functional foods (Bhattacharjee, 2016).

Cushuro (*Nostoc sphaericum*), also known as "murmunta", "llullucha", "crespito", "llayta", is a gelatinous spherical about of blue-green

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alga 10–25 mm in diameter, similar to grapes; majorly grown in Peruvian highlands, over 3000 m altitude as in the departments of: Ancash, Junín, Cajamarca, Huánuco, Cusco and Puno (Chasquibol et al., 2023). They form both microscopic and macroscopic colonies on various lagoons with crystalline and pure waters rich in nitrogen, which favor their growth (Corpus-Gomez et al., 2021). This colonies can be manually harvested, sun-dried, and subsequently sold in local markets (Méndez-Ancca et al., 2023). They have been consumed by people mainly in Peru and Bolivia for a thousand years. The neutral flavor makes cushuro a good ingredient for local dishes, bringing an amazing texture without adding any undesirable flavor and this macroalgae has recently been rediscovered for its potential as an ingredient in restaurant menus or for development of healthier products in the food industry, offering cost-effective alternatives (Pérez-Lloréns & Vergara, 2023). Also, they are also used as natural medicine as well as organic fertilizer (Méndez-Ancca et al., 2023).

This microalgae has gained increasing research interest by high protein content (from 24 to 42%) (Celis-Plá et al., 2021; Choque-Quispe et al., 2024; Hao et al., 2011), ω-3 and ω-6 fatty acids (Macário et al., 2022) and minerals (Méndez-Ancca et al., 2023), as well as all the essential amino acids and vitamins (Corpus-Gomez et al., 2021).The cyanobacteria contain bioactive compounds such as phycobiliproteins (C-phycocyanin) and methyl palmitate (C16:0) (Ruiz-Domínguez et al., 2021). Nostoc sp. contains polyphenols, phycocyanin, and ascorbic acid (Celis-Plá et al., 2021; Li et al., 2020; Xu et al., 2021). They are therefore expected to be a useful tool in fighting world malnutrition issues. Together with their antioxidant capability and anti-diabetic properties reported previously, cushuro has been described as 'the food of future' by some media.

The aim of this paper is to investigate the minerals and protein content, amino acid profiles, along with the protein digestibility of the crude flour and various protein residues from cushuro (*Nostoc sphaericum)* obtained from different extraction steps. We seek to provide an overview on the use of cushuro as a source of minerals and protein for human consumption and for biofunctional food applications.

2. Materials and methods

2.1. Raw material

Cushuro was collected from the lakes of Tacash (Height 4412 masl, Altitude: 9°53′00.74″ S, Longitude: 77°28′44), Llacsha (Height 4512 masl, Altitude:, 09°50′56.40''S, Longitude:77°29′28.72''0) and Ututo (Height 4420 masl, Altitude: 09°51′51′80"S, Longitude: 77°29′24''O); in the district of Cotaparaco, Province of Ancash region-Peru. The samples were washed and dried at 60 °C for 12 h in an infrared dry (IRD D18, Sevilla, Spain), ground in a food grinder (Grindomix GM200/Restch, Haan, Germany) to obtain cushuro flour and stored in aluminized bags at 25 °C. All reagents used were classified and supplied by Merck and Milli-Q water was used.

2.2. Proximal composition

The proximal composition was carried out according to official methods (AOAC, 2012). The moisture content was determined at 110 °C to constant weight using a moisture analyzer Sartorius (MA 30, Gottingen, Germany). The total protein content was determined as g/100 g nitrogen × 6.25 using a Kjeldahl analyzer (UDK 139, VELP, Usmate Velate, Italy). The ash content was determined by incineration at 550 °C for 72 h, in a muffle furnace (Linn High Therm, VMK-80, Germany). The fat content was determined with hexane for 9 h.

2.3. Mineral and heavy metals content

Minerals and heavy metals in flour and lagoon water samples were determined according to NORMA Oficial Mexicana NOM-117-SSA1-1994 (1994). Samples were digested using the Digestion Block (Lab TECH, ED54S, Hopkinton, USA). The ashes were dissolved with 5 mL of 1 mol/L HCl, transferred to a 15 mL flask and filled with the same solvent. The mixture was filtered, and the concentration of minerals and heavy metals was determined by ICP-OES (PerkinElmer, AVIO 550 Max, MA, USA). A calibration curve was performed with Certified Reference Materials (CRMs) (AccuStandar, USA) for minerals (P, Mg, Fe, Ca, Zn, K, Na and Cu) and heavy metals (As, Pb, Hg and Cd).

2.4. Protein solubility curve

The protein solubility curve from cushuro flour was determined according to the method described by Paz et al. (2021) with some modifications. The cushuro flour was dispersed in water (2 g/100 mL) and the pH was adjusted with 1 mol/L NaOH or 1 mol/L HCL and kept under stirring for 1 h at room temperature. At each pH point (2,4,6,8,10,12 and 14), an aliquot was taken in triplicate and centrifugated 15 min at 10000 g. In the recovered supernatants, the nitrogen content was determined in the analyzer (UDK 139 VELP Usmate Velate, Italy). The % soluble protein of each supernatant was determined according to equation (1):

$$\%\ Soluble\ Protein = \frac{Protein\ content\ in\ the\ supernatant\ (g)}{Protein\ content\ in\ cushuro\ flour(g)}\ x100 \quad (1)$$

2.5. Protein fractions

The cushuro flour was mixed with 0.1 mol/L HCL at different solid/solvent ratio ranging from 1/64 (g/mL), which were then transferred into an ultrasound-microwave-assisted extraction (UMAE) (CW-2000, Nade, Shanghai, China), equipped with condensing tube. The optimal UMAE conditions were 90 °C for 20 min, the microwave and ultrasound power were 500W, 50W and 40 Hz, respectively. After cooling, the mixture with pH 1.5 was filtered under vacuum and separated into a supernatant and a residue 1 (R1). The supernatant was deproteinized by adjusting the pH 4 with NaOH 2 mol/L, obtaining the residue 2 (R2). The residue (R1) was resuspended in distilled water and the protein fraction was extracted according to the conditions previously determined: temperature (50 °C), time (1 h), water/raw material 100/1 (mL/g) and pH 8.

The extract obtained was centrifuged at 10000 g × 10 min, obtaining the residue 3 (R3) and a supernatant with pH 8. The supernatant was adjusted to the isoelectric point (pH 3) with HCl, and the precipitate was centrifuged at 10,000 g × 10 min, obtaining the protein isolated (PI). All protein fractions (R1, R2, R3 and PI) were dried by infrared dryer (IRC D18, Irconfort, Sevilla, Spain) at 60 °C for 6 h, then ground in a food shredder (Grindomix GM200, Restch, Haan, Germany) and stored in aluminized bags at 25 °C. All protein fractions (R1, R2, R3 and PI) were obtained according to Wassie et al. (2021) and Peasura et al. (2015) with some modifications, after removed the cushuro polysaccharides.

2.6. Total protein content

The total protein content of cushuro flour (CF) and protein fractions (R1, R2, R3 and PI) were determined by the Dumas's method (Shea & Watts, 1939), with a nitrogen-to-protein-conversion factor of 6.25 in Elementary Vario Max Cube.

2.7. Total and free amino acids

The total and free amino acids were determined according to the method reported by Alaiz et al. (1992). For free amino acids, 200 mg of protein sample were taken and homogenized in 2 mL of 0.01 mol/L HCl solution, then the samples were centrifuged at 2500 g for 15 min and the supernatant was collected for HPLC analysis. For total amino acid 2 mg of protein sample was completely hydrolyzed using 6 mol/L HCl at

110 °C for 24 h under an inert N atmosphere. After hydrolysis the samples were brought to dryness in a rotary evaporator (Buchi rotavapor R 100, BUCHI Labortechnic AG, Flawil, Switzerland) and diluted to 10 mL using 1 mol/L sodium borate buffer. Immediately samples were derivatized with diethyl ethoxy methylene malonate at 50 °C for 50 min under stirring. Amino acids were determined by HPLC using a reversed phase column300 × 3.9 mm i.d. (Novapack C18, 4 μm; Waters) with diode array detector (PDA 2998, Waters, Milford, USA), and 280 nm of wavelength. The following gradient was programmed: solvent A (25 mmol/L sodium acetate with 20 mg/100 mL sodium azide (pH 6.0), and solvent B (acetonitrile), the elution flow rate was set at 0.9 mL/min at 34 °C. with the following gradient: time 0.0–1.39 min, elution with A:B 91:9; time 1.39–5.39 min, linear gradient from A:B 91:9 to A:B 86:14; time 5.39–12.34 min, elution with A:B 86:14; time 12.34–18.60 min, linear gradient from A:B 86:14 to A:B 81:19; time 18.60– 22.78 min, linear gradient from A:B 81:19 to A:B 69:31; time 22.78–26.86 min, elution with A:B 69:31; time 26.86–28.91 min, linear gradient from A:B 69:31 to A:B 91:9 and time 28.91–32 min, elution with A:B 91:9.

Tryptophan was quantified by HPLC after basic hydrolysis (Yust et al., 2004). D, L-α-aminobutyric acid was used as internal standard.

2.8. In vitro protein digestibility

In vitro protein digestibility was measured using the protocol reported by Tinus et al. (2012), with slight modification. Briefly, 10 mL of sample solution containing 62.5 ± 0.5 mg of protein fractions (R1, R2, R3 and PI) were pre-heated to 37 °C, and the pH was adjusted to 8.0 using 0.1 mol/L NaOH. Then, the pH of samples was monitored and maintained at 8.0 for 10 min. Meanwhile, an enzyme cocktail (10 mL) with 16 mg of trypsin (13,000–20,000 BAEE units/mg protein), 31 mg of chymotrypsin (40 units/mg protein) and 13 mg of protease from *Streptomyces griseus* Type XIV (P3.5 units/mg) were prepared, with pH adjusted to 8.0 and kept in a water bath at 37 °C. All enzymes were obtained from Sigma (Sigma Aldrich, Castle Hill, NSW 2154). After that 1 mL of enzyme cocktail was added to 10 mL of sample solution, pH of the protein hydrolysates solution was recorded every 30 s for 10 min. The change in pH (pH 0 min – pH 10 min) during 10 min digestion was used for calculating in vitro protein digestibility (IVPD), using equation (2):

$$IVPD = 65.66 + 18.10 \text{ x (pH 0 min – pH 10 min)} \quad (2)$$

2.9. FPLC-gel filtration chromatography

According to Wang et al., 2023, p. 500 μL of protein fractions (R1, R2, R3 and PI) (0.2 mg/mL protein concentration) was injected for gel filtration chromatography, which was carried out in an AKTA-purifier FPLC system, using Superdex peptide 10/300 GL column (Cat: 17-5176-01, GE Healthcare). 0.75 mol/L ammonium bicarbonate was used for eluent and elution was monitored at 215 nm. Blue dextran (2000 kDa), cytochrome C (12.5 kDa), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da) were used as molecular weight standards.

2.10. Functional properties

2.10.1. Water holding capacity (WHC)

It was determined according to the method described by (Sciarini et al., 2009). 0.25 g of protein fractions (R1, R2, R3 and PI) were weighed in a 15 mL test tube and 10 mL of distilled water with 20 mg/100 mL sodium azide solution was added. The samples were left to rest overnight at 20 °C, then the tubes were centrifuged at 1600 g for 10 min, the supernatant was discarded, and the swollen sample was weighed. The WHC was calculated using equation (3):

$$WHC \text{ (g/g)} = \text{(Weight of the swollen sample – weight of the dry sample)/(weight of the dry sample)} \quad (3)$$

2.10.2. Fat absorption capacity (FAC)

Fat absorption capacity was determined by triplicate using the procedure of Lin et al. (1974) with slight modifications. Samples (0.5 g) were mixed for 1 min with 3 mL of corn oil in a previously weighed 15 mL graduated centrifuge tube. After centrifuging at 4000 g for 30 min, the supernatant was discarded, and the tubes were weighed again. The FAC was calculated using equation (4):

$$FAC \text{ (g/g)} = \text{(weight of fat absorbed per sample/weight of sample)} \quad (4)$$

2.10.3. Foaming capacity (FC)

The FC was determined by the method of Kaewmanee et al. (2014) with slight modifications. 100 mL of aqueous dispersion of the samples al 1 g/100 g (w/V) were homogenized at 10,000 g for 2 min. The FC was calculated using equation (5):

$$FC \text{ (\%)} = \text{(foam volume after homogenization) / (Total volume of the sample) x 100} \quad (5)$$

2.11. Statistical analysis

Results were expressed as mean ± standard deviation. All measurements were determined in duplicate or triplicate. Analysis of variance (ANOVA) was used with a significance level of 95% followed by Tukey's post hoc test to identify significant differences between groups. All analyses were performed using Minitab 19.0 software (Minitab Inc. USA).

3. Results and discussions

3.1. Proximal composition

The proximal composition of cushuro (*Nostoc sphaericum)* flour are shown in Table 1. The protein content varied between 32.4 ± 0.5 g/100 g and 36.9 ± 0.2 g/100 g. These results were higher than those reported by Méndez-Ancca et al. (2023) (28.2 ± 0.3 g/100 g), Hao et al., 2011 (30.4 g/100g) and Corpus-Gomez et al. (2021) (35–42 g/100 g). The carbohydrates content (54.8 g/100 g–57.9 g/100 g); moisture (7.77 ± 0.15 g/100 g to 9.40 ± 0.33 g/100 g) and fat (0.17 ± 0.05 g/100 g to 0.44 ± 0.01 g/100 g) were lower than those reported by Méndez-Ancca et al. (2023) and Corpus-Gomez et al. (2021).

The results obtained indicate that there are physical (pH, conductivity) and chemical (phosphates and sulfates) parameters in the water of the lagoons that determine its nutritional behavior (Méndez-Ancca et al., 2023).

The extraction and characterization of the protein fractions were analyzed for the cushuro flour from the Tacash lagoon due to its higher protein (36.9 ± 0.2 g/100 g) content than the other flours.

Table 1
Proximal composition from cushuro (*Nostoc sphaericum*) flour.

Lagoon	Protein (g/100 g)	Carbohydrates g/100 g)	Moisture (g/100 g)	Fat (g/100 g)
Ututo	32.4 ± 0.5^{b}	57.9 ± 0.5^{a}	9.40 ± 0.33^{a}	$0.26 \pm 0.04^{a,b}$
Tacash	36.9 ± 0.2^{a}	54.8 ± 0.7^{a}	7.77 ± 0.15^{b}	0.44 ± 0.01^{a}
Llacsha	34.3 ± 0.3^{b}	57.7 ± 0.4^{a}	7.90 ± 0.19^{b}	0.17 ± 0.05^{b}

Results are expressed as means ± SD (n = 3).

3.2. Mineral and heavy metals content

Cushuro flour and water lagoons contain significant amounts of macro- and microminerals (Table 2). Calcium content varied between 567 mg/100 g to 1357 mg/100 g, and these results were higher than obtained by Méndez-Ancca et al. (2023) (377 ± 2 mg/100 g). Also, the calcium content was higher than reported by the milk (32 mg/100 g), cooked quinoa (27 mg/100 g), hard-boiled egg (30 mg/100 g), meat chicken (12 mg/100 g), and fish (28 mg/100 g) (Reyes García et al., 2019).

Calcium is the most abundant macromineral in the body, it is the main constituent of teeth and bones, allows normal body movement, participates in heartbeat, muscle contraction and blood clotting; calcium deficiency causes osteoporosis in adults and rickets in children, leading in extreme cases to irreversible changes in bone structure (Lozano Muñoz & Díaz, 2020; Mann & Truswell, 2017). According to the daily requirements for children aged 4–8 years (National Research Council, 2001), a daily ration of 74 g of cushuro flour would cover 100 of the daily requirements.

Likewise, iron had an average value between 13.5 mg/100 g to 26.2 mg/100 g. These values were higher than those reported by Paucar-Menacho et al., 2023 (24.7 ± 0.7 mg/100 g in *Nostoc sphaericum Vaucher ex Bornet & Flahault*), and Méndez-Ancca et al. (2023) (4.76 ± 0.08 mg/100 g in *N. sphericum*), but lower than reported by Corpus--Gomez et al. (2021) (83.3 mg/100 g in *N. sphaericum*). However, the amount of iron is higher in relation to milk (1.3 mg/100 g), quinoa (1.6 mg/100 g), egg (1.1 mg/100 g), meat (1.5 mg/100 g), fish (1.2 mg/100 g) (Reyes García et al., 2013); liver (18.6 mg/100 g), lentils (1–2 mg/100 g), and spinach (3.6 mg/100 g), (Hurrell & Egli, 2010), respectively. Iron is an essential component of hemoglobin, cellular respiration, neurological development and physical growth (Lozano Muñoz and Díaz, 2020). Iron is very important to mitigate anemia, that affects 40% of children aged 6–59 months, (World Health Organization, 2008) and causes gastrointestinal, cognitive, immunological, difficulty concentrating, fatigue and other problems (Camaschella et al., 2015; Domellöf et al., 2014). The recommended dietary allowance of iron for children aged 4–8 years is 10 mg/day (National Research Council, 2001) and a daily ration of 40 g of cushuro flour would cover 100 g/100 g of the daily requirement.

Table 2
Mineral content from cushuro (*Nostoc sphaericum*) flour.

Lagoon Mineral	Ututo (4420 masl)	Tacash (4412 masl)	LLacsha (4512 masl)
Cushuro flour (mg/100 g)			
Phosphorus	25.7 ± 2.9^{a}	21.2 ± 2.4^{a}	20.9 ± 2.4^{a}
Magnesium	118 ± 13^{a}	126 ± 14^{a}	95.4 ± 11^{a}
Iron	21.1 ± 2.4^{a,b}	26.2 ± 2.9^{a}	13.5 ± 1.5^{b}
Calcium	1248 ± 141^{a}	1357 ± 153^{a}	567 ± 64^{b}
Zinc	2.55 ± 0.29^{a}	2.94 ± 0.33^{a}	2.22 ± 0.25^{a}
Sodium	91.2 ± 10.3^{a}	65.5 ± 7.4^{a}	65.1 ± 7.4^{a}
Potassium	99.0 ± 11.2^{a}	34.1 ± 3.9^{b}	48.3 ± 5.5^{b}
Water lagoons (mg/L)			
Aluminum	<0.005 ± 0.00	2.35 ± 0.18^{a}	0.06 ± 0.01^{b}
Boron	0.02 ± 0.00^{b}	<0.002 ± 0.00	0.30 ± 0.02^{a}
Calcium	14.9 ± 1.2^{a}	11.3 ± 0.9^{b}	10.5 ± 0.8^{b}
Copper	0.09 ± 0.01^{b}	3.04 ± 0.24^{a}	<0.0002 ± 0.00
Potassium	<0.04 ± 0.00	1.43 ± 0.11^{a}	<0.04 ± 0.00
Iron	<0.001 ± 0.00	<0.001 ± 0.00	<0.001 ± 0.00
Magnesium	0.93 ± 0.07^{a}	0.80 ± 0.01^{b}	0.20 ± 0.01^{c}
Sodium	9.58 ± 0.76^{a}	0.06 ± 0.00^{b}	<0.004 ± 0.00
Phosphorus	0.05 ± 0.00^{b}	2.78 ± 0.22^{a}	0.01 ± 0.00^{b}
Selenium	0.16 ± 0.01^{b}	0.26 ± 0.02^{a}	0.14 ± 0.01^{c}
Silico	1.60 ± 0.12^{b}	2.58 ± 0.20^{a}	0.29 ± 0.02^{c}
Zinc	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	<0.0001 ± 0.00
Arsenic	<0.002 ± 0.00	<0.002 ± 0.00	<0.002 ± 0.00
Cadmium	<0.0001 ± 0.00	<0.0001 ± 0.00	<0.0001 ± 0.00
Mercury	<0.0001 ± 0.00	<0.0001 ± 0.00	<0.0001 ± 0.00
Lead	<0.002 ± 0.00	<0.002 ± 0.00	<0.002 ± 0.00
Nickel	<0.0003 ± 0.00	<0.0003 ± 0.00	<0.0003 ± 0.00
Chrome	<0.0003 ± 0.00	<0.0003 ± 0.00	<0.0003 ± 0.00

Results are expressed as means ± SD (n = 3).

The magnesium content varied between 95.4 mg/100 g to 126.2 mg/100 g. These values were higher than those reported by Tseng et al. (2021) (50.0 mg/100 g in *Nostoc commune*) *Nostoc commune Nostoc commune*) but lower than the reported by Paucar-Menacho et al. (2023) (215.4 ± 1.2 mg/100 g in *Nostoc sphaericum Vaucher ex Bornet & Flahault*). Of note, the amount of magnesium content was higher in relation to lentils (78 mg/100 g), spinach (50 mg/100 g), prawns (76 mg/100 g), cheese (39 mg/100 g) and fish (23 mg/100 g) (Alimentos ricos en magnesio, 2024). Magnesium is an essential macromineral involved in the metabolism of proteins, lipids, carbohydrates and in the conduction of nerve impulses, muscle contraction and heart rate (Ross et al., 2020). According to the daily requirements for children aged 4–8 years (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1999), a daily ration of 40 g of cushuro flour would cover 100 g/100 g of the magnesium daily requirements.

According to Liang et al. (2022), samples from lower latitudes tend to have a longer wetting time and more suitable temperatures, so they grow faster than those from higher latitudes, which generates faster growth and dilutes the ash content. Therefore, the difference in altitudes could generate differences in mineral content between the analyzed samples.

The presence of heavy metals (As, Pb, Hg and Cd) was not detected neither in the cushuro flour nor in the lagoons water (<0.50 mg/kg) (European Union law, 2023). According to Mouga et al. (2024), the *Nostoc* species requires the presence of iron and magnesium for growth, and concentrations cannot be higher than 0.279 ± 0.120 g/L and 0.277 ± 0.065 g/L per day, because it can cause toxicity. *Nostoc* sp. can absorb metals such as nickel, copper and chromium (Ghorbani et al., 2021); however, no heavy metals were found in the cushuro flours and in the water of the Ututo, Tacash and Llacsha lagoons, respectively.

3.3. Protein solubility curve

The protein solubility curve is shown in Fig. 1. Protein solubility increased with pH. The highest protein solubility was about 19.8 g/100 g at pH 14, this is due to the covalent bonds of proteins with polysaccharides, lipids and phenolic compounds. In addition, the presence of polysaccharides increases the viscosity of the extraction medium and therefore hinders the extraction of proteins (Trigo et al., 2023). The lowest solubility of proteins was around 3.4 g/100 g at pH 2.5 (isoelectric point). The isoelectric point facilitated the purification process to obtain protein fractions from cushuro flour.

Microalgae have a cell wall rich in polysaccharides which decreases the yield of protein extraction, due to ionic interactions between the polysaccharide cell wall and the protein. The pH has a great influence on protein extraction (Fleurence et al., 1995). Many authors have shown that alkaline protein extraction followed by isoelectric precipitation can yield fractions with high protein content (Harrysson et al., 2019).

3.4. Protein content and in vitro protein digestibility (IVPD)

Protein fractions of cushuro determined via micro elemental analyses are shown in Table 3. The cushuro flour contains 36.5 ± 0.5 g/100 g protein, similar to defatted rapeseed canola seeds (37–41 g/100 g protein) (Wanasundara et al., 2016), but higher than egg (raw, 13 g/100 g), beef (26 g/100 g), salmon (22 g/100 g) (USDA, 2018). After the ultrasonic microwave assisted extraction treatment, the protein isolate (PI) presented the highest protein (88.0 ± 1.8 g/100 g) content than Residue 3 (78.0 ± 5.3 g/100 g, Residue 1 (74.5 ± 0.3 g/100 g) and Residue 2 (56.7 ± 2.1 g/100 g).

Compared to cushuro flour, the IVPDs of all four protein fractions were significantly improved after microwave-assisted ultrasonic extraction (Table 3). This could be directly related to the reduction of

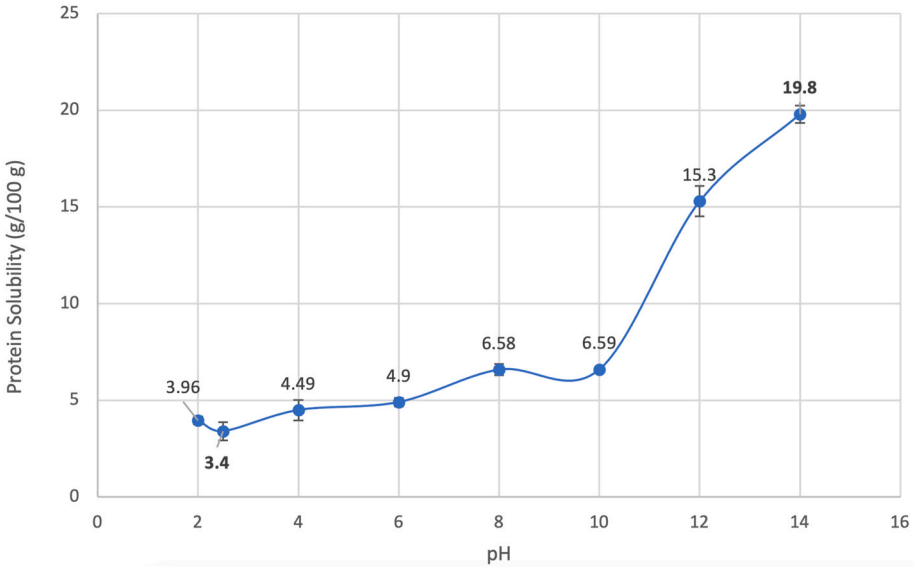

Fig. 1. Protein solubility curve from cushuro (*Nostoc sphaericum*) flour.

Table 3
Protein content (g/100 g) and in vitro protein digestibility from cushuro (*Nostoc sphaericum*) protein fractions.

Cushuro samples	Protein content (g/100 g)	*In vitro* protein digestibility (IVPD g/100 g)
Cushuro flour	36.5 ± 0.5[a]	75.9 ± 1. 5[a]
Residue 1	74.5 ± 0.3[b]	82.5 ± 1.3[a,b]
Residue 2	56.7 ± 2.1[c]	86.6 ± 4.0[a,b]
Residue 3	78.0 ± 5.3[b]	81.5 ± 0.1[a,b]
Protein isolate	88.0 ± 1.8[b,c]	89.0 ± 0.9[b]

Results are expressed as means ± SD (n = 3).

dietary fiber (Mišurcová et al., 2010), tannins (Babiker et al., 1995) and trypsin inhibitors. In addition, ultrasound, together with microwave (high temperature, 90 °C) may also increase the IVPD values by modifying protein structure (e.g., reduction of β-sheet content in the secondary structure) and protein degradation (Embaby, 2010; Pan et al., 2020).

The IVPD of cushuro protein isolates and residues ranges from 81.5 to 89.0 g/100 g, which is higher than that of cowpeas (around 70 g/100 g) (Teka et al., 2020), faba bean (80.1 g/100 g) (Elsheikh et al., 1999) and soybean (75.3 g/100 g), but lower than that of casein (93.2 g/100 g) (Guzmán-Ortiz et al., 2015). Excellent digestibility of algae protein has also been reported in other publications. For instance, the protein digestibility of *Ulva* and *Gracilaria* spp. L. is 89 g/100 g (Kazir et al., 2019) and the one of *Undaria pinnatifida* L. is 85 g/100 g (Taboada et al., 2013).

The low percentage of IVPD (75.9 ± 1.5 g/100 g) in cushuro flour could be related to the presence of antinutritional compounds (Hori et al., 1990). In this research, isoelectric precipitation allowed the separation of protein isolate (PI) from other compounds such as carbohydrates, pigments and antinutritional compounds, obtaining a PI with the highest in vitro protein digestibility (89.01 ± 0.94 g/100 g). Therefore, a high content of IVPD could be associated with a better availability to be metabolized and utilized by the digestive system, favoring the adsorption of amino acids, particularly the essential ones, and their transport to the blood.

3.5. Free amino acid and total amino acid profiles

Free amino acids refer to the amino acids that do not interact with other protein, polypeptide, peptide, or amino acid. They contribute to the food taste. Table 4 presents the free amino acid composition of cushuro flour, Residue 1, Residue 2 and Protein isolated (PI) and It is obvious that there are significant differences in the amino acid composition of the four samples, though they are made from the same raw materials. The highest content of free amino acids was found in cushuro flour (414.13 mg/100 g), much higher than those in other samples. The highest arginine in cushuro flour (200.9 mg/100 g), aspartic acid and

Table 4
Free amino acids from cushuro (*Nostoc sphaericum*) flour and protein fractions (mg/100 g dry sample).

Amino Acid	Cushuro flour	Residue 1	Residue 2	PI
Aspartic acid	78.96 ± 2.05[a]	14.44 ± 0.07[b]	13.50 ± 3.20[b]	5.12 ± 0.21[c]
Glutamic acid	66.30 ± 0.09[a]	0.90 ± 0.00[c]	4.73 ± 0.09[b]	0.42 ± 0.40[c]
Asparagine	3.06 ± 0.02[a]	0.10 ± 0.01[c]	0.67 ± 0.19[b]	0.03 ± 0.01[c]
Serine	9.10 ± 0.71[b]	1.14 ± 0.01[c]	12.81 ± 0.58[a]	0.39 ± 0.03[d]
Glutamine	2.07 ± 0.56[a]	–	–	–
Histidine	2.90 ± 0.12[a]	0.07 ± 0.02[c]	0.81 ± 0.44[b]	0.02 ± 0.03[c]
Glycine	11.34 ± 3.13[a]	1.15 ± 0.01[b]	9.68 ± 0.06[a]	0.64 ± 0.07[c]
Threonine	10.70 ± 0.15[b]	0.67 ± 0.24[c]	25.88 ± 0.51[a]	0.31 ± 0.13[c]
Arginine	100.90 ± 1.14[a]	2.59 ± 0.08[c]	7.27 ± 0.31[b]	0.19 ± 0.04[d]
Alanine	30.35 ± 0.18a	1.14 ± 0.11[c]	6.61 ± 0.41[b]	0.59 ± 0.01[d]
Proline	–	–	–	–
Tyrosine	6.87 ± 2.26[a]	0.31 ± 0.12[c]	1.33 ± 0.21[b]	0.22 ± 0.04[c]
Valine	20.91 ± 0.17[a]	–	1.23 ± 0.16[b]	–
Methionine	1.63 ± 0.13[a]	–	–	–
Cystine	0.87 ± 0.02[a]	–	–	–
Isoleucine	15.26 ± 0.03[a]	0.56 ± 0.16[c]	1.45 ± 0.02[b]	0.45 ± 0.04[c]
Tryptophan	1.64 ± 0.04[a]	0.18 ± 0.04[b]	–	–
Leucine	22.62 ± 0.97[a]	0.26 ± 0.10[d]	1.92 ± 0.23[b]	0.77 ± 0.01[c]
Phenylalanine	15.58 ± 0.20[a]	1.54 ± 0.34[b]	2.12 ± 0.31[b]	0.64 ± 0.47[c]
Lysine	13.08 ± 0.03[a]	0.46 ± 0.18[c]	1.30 ± 0.53[b]	0.55 ± 0.00[c]
Total	414.13	25.53	91.31	10.33

PI=Protein Isolated. Results are expressed as means ± SD (n = 3).

glutamic acid constitute the major free amino acids in the four samples. Both amino acids are related to umami taste (Zhang et al., 2013). Moreover, the samples were not found to have a high amount of proline, valine, leucine, phenylalanine and tryptophan, which have been reported to be closely linked to bitterness (Aluko, 2017). Moreover, the content of glycine, alanine and serine, which contributes to sweet taste (Schiffman et al., 1981), were found to be quite low as well. Taken together, all cushuro products present a favorable taste.

The total amino acid composition is summarized in Table 5. Cushuro flour has the highest free amino acid composition, but the lowest total amino acid content (37.23 g/100 g sample), while residue 2 had the most abundant amino acid (92.52 g/100 g sample). In the four cushuro protein products, all essential amino acids were found, though histidine, methionine, tryptophane are all in lower levels (<1g/100 g) compared to other amino acids. The most abundant amino acids in cushuro were Aspartic + Asparagine, threonine, valine, leucine and phenylalanine. The amino acid composition of the proteins showed a balanced profile, as it conforms to the FAO/WHO recommendations for healthy nutrition.

The ratio of essential amino acids (EAA)/non-essential amino acids (NEAA) in the four samples are 0.85, 0.81,0.77 and 0.85 respectively (Table 5). Terriente-Palacios and Castellari (2022) also reported that the ratio of EAA/NEAA in green algae ranges from 0.47 to 0.96. Meanwhile, Romano et al. (2019) suggested that an EEA/NEAA ratio of less than 0.9 is preferred. Hydrophobic amino acids were responsible for the protein or peptides' antioxidant capability (Udenigwe & Aluko, 2011). In the four samples respectively, 43.03 g/100 g, 39.53 g/100 g, 40.82 g/100 g and 41.01 g/100 g amino acid residues are hydrophobic. Therefore, these products are expected to have rather potent antioxidant capability. To the best of our knowledge, this is the first study that provides an analysis of the amino acid composition in cushuro (*Nostoc sphaericum*) flour and its protein fractions.

3.6. Fast protein liquid chromatography

Fig. 2 shows the chromatogram of 4 fractions of cushuro samples. The cushuro flour presented a single signal (blue peak) with a low M_w of 12.72 kDa occupying 100 % of the chromatogram.

After acid extraction of the polysaccharide by UMAE, the cushuro protein was fragmented, showing a main signal (golden peak) corresponding to Residue 1 with a lower M_w of 4.73 kDa. After the alkaline extraction of protein from Residue 1, a Residue 3 was obtained that presented three signals (green peak), one of them of greater intensity, with a M_w of 15.66 kDa, this occurs due to different phenomena of aggregation-disaggregation by effects of alkaline pH, temperature and drying. Finally, the intense signal (red peak) with an M_w of 24 kDa corresponds to the Protein isolated (PI) and represented almost 100% of the chromatogram and did not present another peak, indicating that a more purified sample was obtained in comparison with the protein residues and the cushuro flour.

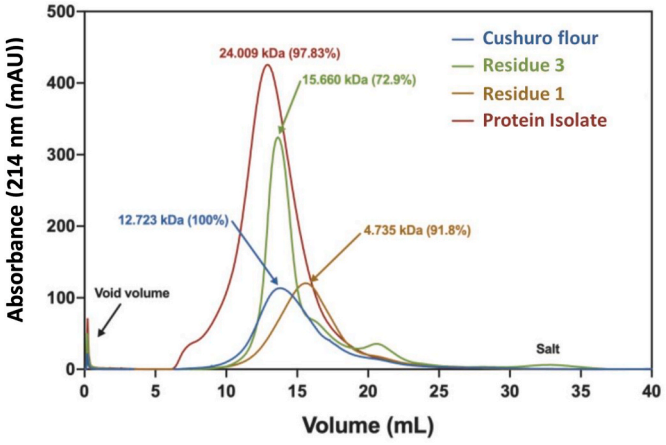

Fig. 2. FPLC Elution profiles from cushuro (*Nostoc sphaericum*) flour and protein fractions.

Table 6
Functional properties from cushuro (*Nostoc sphaericum*) flour and protein fractions.

	CF	R1	R2	R3	PI
WHC (g water/ g protein)	26.3 ± 0.5^{a}	2.8 ± 0.1^{b}	3.0 ± 0.2^{b}	8.2 ± 0.2^{c}	4.9 ± 0.4^{d}
FAC (g oil/g protein)	4.00 ± 0.3^{a}	1.5 ± 0.0^{b}	2.3 ± 0.3^{b,c}	3.7 ± 0.4^{a,c}	1.80 ± 0.0^{b}
FC (%)	13.0 ± 0.3^{a}	112±2^{b}	24.9 ± 0.4^{c}	34.2 ± 0.9^{d}	57.1 ± 1.2^{e}

WHC: Water holding capacity; FAC: Fat absorption capacity; FC: Foaming capacity.

Table 5
Total amino acid from cushuro (*Nostoc sphaericum*) flour and protein fractions.

Amino Acid	Cushuro flour		Residue 1		Residue 2		PI	
	g/100g sample	g/100g protein	g/100g sample	g/100g protein	g/100g sample	g/100g protein	g/100g sample	g/100g protein
Aspartic + Aspargine	5.62 ± 0.04	15.10 ± 0.12	10.28 ± 0.13	13.79 ± 0.06	13.85 ± 0.09	14.98 ± 0.08	13.34 ± 0.08	15.31 ± 0.05
Glutamic + Glutamine	2.99 ± 0.02	8.03 ± 0.17	6.00 ± 0.04	8.05 ± 0.09	7.44 ± 0.21	8.05 ± 0.32	7.12 ± 0.03	8.17 ± 0.06
Serine	1.95 ± 0.00	5.23 ± 0.06	4.46 ± 0.01	5.99 ± 0.09	5.48 ± 0.05	5.92 ± 0.02	5.46 ± 0.04	6.26 ± 0.03
Histidine	0.09 ± 0.00	0.25 ± 0.00	0.28 ± 0.01	0.37 ± 0.02	0.21 ± 0.01	0.22 ± 0.01	0.72 ± 0.01	0.82 ± 0.01
Glycine	2.40 ± 0.02	6.46 ± 0.04	5.44 ± 0.07	7.30 ± 0.03	5.30 ± 0.10	5.73 ± 0.04	6.45 ± 0.07	7.41 ± 0.10
Threonine	4.41 ± 0.07	11.84 ± 0.02	7.27 ± 0.13	9.75 ± 0.01	11.28 ± 0.17	12.19 ± 0.04	7.60 ± 0.08	8.72 ± 0.07
Arginine	2.23 ± 0.01	5.98 ± 0.05	3.44 ± 0.02	4.62 ± 0.06	4.15 ± 0.09	4.48 ± 0.04	3.91 ± 0.02	4.49 ± 0.01
Alanine	2.97 ± 0.01	7.99 ± 0.10	5.59 ± 0.00	7.50 ± 0.12	8.07 ± 0.13	8.73 ± 0.04	6.61 ± 0.01	7.59 ± 0.00
Proline	1.76 ± 0.11	4.73 ± 0.22	3.14 ± 0.02	4.21 ± 0.04	3.97 ± 0.26	4.29 ± 0.23	4.05 ± 0.24	4.64 ± 0.29
Tyrosine	0.60 ± 0.01	1.60 ± 0.01	1.82 ± 0.02	2.45 ± 0.02	1.65 ± 0.03	1.78 ± 0.01	1.64 ± 0.01	1.89 ± 0.01
Valine	3.70 ± 0.20	9.93 ± 0.38	6.62 ± 0.56	8.88 ± 0.60	9.60 ± 0.13	10.38 ± 0.01	6.40 ± 0.21	7.35 ± 0.22
Methionine	0.07 ± 0.00	0.18 ± 0.00	0.22 ± 0.00	0.29 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.19 ± 0.01
Cystine	0.03 ± 0.00	0.09 ± 0.01	0.14 ± 0.00	0.18 ± 0.00	0.10 ± 0.00	0.11 ± 0.00	0.51 ± 0.01	0.58 ± 0.01
Isoleucine	2.13 ± 0.02	5.73 ± 0.03	4.74 ± 0.06	6.36 ± 0.03	5.35 ± 0.06	5.78 ± 0.00	5.49 ± 0.00	6.30 ± 0.02
Tryptophan	0.39 ± 0.03	1.05 ± 0.07	0.76 ± 0.13	1.01 ± 0.16	0.80 ± 0.01	0.86 ± 0.00	0.87 ± 0.02	0.99 ± 0.02
Leucine	2.45 ± 0.02^{d}	6.57 ± 0.05^{D}	6.96 ± 0.04^{b}	9.35 ± 0.10^{B}	5.92 ± 0.07^{c}	6.40 ± 0.00^{C}	8.38 ± 0.03^{a}	9.62 ± 0.06^{A}
Phenylalanine	2.41 ± 0.01	6.47 ± 0.05	5.37 ± 0.05	7.20 ± 0.06	6.41 ± 0.09	6.93 ± 0.02	6.00 ± 0.18	6.88 ± 0.19
Lysine	1.03 ± 0.01	2.75 ± 0.02	2.00 ± 0.00	2.69 ± 0.04	2.79 ± 0.04	3.01 ± 0.01	2.42 ± 0.00	2.77 ± 0.01
Total	**37.23**	**100**	**74.51**	**100**	**92.52**	**100**	**87.14**	**100**

PI=Protein Isolate. Results are expressed as means ± SD (n = 3).

3.7. Functional properties

Table 6 shows the functional properties from cushuro flour and protein fractions. Water holding capacity (WHC) varied from (2.8 ± 0.1–26.3 ± 0.5 g water/g protein) and it was higher in cushuro flour (26.3 ± 0.5 g water/g protein) than protein fractions, because the WHC decreased by extraction of the polysaccharide, and other compounds by UMAE. WHC in PI (4.9 ± 0.4 g water/g protein) was like protein isolate from *Spirulina platensis* (5.1 g water/g protein) (Yücetepe et al., 2019) and higher to protein isolate from *Kappaphycus alvarezii* (2.2 ± 0.0 g water/g protein) (Kumar et al., 2014). WHC defines the ability of a food to retain water in its structure and is related with the viscosity properties of the food (Bleakley and Hayes, 2021).

Fat absorption capacity (FAC) varied from 1.5 ± 0.0–4.0 ± 0.3 g oil/g protein and it was high in cushuro flour (4.0 ± 0.3 g oil/g protein), R3 (3.7 ± 0.4 oil/g protein) than PI (1.8 ± 0.0 g oil/g protein). The FAC in PI was lower than protein isolate of *Spirulina platensis* (10.2 g of oil/g protein) (Yücetepe et al., 2019) and seaweed *H. elongata* protein concentrate (8.1 g oil/g protein) (García-Vaquero et al., 2017). A high FAC value is desirable because it preserves the flavor and improves the palatability of food products (Bleakley and Hayes, 2021; Waghmare et al., 2016).

Foaming capacity (FC) varied from (13.0 ± 0.3–112 ± 2 %) and it was higher in residue 1 (112 ± 2 %) than PI (57.1 ± 1.2 %), also FC in PI was higher the *Arthospira platensis* (41.3 %) (Mahali & Sibi, 2019). Similar results were reported for *H. elongata* protein concentrate (71.5 ± 4.8 %) (García-Vaquero et al., 2017), 54 % for *Kappaphycus alvarezii* (Kumar et al., 2014). The FC improves lightness, softness and allows the volatilization of aromas, improving the palatability of food products (Ngoc et al., 2012).

4. Conclusions

The results demonstrated that cushuro (*Nostoc sphaericum*) contain significant amounts of minerals (Ca, Fe, Mg, K, Na, P, Zn) and protein. The protein fractions of cushuro present an acceptable digestibility that is improved with respect to the initial flour. The total amino acid profile is complete with a notable amount of essential amino acids and hydrophobic amino acids. The predominant free amino acids that were detected are related to the umami flavor, so sensorially it is anticipated that cushuro and its protein fractions have good sensory acceptability, and functional properties that could be used in the development of different foods. It is recommended to continue with the study of the biological activities of the protein fractions of cushuro and its peptides, based on their antioxidant properties and in comparison with other protein sources.

CRediT authorship contribution statement

N. Chasquibol: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **A. Sotelo:** Methodology, Investigation, Formal analysis. **M. Tapia:** Writing – original draft, Investigation, Formal analysis. **F.M. Goycoolea:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **A.J. Hernández-Álvarez:** Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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