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RESEARCH

Optimization of Protein Extraction from Duckweed Using Different Extraction Processes

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Abstract

Despite its recent commercialization of duckweed, the extraction of high-quality protein presents challenges primarily in the efficiency of the extraction processes. In this study, conventional, ultrasound-assisted extraction (UAE), and ultrafiltration (UF) extraction techniques, were evaluated showing significant enhancements in protein yield, amino acid profile, and chlorophyll extraction. The highest protein release of 80.83% was achieved with a 40% amplitude applied for 10 min. UAE increased the protein yield substantially compared to conventional methods, achieving the highest yield of 41.30% with a moderate protein content of 50.91% in *Lemna gibba* protein concentrate. However, it comes at the cost of reduced protein purity and altered protein profiles due to co-extraction of other plant components. SDS-PAGE analysis revealed that ultrasound methods improved the extraction of chlorophyll-binding proteins (LHCB1) but decreased the efficiency of extracting RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) subunits compared to non-ultrasound methods. Lysine and tryptophan were identified as limiting essential amino acids (EAA), except when ultrasound methods were applied, which raised the levels partially above the recommended amino acid score (AAS) threshold of 1 for adults. The study established a nitrogen-to-protein conversion factor for duckweed at ~ 5.8, aligning with RuBisCO-based calculations and providing a more accurate tool for future nutritional assessments of duckweed proteins.

Keywords *Lemna gibba* · Response surface methodology · Ultrasound-assisted extraction · Ultrafiltration · Mild protein extraction

Introduction

Duckweed also known as water lentil was rediscovered in the 1980s (Muller et al., 2023) as a potential alternative protein source. As a small aquatic plant that grows in calm or slow-flowing freshwater, duckweed belongs to the Lemnaceae family, which has five genera (*Spirodela*, *Lemna*, *Landoltia*, *Wolfiella*, and *Wolffia*) and 38 species (William. S. Hillman 1961; Pagliuso et al., 2022). Depending on the species, total protein content ranges from ~ 20 to 35% based on dry weight, making it a promising candidate for food protein fortification (Appenroth et al., 2017). As of April 9, 2024, the European Food Safety Authority authorized the marketing and commercialization of a protein concentrate from *L. gibba* and *L. minor* as a novel food in accordance with European Parliament regulation 2015/2283, for a private company (The European Commission 2024; Turck et al., 2023).

Duckweed is a fast-growing plant, capable of doubling its biomass in less than 96 h (Ziegler et al., 2015), and grows in

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a wide range of conditions (pH, temperature) (J. P. Goopy and P. J. Murray 2003). It has a low ecological footprint, with 0.4 kg CO_2 equivalent per kg of duckweed, compared to 27 kg CO_2 equivalent per kg of beef (Beukelaar et al., 2019). Its nutritional value comes from its high protein content, balanced amino acid profile, and the presence of essential amino acids (EAA) and non-EAA, which meet WHO/FAO recommendations (FAO, 2011; WHO, 2007). The EAA content in duckweed often matches or surpasses FAO reference values, e.g. *L. minor*, *L. gibba*, *S. polyrhiza*, *L. punctata*, and *W. columbiana* contain leucine, phenylalanine, and valine in quantities higher than the FAO, 2011 reference (Xu et al., 2021). Duckweed is also highly digestible, as feed (Pagliuso et al., 2022) providing valuable n3 and n6 fatty acids, with a higher content of polyunsaturated than monounsaturated fatty acids. It has high concentrations of antioxidants such as xanthine, chlorophylls, and carotenoids, including lutein, which has health-promoting properties to prevent age-related macular degeneration (Appenroth et al., 2017). In addition, RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase), a major cytoplasmic protein fraction within chloroplasts, represents almost 50% of all proteins in fresh duckweed (Nieuwland et al., 2021). RuBisCo is described as having a neutral odor and taste and being white or cream in color (Roberto Fiorentini & Carlo Galoppini, 1983). Given their nutritional benefits, various extraction processes have been studied for isolating green leaf proteins.

Conventional (wet-chemical) extraction processes are frequently used to produce protein concentrates or isolates by protein solubility and subsequent precipitation at the isoelectric point (Yang & Sagis, 2021; Yang et al., 2022). A key challenge in extracting proteins from green leafy plants is the separation of green pigments, primarily chlorophyll, to produce a light-colored protein powder, enabling greater versatility in food product applications. Several scientific studies have addressed this issue and applied different extraction methods and processes. Kobbi et al. (2017) added ammonium sulfate solution to fresh alfalfa leaves to separate soluble and insoluble fractions, followed by triple solubility at an alkaline pH of 10. Acidification at pH 3 precipitated proteins, leading to a light-brown protein juice with a protein content of 69 to 73% (Kobbi et al., 2017). There are a number of studies in which heat coagulation has been used to extract proteins from green-leafy plants, with a particular focus on RuBisCo. Recent studies from Muller et al. (2023) recovered RuBisCo from duckweed powder at 80 °C, followed by purification at pH 4, using HCL, yielding a protein yield of 60% and a total protein content of 57.9%. Contrary to these findings, Nynäs et al. (2021) found RuBisCo significantly degrades above 55 °C. Various factors, such as cell wall thickness, the maturity of the plants, or factors, such as the thermal sensitivity of RuBisCo, can differ between the species and must be taken into account. Although the application of heat to green leafy plants during extraction can be advantageous, for example, to break down the chlorophyll (> 50 °C), which can result in less intense green colored protein powder, higher temperatures can lead to a loss of native protein conformation and denaturation of the proteins (Carmen Merodio, Mercedes Martin, and Bartolomé Sabater 1983; Chiesa & Gnansounou, 2011); therefore, different mild extraction approaches should be considered and investigated to examine their effects and benefits.

Research on duckweed as a novel green protein source and mild extraction methods is limited due to the complex cell matrix of green-leaved plants. This study focuses on mild extraction methods, without the use of heat, by applying ultrafiltration and ultrasound-assisted extraction (UAE) for cell disruption and protein release. Ultrasonication, using low frequency (20–100 kHz) and high power (amplitude > 30%), is an effective technology for protein extraction (Vernès, Vian and Chemat 2020; Yusoff et al., 2022). UAE offers advantages such as reduced extraction time, temperature regulation, decreased solvent volume, and increased yield of target compounds. Ultrasound waves produce acoustic cavitation bubbles that disrupt the cell walls of plants, releasing bioactive compounds like proteins (Vernès, Vian and Chemat 2020). The application of UAE is being intensively researched in various plants from seeds, legumes, or algae in order to extract proteins and antioxidants, improve functional properties, reduce the immunoreactivity of allergens, or increase digestibility for humans, e.g., in rapeseed, yeast cells, *Chlorella vulgaris*, soy, chickpea, and faba bean (Dong et al., 2011; Liu et al., 2013; Hildebrand et al., 2020; Zhang et al., 2021; Ravindran, Akshaya and Ramaswamy, Hosahalli S 2023; Wang et al., 2022; Badjona et al., 2024; Aghababaei et al., 2024). The improved solubility of, e.g., proteins after ultrasound treatment is attributed to mechanical forces, including shear- and microcurrents due to cavitation, which can lead to the opening and partial unfolding of protein structures. Such effects could lead to a break-down of non-covalent bonds, including hydrophobic and electrostatic interactions, as well as hydrogen bonds, that promote stronger interactions between proteins and water molecules (Silventoinen & Sozer, 2020).

There is limited research on duckweed regarding UAE of proteins. Duangjarus et al. (2022) investigated UAE on Duckweed species *Wolffia globosa*, by also combining different protein extraction methods with it and obtained total protein contents between 20 and 60%. However, information on the protein yield of ultrasound-assisted protein extraction and the comparison with non-ultrasound-treated samples is lacking, which means that further studies are needed to emphasize the effect of ultrasound. Recent studies by Mirón-Mérida et al. (2024) and Nitiwuttithorn et al. (2024) showed differences in the optimization of UAE with optimal protein extraction at alkaline conditions of pH 11.5 for *L. minor* and at a lower pH of 8.5 for *W. arrhiza*, respectively. In addition,

ultrafiltration has been successfully used to concentrate proteins, e.g., from sugar beet leaves (De Jong, Govardus, Adrianus, Hubertus et al. 2014 2014; Martin et al., 2019) or duckweed (*Lemna minor*) (Martens et al., 2021) and has proven to be effective in removing polyphenols and chlorophyll in combination with salts and increased protein yields. This research aims to establish an eco-friendly method to improve the extraction of duckweed protein. Integrating UAE and ultrafiltration (UF) could enhance protein content and yield, resulting in nutrient-rich concentrates with balanced amino acid profile. Avoiding thermal treatment allows the evaluation of ultrasonication's impact on protein structure, preserving heat-sensitive compounds and potentially improving nutritional value and extract quality.

Materials and Methods

Raw Material

Lemna gibba (LG), Clone No. 7796, was originally obtained from the University of Jena, Germany. It is cultivated in a greenhouse at the Institute of Horticulture, Weihenstephan-Triesdorf University of Applied Sciences, using natural light in summer and artificial light in winter, with a 16-h light period, and temperatures are controlled around 20 °C. According to a defined SOP, it is washed, frozen, freeze-dried (FD260, Wave, Austria), vacuum-sealed, and stored at 4 °C until further use. For protein extraction, a large batch of fine powder was produced using a ball mill (PM 100 Retsch, Germany) and stored in an airtight container at − 25 °C until further processing.

Optimization of Protein Extraction Using Response Surface Methodology

Response surface methodology (RSM) was applied, using a central composite design (CCD) for the optimization of protein solubility and protein precipitation. The applied type CCD is often used for designing sequential experiments until an optimum accuracy of variables is achieved by adding center and axial points. To identify the optimal conditions for maximum protein solubility, three variables were selected: pH, extraction time, and the powder concentration in wt%. Protein precipitation focuses on adjusting the pH and extraction time. A multiple regression model (a second-order polynomial equation) was proposed for the response, according to Eq. 1 with three variables ($n=3$) and $\alpha=1.68$. The factors were chosen to be applied in a 2^3 full factorial design.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i<j=1}^{3}\beta_{ij} X_i X_j \quad (1)$$

The experimental design allows the analysis of various operating conditions simultaneously and identifies the most significant extraction parameters for protein solubility and precipitation, respectively.

Table 1 Protein solubility parameter

Runs	pH	Extraction time in min	Conc. in wt%
14	6.3*–10.0	60–90	0.5–5

*pH 6.3 is the natural pH of LG solubilized in deionized water

Table 2 Protein precipitation parameter

Runs	pH	Extraction time in min
19	2–4	20–40

Protein Solubility

In the experimental design, 14 runs with different parameter settings were tested based on preparations with total quantities of 25 g (Table 1). For each run, three samples were prepared and measured in triplicate. In detail, an aqueous solution was prepared on an analytical balance (Precisa, $d=0.0001$ g, Switzerland) according to the parameters defined in the RSM model, subsequently stirred on a magnetic stirrer after pH adjustment, using 0.5, 1, 2, or 5 M NaOH solution, and then centrifuged at 4600 rpm for 20 min. The protein content of the supernatant was measured with the BCA-Protein-Assay. Bovine-serum albumin (BSA) (Thermo Scientific™ Pierce, USA) was used as standard, and the results were calculated as mg equivalent of BSA per g of dry biomass and expressed in percentage.

Protein Precipitation

The protein precipitation at the isoelectric point (IEP) was optimized in 19 runs (see Table 2). The range of pH values for the precipitation of the proteins was selected using the solubility curve from the "Protein Solubility Curve" section, and the respective runs were defined in the RSM design, taking the extraction time into account. The optimized powder concentration of 0.5 wt% was maintained from the previous protein solubility experiments. In order to analyze the optimal IEP, the supernatant 1 (S1) of the protein solubility obtained after centrifugation was stirred on a magnetic stirrer and adjusted to the respective pH using 0.5, 1, 2, or 5 M HCL, to determine the optimal precipitation point. After precipitation, the samples were centrifuged, and the supernatant 2 (S2) was analyzed for protein content by applying the BCA-Protein-Assay using BSA (Thermo Scientific™ Pierce, USA) as standard, and the results were calculated as mg equivalent of BSA per g of dry biomass and expressed in percentage.

A multiple regression model (a second-order polynomial equation) was proposed for the response according to Eq. 2, with two variables ($n = 2$) and $\alpha = 1.41$. The factors were chosen to be applied in a 2^2 full factorial design.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i<j=1}^{2} \beta_{ij} X_i X_j \quad (2)$$

Ultrasound-Assisted Protein Release

A high-intensity probe ultrasound generation system of max. Power 400 W, 24 kHz was used. The instrument was the model UP400St from Hielscher Ultrasonics GmbH (Teltow, Germany) for which amplitude (10–100%), and time settings were used. The optimization of the ultrasound parameters was carried out and determined before the optimum of the conventional protein extraction was found ("Optimization of Protein Extraction Using Response Surface Methodology"). The treatment was carried out in an ice bath; the maximum temperature was set at 48 °C with a restart temperature difference of 5 °C, to prevent protein denaturation. A suspension of duckweed powder in a concentration of 2.5 wt% and deionized water was mixed in a plastic beaker with a capacity of 100 ml and placed on a magnetic stirrer for 15 min, starting with a natural pH value of 6.3. The beaker was placed in an ice bath and fixed to allow complete immersion. The sonotrode (diameter of 22 mm) and a thermometer were immersed in the center of the sample, to a depth of approximately 2 cm into the beaker. In consideration of the diameter of 22 mm of the sonotrode, a setting of, e.g., 40% power amplitude means an amplitude of 40 µm peak-to-peak. After centrifugation, the protein content of the supernatant (10 ml) was measured in triplicate using the Kjeldahl method ($N \times 5.8$, see "Specific Nitrogen Conversion Factor" section).

Particle Size Distribution

Particle size analyzer S3500 from Microtrac (USA), consisting of a Tri-Laser-System, using the laser diffraction technique was applied to determine the particle size distribution. The S3500 is equipped with a wet dispersion module operating in a measuring range between 0.1 and 1000 µm. The results of the particle size distribution were displayed as a cumulative distribution function which is characterized by the percentile values d10, d50, and d90. The measurements were used to evaluate the optimization of the ultrasound process concerning protein release ("Ultrasound-Assisted Protein Release" section). In addition to that, the width of particle size distribution was calculated (span in %) using Eq. 3.

$$\text{Span in } \% = \frac{(\text{d90} - \text{d10})}{\text{d50}} \quad (3)$$

Protein Solubility Curve

To evaluate the protein solubility profile, two different protein solubility curves at a series of pH values from pH 2 to 12 were performed. Accordingly, freeze-dried duckweed *L. gibba* was ground to a powder, and the samples were suspended in deionized water at a concentration of 2.5 wt% and set to the relevant pH value using a pH meter (pH 1100 H, accuracy ≤ 0.005 pH ± 1 digit, VWR, Germany). The samples were then stirred for 30 min and centrifuged at 4600 rcf for 30 min at RT. The second protein solubility curve was generated in the same way, except with additional ultrasound treatment using the optimum parameters determined in the "Ultrasound-Assisted Protein Release and Particle Size Distribution" section. The protein content of the supernatant was measured in triplicate by using the Pierce BCA-Protein-Assay method using BSA (Thermo Scientific™ Pierce, USA) as standard, and the results were calculated as mg equivalent of BSA per g of dry biomass and expressed in percentage.

Protein Extraction Processes

Based on the optimized extraction parameter from the RSM design of conventional protein extraction (see "Optimization of Protein Extraction Using Response Surface Methodology"), four different extraction processes were tested including UAE for protein release and ultrafiltration (UF) instead of protein precipitation. In Fig. 1, all four extraction processes are illustrated in a flowchart: process A, conventional with wet chemical extraction (LG-C); process B, with prior additional UAE (LG-US-C); process C, use of ultrafiltration instead of acidic precipitation (LG-UF); process D, with prior UAE and UF (LG-US-UF). UAE was carried out with the optimized parameters from the "Ultrasound-Assisted Protein Release and Particle Size Distribution" section, and UF was conducted using 100 kDa (Hydrosart® Membrane, Sartorius AG, Germany) at 2.5 bar transmembrane pressure and a flow rate between 340 and 360 ml/min. The protein concentrate obtained from IEP precipitation was neutralized to pH 7 as well as for the samples that had been concentrated by UF. All samples were finally freeze-dried to obtain a powder. The crude protein content of extracted duckweed concentrate was measured by the method of Dumas with Dumatherm N Pro from C. Gerhardt GmbH, Germany, using protein (*N*) calculation factor 5.8. The protein yield was quantified as the pure mass of the resulting protein concentrate (m_P), considering the specific protein content (c_P) in relation to the total mass of the duckweed flour (m_R) and its initial protein content (c_R), used for extraction (Eq. 4).

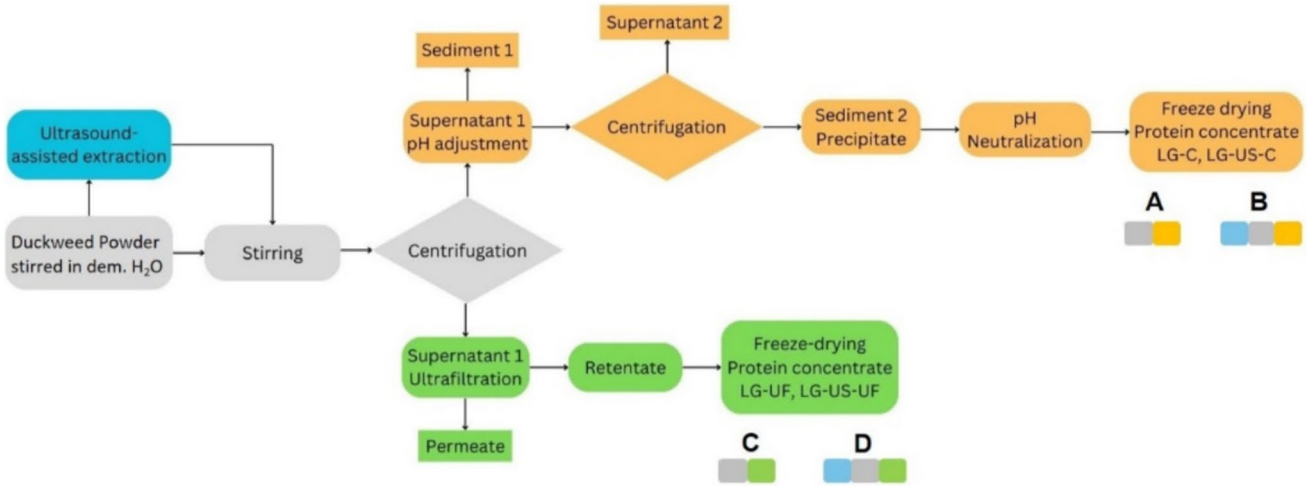


Fig. 1 Protein extraction processes

$$\text{Protein Yield in } \% = \frac{(m_P * c_P)}{(m_R * c_R)} * 100 \tag{4}$$

L*a*b* Color Measurement

Digital capturing and visualization of color differences have been performed by using a DigiEye (VeriVide, UK) in diffuse mode to remove the specular reflection. A standardized light source CIE D65 was chosen, as most food is viewed in daylight. The angle of observation was set to 10° to simulate a wide field of view. The L*a*b* values were determined, which stand for coordinates in the CIELAB color room. When comparing two different colors, differences in the color coordinates are formed. These are marked with delta ΔE. The ΔE indicates the total color difference with regard to the initially used *Lemna gibba* flour to protein concentrates, without direction. The letter X in the equation stands for the respective value from LG flour. The total distance ΔE was calculated using Eq. 5.

$$\Delta E = \sqrt{(X - \Delta L^*)^2 + (X - \Delta a^*)^2 + (X - \Delta b^*)^2} \tag{5}$$

Determination of Chlorophyll Content

A chlorophyll (Chl) a and b double extraction was carried out according to Castle et al. (2011) and Caesar et al. (2018) using calculation coefficients for Chl a and b from Ritchie, 2006. Duckweed protein concentrate or dried duckweed was ground to a fine powder from which 20 mg and a spatula tip of ~ 4 mg $MgCO_3$ (Sigma-Aldrich Chemie GmbH, Germany) was mixed with 6 ml of ethanol ≥ 96% (Carl Roth GmbH + Co. KG, denatured) which was stirred for 5 min at 350 rpm. Subsequently, the incubation of the suspension was carried out in a water bath at 80 °C for 5 min. For this purpose, the samples were first transferred into centrifuge tubes and sealed with parafilm to prevent evaporation of the ethanol while allowing gas to escape. Next, the samples were put for 20 min on an orbital shaker. The samples were then centrifuged for 10 min at 4000 rpm, and the supernatant 1 was transferred to a beaker, while the pellet was subjected to a second extraction cycle. After the pellet had undergone a second extraction cycle under the same extraction steps described above, the second supernatant was combined with the first, and a 1:1 dilution with ethanol 96% was prepared before measurement. The blank sample was prepared with ~ 4 mg $MgCO_3$ mixed in 6 ml ethanol 96% and was subjected to the same pre-treatment steps. As a control, a cuvette with 96% of ethanol was filled and measured. The absorptions were measured with a spectrophotometer SPECORD PLUS 210, Jena Germany, at respective wavelengths 649, 665, and 750 nm. The calculation of the Chl a and b content is provided in Eqs. 6 and 7 expressed in mg per g sample (dry weight). To calculate, the values of absorption (A_x), dilution factor (DF), the amounts of solvent (S) in ml, and sample weight (W) in g are needed. The total Chl a + b content is the sum of the results obtained from Eqs. (6) and (7), applied according to Caesar et al. (2018) and Ritchie (2006.

$$Chla\left[mg/g\right] = \left(\frac{\left(13.5275 * \left(A_{665} - A_{750}\right) - 5.2007 * \left(A_{649} - A_{750}\right)\right) * DF * S}{W}\right)/1000 \tag{6}$$

$$Chlb\left[mg/g\right] = \left(\frac{\left(22.4327 * \left(A_{649} - A_{750}\right) - 7.0741 * \left(A_{665} - A_{750}\right)\right) * DF * S}{W}\right)/1000 \quad (7)$$

Antioxidant Capacity and Phenolic Content

Preparation and Extraction

The absorptions of all three antioxidant methods were measured spectrophotometrically with the SPECORD 210 Plus in triplicate. To prepare antioxidant determinations, duckweed was ground and extracted according to the method of Stratil et al. (2006), with slight adaptions. First, an acidified methanol solution was prepared, which is composed of 240 ml methanol (> 99.8%, ultrapure, spectrophotometric grade, Thermo Scientific, USA), 57 ml distilled water, and 3 ml acetic acid (99–100%, Sigma-Aldrich, USA). A quantity of 0.5 g duckweed sample was weighed into an Erlenmeyer flask and mixed with 20 ml of acidified methanol solution, which was then covered with parafilm and incubated at 40 °C for 30 min under constant stirring. The sample was transferred to a centrifuge tube and centrifuged at 4000 rpm for 10 min and then filtered with a Whatman filter Grade 1 V. The filtrate was the starting sample for the determination of the antioxidant capacity (FRAP, DPPH) and the phenolic content (Folin-Ciocalteu).

FRAP

The FRAP (ferric reducing antioxidant power) method measures the reduction of complex ferric ions Fe^{3+}-ligand (colorless) to Fe^{2+} which appear in a blue ferrous complex. The antioxidant reaction was carried out under acidic conditions (pH 3.6) to maintain iron solubility. It possesses a high sensitivity and precision, which analyses predominantly hydrophilic antioxidants (Munteanu & Apetrei, 2021; Rumpf et al., 2023). The FRAP test was performed according to the methods from Benzie (1996), Stratil et al. (2006), and Tzima et al. (2021) with some adaptions. The calibration curve ($R^2 = 0.9919$) was generated (see supplementary materials SFig. 3) using a standard dilution series in the optimum measuring range of 0.1–0.8 mg mL^{-1} with a stock solution of Trolox 1 mg/ml (Merck Millipore, USA) in diluted methanol 76% (> 99.8%, ultrapure, spectrophotometric grade, Thermo Scientific, USA; demineralized water). To prepare the FRAP-Reagent, 12.4 g of TPTZ (2,4,6-Tri-(2-pyridyl)-s-triazin)-reagent (Cayman Chemical, USA) was solved in 4 ml HCL 35% (VWR Chemicals, France). A ferric-chloride ($FeCl_3$) solution was separately prepared by dissolving 129.8 mg $FeCl_3$ (97%, Sigma-Aldrich, USA) in 40 ml deionized H_2O. The freshly produced FRAP-Reagent contained 10 mM TPTZ solution, 20 mM $FeCl_3$ solution, and 300 mM acetate buffer pH 3.6 (Morphisto GmbH, Germany) were mixed in a ratio of 1:1:10. The FRAP reagent was covered in an aluminum foil to prevent light oxidation and was incubated for 10 min at 37 °C. If the value of absorption exceeded the linear range of the Trolox standard curve, the sample was diluted with methanol 76% and was then mixed with FRAP reagent 1:10. The mixture underwent an incubation of 40 min at 37 °C in the dark, and the absorption was measured at 593 nm.

DPPH

The DPPH method is a neutralization test, based on the release of electrons by the antioxidants to neutralize the DPPH radical (radical scavenging). The method is based on a discoloration from deep purple to pale yellow, as an indicator of increasing antioxidant capacity by radical scavenging. DPPH (2,2-diphenyl-1-picrylhydrazil) is an organic radical and primarily measures hydrophobic antioxidants (Hu et al., 2022; Munteanu & Apetrei, 2021) and in particular small antioxidant molecules, as these have better access to the radical site of DPPH (Magalhães et al., 2008). The DPPH test was performed according to the manuscript of W. Brand-Williams (1995), Lee et al. (2014), and Akar et al. (2017) with some adaptions. For the calibration curve of the method ($R^2 = 0.9966$, see supplementary materials SFig. 4), a standard dilution series in the optimum measuring range of 0.01–0.35 mg mL^{-1} with Trolox 0.5 mg/ml (Merck Millipore, USA) was prepared. The solvent solution was prepared by dissolving 1 mg DPPH (95%, Thermo Scientific, USA) in 40 ml methanol > 99,8% (ultrapure, spectrophotometric grade, Thermo Scientific, USA) and was wrapped in aluminum foil. Finally, a mixture of 51 µl extracted sample was mixed with 1.9 ml solvent solution in small centrifugal reaction tubes (2 ml, plastic, sterile, Brand GmbH + Co. KG, Germany) and vortexed to homogeneity. After the final incubation time of 90 min at RT, the absorbance values were recorded at 517 nm.

Folin-Ciocalteu (FC)

The phenolic content was analyzed through the Folin-Ciocalteu (FC) method. The FC method is based on the reduction of the FC reagent by phenolic molecules in an alkaline environment (~ pH 10), after the addition of a Na_2CO_3 solution, which induces a color change from yellow to blue chromophores. The FC method predominantly measures hydrophilic antioxidants (Munteanu & Apetrei, 2021). The

FC method was performed according to Vernon L. (1999) with slight modifications. The total phenolics are expressed as mg gallic acid equivalent (mg GAE/mL) (97.5–102.5% titration, Sigma-Aldrich, Germany) using a standard calibration curve ($R^2 = 0.9995$, see supplementary materials SFig. 5), which is prepared from 0.005 to 0.07 mg mL^{-1} (Lee et al., 2014). The FC reagent was diluted 1:10 with methanol 6% (> 99.8%, ultrapure, spectrophotometric grade, Thermo Scientific, USA) and wrapped in aluminum foil. Afterward, the 120 µl extracted sample was mixed with 900 µl FC reagent (Merck KGaA, Germany) in a centrifugal tube (2 ml, plastic, sterile) and incubated for 5 min at RT in the dark. Then 900 µl Na_2CO_3 solution (Merck KGaA, Germany) was added to raise the pH to alkaline conditions for an optimal reaction and the incubation lasted 90 min at RT in the dark. After incubation, the absorption is measured at 760 nm (Singleton & Orthofer, 1974; Gülçin, 2011).

SDS-PAGE Electrophoresis

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed as described by Prandi et al. (2021) with some modifications. The amount of sample containing 60 µg of protein was weighed into tubes and dissolved with 25 µL of sample buffer (17.5 µL ultrapure water, 6.25 µL of sample buffer 4×, and 1. 25 µL of reducing agent 20×— BIO-RAD, Hercules, CA, USA). Samples were thermocycled at 95 °C for 5 min to denature proteins and loaded onto 10% Criterion XT Bis–Tris Gel (BIO-RAD, Hercules, CA, USA); a protein marker was also loaded for the identification of molecular weights. The run was performed in an MES buffer (BIO-RAD, Hercules, CA, USA), for 45 min at 150 V using the PowerPac™ (BIO-RAD, Hercules, CA, USA). At the end of the run, the gel was stained with the staining solution (50% ultrapure water, 40% methanol, 10% glacial acetic acid, 1 g/L Comassie Brilliant Blue — BIO-RAD, Hercules, CA, USA) for 2 h at 20 rpm. After 4 washes (20 min at 20 rpm each) with the destaining solution (50% ultrapure water, 40% methanol, 10% glacial acetic acid), the gel was scanned using a GS-800 calibrated imaging densitometer (BIO-RAD, Hercules, CA, USA). The identification of the bands was performed, with the help of the protein marker, for comparison with the literature.

Amino Acid (AA) Analysis

The determination of total amino acids was performed in triplicate as described by Cutroneo et al. (2023). Briefly, total amino acids were determined with acid hydrolysis — 23 h, 110 °C, in 6 N HCl with the use of nor-leucine (Sigma-Aldrich, St. Louis, MO, USA) as internal standard (50 mM in 0.1 N HCl) — with the exception of sulfur-containing amino acids and tryptophan. Sulfur-containing amino acids (cysteine and methionine) were subjected, indeed, to a phase of pre-oxidation with freshly prepared performic acid (95% formic acid and hydrogen peroxide in ratio 90:10). Subsequently, they underwent the acid hydrolysis with the same conditions as for total amino acids. For the quantification, a calibration curve was performed by mixing the 2.5 mM AA standard mixture (Waters, Milford, MA, USA) with a 2.5 mM mixture of AA (nor-leucine, cysteic acid, methionine sulfone — Sigma Aldrich, St. Louis, MO, USA) in the ratio of 1:1. Five different subsequential dilutions were performed in triplicate obtaining 6 concentration points: 1.25 mM, 0.625 mM, 0.3125 mM, 0.156 mM, 0.078 mM, 0.039 mM. Prior to analysis, samples were derivatized using the AccQ-Fluor reagent kit (Waters, Milford, MA, USA) following the guidance material. Tryptophan determination was performed with an alkaline analysis (6 h, 100 °C, in 4 N NaOH), using α-methyl-tryptophan (50 mg/100 mL — Sigma Aldrich, St. Louis, MO, USA) as internal standard. For the quantification, a response factor was performed in triplicate mixing α-methyl-tryptophan (50 mg/100 mL) and tryptophan (50 mg/100 mL — Sigma Aldrich, St. Louis, MO, USA) in a ratio 1:1, in the same concentration of the sample.

All samples (total amino acids, sulfur-containing amino acids, calibration curves, tryptophan, and response factor) were analyzed using an ACQUITY system coupled with an ACQUITY SQ ESI–MS system (Waters, Milford, MA, USA), with an ACQUITY UPLC Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm 170×150 mm) column (Waters, Milford, MA, USA) and an ACQUITY UPLC Peptide BEH C18 VanGuard™ (300 Å, 1.7 µm, 2.1 mm×5 mm) pre-column (Waters, Milford, MA, USA). Samples were analyzed as described by Cutroneo et al. (2023) and acquired in SIR mode. Finally, data acquisition and data processing were performed using MassLynxTM V4.0 (Waters, Milford, MA, USA).

N Response Factor Estimation

The response factor for N calculation was estimated from the total amino acids. In detail, it was estimated as the percentage of the average nitrogen content of the amino acid residues in duckweed protein.

Amino Acid Score (AAS) Estimation

The AAS was estimated for all the essential amino acids (EAA) as described by Cutroneo et al. (2024). It was calculated as the ratio between the mg/g of protein of the amino acid content and the requirement for that amino acid. The requirements were retrieved from the values set by FAO, 2011 for children (from 3 to 18 years) and adults.

Table 3 Model runs of protein solubility

Runs	pH	Time in min	Conc. in wt %	Protein solubility in %	
				Experimental	Predicted
1	10.0	70	1.00	28.93 ± 1.1^{c}	29.02
2	8.5	60	1.50	38.73 ± 0.3^{a}	37.24
3	8.5	60	0.65	38.93 ± 1.1^{a}	39.17
4	6.4	70	3.00	$31.88 \pm 0.9^{b,c}$	33.05
5	6.4	80	3.00	$32.36 \pm 1.0^{b,c}$	32.28
6	6.3	90	3.00	$31.70 \pm 1.2^{b,c}$	31.34
7	6.4	80	5.00	$30.05 \pm 1.4^{b,c}$	29.13
8	6.4	90	5.00	$29.97 \pm 1.4^{b,c,c}$	30.51
9	8.5	80	3.00	$32.03 \pm 1.2^{b,c}$	32.29
10	8.5	60	3.00	32.64 ± 2.3^{b}	33.83
11	8.5	60	5.00	$29.79 \pm 1.2^{b,c}$	29.29
12	7.0	90	1.00	$32.52 \pm 0.7^{b,c}$	32.42
13	7.0	70	0.50	38.33 ± 0.2^{a}	39.37
14	6.5	70	0.50	40.41 ± 1.4^{a}	39.67

Table 4 Analysis of variances

Source	DF	Cor SS	Cor MS	*F*-value	*p*-value
Model	6	544.91	90.819	47.78	0.000
Linear	3	40.80	13.599	7.15	0.001
pH	1	24.39	24.387	12.83	0.001
Time	1	25.94	25.939	13.65	0.001
Conc	1	23.13	23.125	12.17	0.001
Quadratic	1	18.84	18.839	9.91	0.003
pH*pH	1	18.84	18.839	9.91	0.003
2-Factor-Interaction	2	66.38	33.190	17.46	0.000
pH*Conc	1	14.38	14.379	7.56	0.009
Time*Conc	1	56.44	56.438	29.69	0.000
Error	34	64.63	1.901		
Lack of fit	8	26.27	3.283	2.23	0.059
Pure error	26	38.37	1.476		
Total	40	609.55			
$R^2 = 89.40\%$					

Statistical Analysis

Statistical analysis was performed based on appropriate procedures and methods using Minitab Statistical Software 21©. This includes standard procedures in the order of the test of normal distribution (Kolmogorov–Smirnov Test). The obtained results were then statistically analyzed with the analysis of variance (one-way ANOVA) using a 95% confidence level and a Tukey post-hoc test to verify the statistical significance of the data collected. For the evaluation of the RSM-CCD model, the Minitab Statistical Software 20© was also used to draw the responses. After the test of normal distribution and test of equal variances (test of homoscedasticity) with the Levene test, the obtained results were statistically analyzed as mentioned above with one-way ANOVA and Tukey post-hoc test. The regression coefficient, the lack of fit, and the *F*-value obtained from the ANOVA were evaluated to determine the model's adequacy. The ANOVA-Welch test used a 95% confidence level, and the Games-Howell test was used for ultrasound-assisted extraction and optimization of parameters ("Ultrasound-Assisted Protein Release and Particle Size Distribution") since normal distribution was not provided. The statistical analysis for amino acids was carried out using Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM Corp., Chicago, IL). Specifically, one-way ANOVA with Tukey's-*b* multiple pairwise comparisons test was used and performed at $\rho < 0.05$ of the significance level.

Results and Discussion

Optimization of Protein Extraction Using Response Surface Methodology

Protein Solubility

The empirical relationship between protein solubility and the variables pH, extraction time, and powder concentration in wt% is described in the regression Eq. 8 in uncoded units.

$$\text{Protein Solubility} = 38.2 + 9.99\text{pH} - 0.3989\text{Time} - 14.59\text{Conc} - 0.809\text{pH} * \text{pH} + 0.691\text{pH} * \text{Conc.} + 0.1073\text{Time} * \text{Conc} \quad (8)$$

The experimental results of protein solubility and predicted values are shown in Table 3). The statistical analysis of variance is listed in Table 4. Here, the linear effects of pH, time, and Conc. are significant ($\rho < 0.05$), while pH and Conc. as well as time and Conc. strongly correlate with each other (2-factor interaction). Compared to the results of Muller et al. (2023), also using the RSM-CCD design for protein extraction from duckweed (*Lemna* species powder from *Lemnature AquaFarms*, USA, and *Seta organic*, Canada), the interaction of concentration and pH had no significant effect on protein solubility, and different extraction times were not considered. In this study, the highest protein solubility value obtained experimentally was 40.4% in run 14, which represented

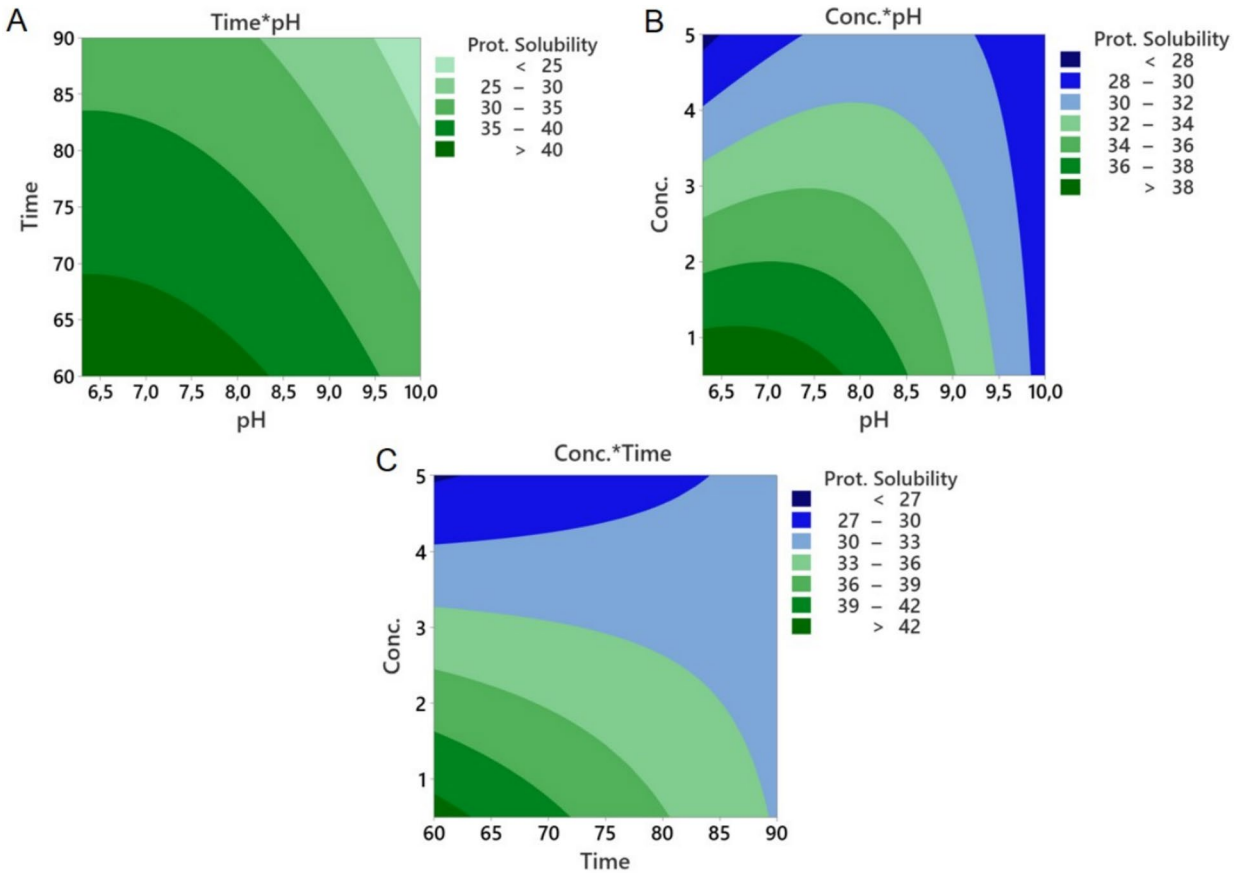


Fig. 2 Contour plots of extraction time vs. pH (**A**), powder concentration vs. pH (**B**), and powder concentration vs. time (**C**)

the optimized parameter at pH 6.5 with an extraction time of 70 min and a powder concentration of 0.5 wt%. The contour plots A, B, and C in Fig. 2 (Time*pH, Conc.*pH, Conc.*Time) serve to illustrate the statistical analyses of the results. The R^2 value (Table 4) of the linear model fit is ~ 90% indicating that the model sufficiently explains the variation in the data set (further data, see supplementary materials ST1). The model did not show a lack of fit ($\rho > 0.05$). The signal-to-noise ratio is calculated as 11.34. A normality test was carried out without eliminating insignificant data (see supplementary materials SFig. 1). However, one-way ANOVA and Tukey test did not show a significantly higher protein solubility for run 14 compared to runs 2, 3, and 13, in some of which higher concentrations (wt%) and slightly higher pH-values were used. The target size optimization (see supplementary materials ST2), which is at pH 6.4 (6.37), 60 min, and 0.5 wt% with a predicted protein solubility value of 43.13%, was tested again and showed a protein content of 39.07% (see supplementary materials ST3), which is lower than run 14. Taking into account the protein solubility curve ("Protein Solubility Curve" section) that demonstrates better solubility at pH 6.5, the parameters from run 14

Table 5 Model runs of protein precipitation

Runs	pH	Time in min	Protein solubility in %	
			Experimental	Predicted
1	2.0	40	33.41	32.37
2	4.0	40	20.27	23.21
3	3.0	30	22.50	19.08
4	2.0	20	26.97	26.42
5	3.0	30	19.11	19.08
6	1.6	30	35.85	36.74
7	4.4	30	25.18	23.92
8	3.0	30	18.79	19.08
9	4.0	20	19.08	17.26
10	3.0	44	26.29	23.24
11	2.5	30	19.38	22.80
12	2.5	30	22.00	22.80
13	2.5	30	24.91	22.80
14	3.0	30	18.50	19.08
15	3.0	30	18.15	19.08
16	3.0	30	18.32	19.08
17	3.5	30	16.83	18.22
18	3.5	30	16.52	18.22
19	3.5	30	18.63	18.22

Table 6 Analysis of variance of protein precipitation

Source	DF	Cor SS	Cor MS	*F*-value	*p*-value
Model	3	468.95	156.318	38.83	0.000
Linear	2	248.66	124.328	30.89	0.000
pH	1	197.41	197.409	49.04	0.000
Time	1	51.25	51.247	12.73	0.003
Quadratic	1	240.71	240.714	59.80	0.000
pH*pH	1	240.71	240.714	59.80	0.000
Error	15	60.38	4.025		
Lack of fit	6	29.05	4.842	1.39	0.315
Pure error	9	31.33	3.481		
Total	18	529.34			
$R^2 = 88.59\%$					

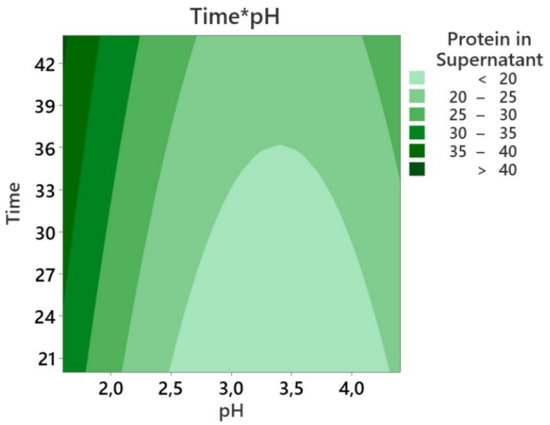


Fig. 3 Contour plot of precipitation time vs. pH

were applied to all subsequent experiments to enhance protein precipitation.

Protein Precipitation

Experimental and predicted results from protein precipitation of 19 runs are listed in Table 5. To facilitate practical implementation, the protein content of the supernatant (S2) was measured after precipitation. This means that the lower the value, the better the protein precipitation, as proteins should be in the precipitate.

The empirical relationship between protein solubility and the variables pH and extraction time is described in the regression Eq. 9.

$$\text{Protein Solubility} = 75.55 - 39.02\text{pH} + 0.2975\text{Time} + 5.740\text{pH} * \text{pH} \quad (9)$$

The linear effects of the analysis of variance (Table 6) show significant effects ($\rho < 0.05$) in pH and precipitation time. The quadratic effects are significant only for pH, suggesting that the response surface features curvature. Similar results have been researched by Muller et al. (2023) who investigated an IEP of pH 4 but used higher powder concentrations of 2 and 4%, who observed significant linear and quadratic effects and a significant effect of the interaction between pH and concentration. According to the results of this study, the pH is decisive but was not dependent on the extraction time to precipitate proteins. The R^2 value (Table 6) of the linear model fit is ~ 90% indicating that the model sufficiently explains the variation in the data set (further data, see supplementary materials ST4). The model did not show a lack of fit ($p > 0.05$). The signal-to-noise ratio is calculated as 13.74. A normality test was carried out without eliminating insignificant data (see supplementary materials SFig. 2). The evaluation of the model based on the experimental results, including the contour plot (Fig. 3) and the target value optimization, yields precipitation parameters at pH 3 for 30 min in the experimental trials. However, the calculation of the target size optimization (see supplementary materials ST5) revealed a pH of 3.4 for 20 min which is close to the experimental trials. To finalize the protein extraction parameters, four different parameter settings for protein solubility and precipitation were tested, resulting in the lowest protein content in the supernatant in samples no. 1 and 4 (Table 7). The protein yield of the precipitated protein of sample no. 4 was higher (26.98%) than that of sample no. 1 (23.78%) but was not significantly different from each other, nor was the protein content in the supernatant. The IEP at pH 3, which resulted in the lowest protein content in the supernatant, was selected based on the protein solubility curve presented in the "Protein Solubility Curve" section. Subsequent experiments were conducted using these optimized parameters identified through both RSM designs.

Ultrasound-Assisted Protein Release and Particle Size Distribution

After preliminary testing, the most promising parameters for ultrasound-assisted extraction (UAE) for protein release were evaluated (Table 8). A setting with a low frequency of 24 kHz, amplitudes of 40, 50, and 60%, and treatment durations of 4 and 10 min per 100 g sample was applied. The highest protein release of 80.83% was achieved with an amplitude of 40% (40 µm peak-to-peak) applied for 10 min. Higher amplitudes (50 and 60%) did not result in higher protein release for the same treatment time. Additionally, a significantly lower energy input of 411.37 Ws/g than the treatment settings 50/10 and 60/10 confirmed the choice of a 40/10 setting for further extraction experiments. The

Table 7 Final parameters for protein extraction

Sample no	Solubility			Precipitation			Protein in supernatant after precipitation
	pH	Time in min	Conc. in wt%	pH	Time		Protein content in %*
1	7.0	70	0.5	3.5		30	15.79 ± 0.08^{c}
2	7.0	70	0.5	3.0		30	21.41 ± 0.58^{a}
3	6.5	70	0.5	3.5		30	17.48 ± 0.46^{b}
4	6.5	70	0.5	3.0		30	15.05 ± 0.28^{c}

*Values with different lowercase letters show a significant difference at $\rho < 0.05$, according to the Tukey post-hoc test

50/4 setting resulted in the lowest energy input in relation to a shorter treatment time and released the lowest amount of protein. In another study, an amplitude of 60%, 20-min treatment time at pH 11.5 resulted in a protein release of 41.30% for *Lemna minor* flour suspension (Mirón-Mérida et al., 2024). Jiang et al. (2014) emphasized that using low to medium ultrasound power can enhance the negative surface charge on proteins, intensify electrostatic repulsions, disintegrate protein aggregates, prevent further aggregation, and stabilize protein dispersions, leading to enhanced protein solubility. This observation may explain the results of this study with the best protein release at a lower amplitude of 40% and moderate treatment time. On the other hand, an increased ultrasound intensity and duration can enhance protein solubility to a certain extent, which can be attributed to smaller particle size distribution, partial unfolding, and increased negative surface charge of protein molecules resulting in greater interaction with water molecules. However, when optimum ultrasound intensity and treatment time are exceeded, protein solubility can also decline due to the formation of macromolecular aggregates (re-polymerization) by non-covalent interactions (Jiang et al., 2014) and protein denaturation (Inguanez et al., 2023). Therefore, the right balance of ultrasound intensity and duration for protein release needs to be studied individually (Jiang et al., 2014). The particle size distribution before and after ultrasound treatment can be seen in (Table 8) as "no US" and "US," respectively. The particle size was smaller after ultrasound treatment for each percentile (d10, d50, d90) across all samples. The d10 percentile value revealed no variation in particle size following ultrasound treatment across all tested amplitudes. However, significant changes were detected in the median (d50) and for d90 percentiles by ultrasound treatment at different amplitudes. Defined treatment times are therefore necessary to generate a reduction in particle size via ultrasound. The span, calculated in percentage (%), was consulted to quantify the particle size distribution. The smaller the span in %, the narrower the particle size distribution. The particle sizes generally shifted toward smaller values, but ultrasound treatment also slightly widened the particle size distributions across all samples, without affecting the protein release negatively. Samples 40/10 and 60/10 exhibited the smallest span (%) after US treatment. A narrower span within a range of smaller particles aligns with increased solubility, whereas larger particles indicate insoluble agglomerations of proteins, carbohydrates, or fibers (Preece et al., 2017).

Protein Solubility Curve

A protein solubility curve (blue line) and a US-assisted protein solubility curve (orange line) with LG flour were carried out (Fig. 4). The pH of 12 was a potential option for protein solubility but was excluded from this study, due to its high chemical demand and limited application in the food industry.

The protein solubility curve obtained using LG flour (blue line) without ultrasound treatment shows an optimum solubility at pH 6.5 and an isoelectric point (IEP) at pH 3. Nieuwland et al. (2021), who also studied the protein solubility of *Lemna gibba*, observed the highest protein solubility at pH 7 (with a subsequent plateau), which is consistent with the present study's result (pH 6.5). However, they reported a different IEP at pH 5. A recent study on duckweed protein extraction using *Wolffia arrhiza* found an IEP at pH 4 and observed a rapid increase in protein solubility up to pH 7. Beyond this point, solubility increased only slightly, reaching a plateau (Nitiwuttithorn et al., 2024). It is important to note that the IEP should not be assumed solely based on literature values for protein precipitation, as it can vary depending on the specific protein source and extraction conditions. Researchers should determine the IEP for the particular protein type under investigation to ensure accurate analysis. The protein solubility can be influenced by several factors, such as pH adjustment, dispersion conditions (e.g. duration, incubation temperature), and measurement methods used for comparative analysis. Additionally, protein solubility in plant protein flours may exhibit greater variability compared to protein isolates or concentrates from the same source, likely due to lower selectivity and the presence of other nutrients in flours (e.g., carbohydrates, fibers), as observed in studies like that of green lentil flour (Ma et al., 2022).

Table 8 Correlation of protein release, energy input, and particle size distribution from *Lemna gibba* after US

Amplitude in %/Time in min	UAE			Particle size distribution in µm							
	Protein release in %[1]	Energy in Ws/g[2]	Total energy in Ws[2]	D10[3]		D50[3]		D90[3]		Span in %[4]	
				no US	US	no US	US	no US	US	no US	US
40/10	$80.83^{a} \pm 2.16$	$411.37^{a} \pm 5.45$	$20591^{a} \pm 261$	$7.54^{b} \pm 0.13$	$2.49^{e} \pm 0.49$	$25.33^{b} \pm 0.82$	$17.94^{d,e} \pm 5.10$	$85.02^{c} \pm 7.17$	$70.24^{c,d} \pm 3.53$	3.06	3.78
50/4	$54.14^{c} \pm 0.46$	$213.53^{b} \pm 3.62$	$10685^{b} \pm 176$	$7.05^{b} \pm 0.19$	$2.18^{e} \pm 0.32$	$23.96^{b} \pm 0.59$	$13.13^{e} \pm 0.68$	$70.62^{c,d} \pm 1.06$	$63.96^{d} \pm 2.79$	2.65	4.70
50/10	$78.30^{b} \pm 0.52$	$500.67^{c} \pm 13.92$	$25069^{c} \pm 678$	$9.53^{a} \pm 0.33$	$2.95^{e} \pm 0.14$	$34.02^{a} \pm 1.43$	$22.95^{b,c} \pm 1.37$	$125.00^{a} \pm 1.28$	$98.96^{b} \pm 6.27$	3.39	4.18
60/10	$77.02^{b} \pm 0.77$	$642.30^{d} \pm 34.30$	$32216^{d} \pm 1658$	$9.39^{a} \pm 0.16$	$2.65^{e} \pm 0.08$	$33.25^{a} \pm 0.95$	$18.84^{c,d} \pm 1.27$	$125.17^{a} \pm 7.83$	$71.51^{c,d} \pm 3.61$	3.48	3.66

[1]Values with different lowercase letters in the same column show a significant difference at $\rho < 0.05$, according to Games-Howell test. [2]Values with different lowercase letters in the same columns show a significant difference at $\rho < 0.05$, according to Tukey post-hoc test. [3]Values with different lowercase letters in the same respective columns for D10, 50, and 90 and for each amplitude setting show a significant difference at $\rho < 0.05$, according to Tukey post-hoc test and Games-Howell test for D10. [4]The span in percentage (%) was analyzed according to a two-sample *t*-test at $\rho < 0.05$

The ultrasound-assisted protein solubility curve (orange line) exhibited a shift in the IEP from pH 3 to pH 3.5. This could be due to a change in the net charge on the protein surface (Nitiwuttithorn et al., 2024). From pH 7 onward, solubility increased slightly, with no significant difference observed among pH 8, 9, and 10 ($\rho < 0.05$). Similar results on ultrasound-assisted protein solubility were reported by Nitiwuttithorn et al. (2024), who found an IEP at pH 4 and a rapid increase up to pH 6.5, with a minimal increase (~ 10%) up to pH 9. The optimum ultrasound parameters found in the "Ultrasound-Assisted Protein Release and Particle Size Distribution" section were tested with the optimum protein extraction parameters investigated in the RSM-Design ("Protein Solubility" and "Protein Precipitation" sections) as well as with the most promising pH values (8 and 10) from the US-protein solubility curve. The results are shown in Table 9. All experiments were performed with the same extraction time and concentration (0.5 wt%), which differs from the concentration used in the protein solubility curves (2.5 wt%). The results show no significant differences in protein solubility within the tested pH values, leading to the decision to use the optimal pH of 6.5 determined in the RSM design for the US-assisted protein extraction.

Comparison of Protein Extraction Processes

Different protein concentrates were extracted according to the extraction procedures described under "Protein Extraction Processes" and diverged significantly in protein content and yield ($\rho < 0.05$). Conventional extraction (LG-C) achieved the highest protein content at 64.54% but resulted in the lowest protein yield at 13.60% (Table 10). When UAE was applied, the protein yield increased significantly to 28.90% (LG-US-C) though the protein content declined to 48.59%. The application of ultrafiltration instead of IEP precipitation (LG-UF), aiming to concentrate proteins, resulted in a protein content of 54.59%, being lower than LG-C and resulting in a significantly higher protein yield of 24.50%. Among all protein extracts, LG-US-UF exhibited the highest protein yield at 41.30%, with a corresponding protein content of 50.91%. These results demonstrate that UAE-treated samples consistently achieved higher protein yields but lower protein contents compared to non-UAE-treated samples. UAE can stimulate protein–protein aggregation caused by disulfide bonds or protein-carbohydrate interactions which may influence protein purity showing reduced protein contents (Inguanez et al., 2023; Nitiwuttithorn et al., 2024). Similar studies were carried out on *Wolffia arrhiza* (Nitiwuttithorn et al., 2024), comparing ultrasound-assisted alkaline extraction (UAAE), ultrasound-assisted water extraction (UAWE), and alkaline extraction (AE) on protein extractability. The highest protein yield

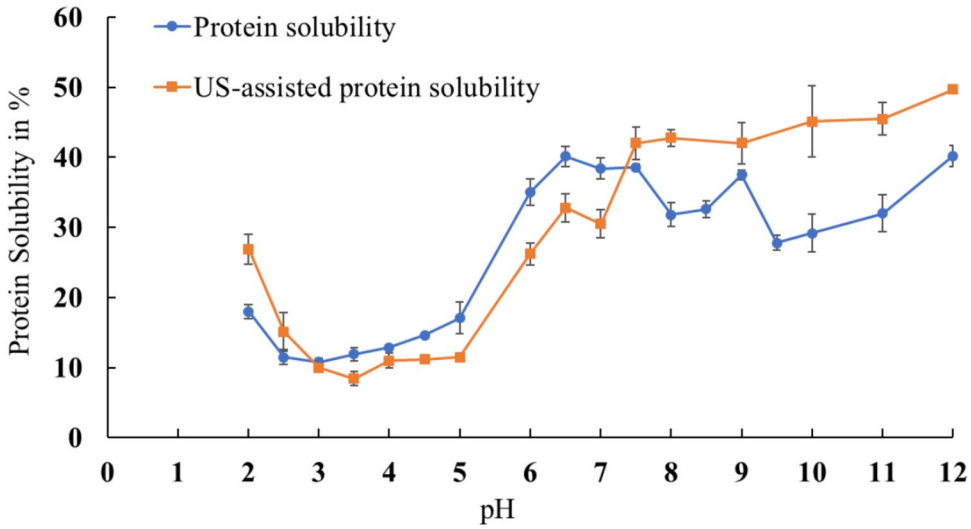


Fig. 4 Protein solubility curve (blue line, no US) and a US-assisted protein solubility curve (orange line) at a series of pH-values of *Lemna gibba*

Table 9 Ultrasound-assisted protein solubility

Sample	pH	Time	Conc. in wt%	Protein solubility in %*
US-pH 6.5	6.5	70	0.5	76.96 ± 1.57 [a]
US-pH 8	8.0	70	0.5	72.85 ± 5.82 [a]
US-pH 10	10.0	70	0.5	72.17 ± 4.94 [a]

*Values with different lowercase letters show a significant difference at $\rho < 0.05$, according to Tukey post-hoc test

achieved by UAAE (34.2%) followed by UAWE (24.2%) and AE (17.1%). UAAE resulted in the lowest protein content (64.19%) and AE in the highest (72.96%). Ultrasound-treated samples also resulted in higher yields than non-ultrasound-treated samples which is in line with the findings of this study. Ultrasonication also leads to co-extraction of other components, such as antioxidants (see "Antioxidant Capacity and Phenolic Content" section). Moreover, the color of the obtained duckweed protein concentrates varies significantly, whereby ultrasound-treated concentrates have a visibly greener color than the protein concentrates not treated with ultrasound, which is discussed in detail in the "Chlorophyll and CIE LAB Color Space Measurement" section. This opens new avenues for further research into UAE to address existing challenges and to explore the mechanisms and biochemical reactions involved. Investigating processing steps or pre-treatments (such as defatting) could enhance extraction yields (Kumar et al., 2021), even though duckweed *L. gibba* contains only approximately 5% lipids (Appenroth et al., 2017). Additionally, moderate temperatures between 30 and 45 °C have been shown to improve extraction yields, whereas higher temperatures reduce yields due to weakened cavitation effects (Kumar et al., 2021). A combination of innovative process technologies, such as ultrasound with pulsed electric field (PEF) or microwave-assisted extraction has been proposed as a future research perspective to potentially increase extraction yields of bioactive compounds (Meena et al., 2024). It may give the opportunity also to enhance the protein content and yield of duckweed extracts.

Table 10 Protein content and yield of protein concentrates. [1,2]Values of the protein concentrates LG-C, LG-US-C, LG-UF, and LG-US-UF (LG flour excluded), see "Protein Extraction Processes" section, with different lowercase letters in the same line show a significant difference at $\rho < 0.05$, according to Tukey post-hoc test

Sample	LG flour	LG-C	LG-US-C	LG-UF	LG-US-UF
Prot. Cont. in %[1]	36.96 ± 0.12	64.54 [a] ± 0.52	48.59 [b] ± 0.03	54.59 [c] ± 0.53	50.91 [d] ± 0.70
Prot. Yield in %[2]	-	13.60 [a] ± 1.31	28.90 [b] ± 1.25	24.50 [c] ± 0.46	41.30 [d] ± 0.52

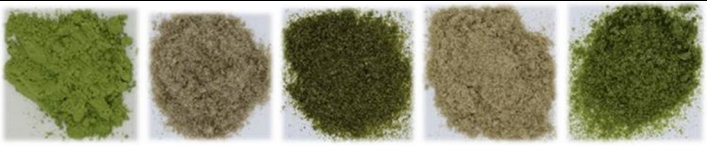


Table 11 Chlorophyll and CIE LAB color space measurement. [1,2]Values with different lowercase letters in the same columns show a significant difference at $\rho < 0.05$, according to Tukey post-hoc test

	Chlorophyll Content [mgxg^{-1}] [1]			CIE LAB Colour Space [2]					
Sample	**Chl a**	**Chl b**	**Total Chl**	**L***	**a***	**b***	**Yellow -ness**	**ΔE**	**Colour Space**
LG flour	10.11 [c] ± 0.05	2.52 [c] ± 0.00	12.63 [c] ± 0.05	46.01 [b] ± 0.02	-13.64 [e] ± 0.01	34.60 [a] ± 0.01	71.88 [c] ± 0.02	-	
LG-C	0.69 [d] ± 0.00	0.07 [d] ± 0.00	0.76 [d] ± 0.00	43.03 [c] ± 0.27	4.18 [a] ± 0.00	16.55 [d] ± 0.04	46.63 [e] ± 0.04	25.54	
LG-US-C	15.44 [a] ± 0.24	4.90 [a] ± 0.08	20.34 [a] ± 0.32	21.71 [e] ± 0.38	-8.13 [c] ± 0.06	21.63 [c] ± 0.09	72.98 [b] ± 0.17	28.09	
LG-UF	0.42 [d] ± 0.00	0.09 [d] ± 0.00	0.50 [d] ± 0.00	51.67 [a] ± 0.06	3.66 [b] ± 0.01	21.74 [c] ± 0.04	51.04 [d] ± 0.03	22.29	
LG-US-UF	10.51 [b] ± 0.05	3.44 [b] ± 0.04	13.95 [b] ± 0.09	27.51 [d] ± 0.05	-13.19 [d] ± 0.07	29.42 [b] ± 0.06	79.59 [a] ± 0.12	19.22	

Chlorophyll and CIE LAB Color Space Measurement

The evaluation of chlorophyll content involved measuring Chl a and b, followed by calculation of the total chlorophyll content. Among the protein concentrates obtained, LG-UF had the lowest total chlorophyll content of 0.50 mg × g^{-1}, indicating that ultrafiltration may effectively separate green pigments to a certain extent (Table 11). Acidic precipitation was also effective in reducing the green color, mainly coming from chlorophylls, in LG-C. The reduction in chlorophyll content in LG-C can be explained by the acid sensitivity of chlorophyll (Caesar et al., 2018; Hynninen et al., 1973), transforming chlorophyll into pheophytin that occurs in an olive-green color. Furthermore, it could be observed that LG-C has a higher amount of Chl a and a lower amount of Chl b than LG-UF. Since Chl a has a blue-green color, due to the methyl group (-CH3), Chl b has a formyl group attached to one pyrrole ring instead, appearing yellow-green (Leite et al., 2018). That may explain the slightly brighter color of LG-UF samples. In contrast, the application of UAE in LG-US-C and LG-US-UF resulted in a remarkable increase in total chlorophyll content, indicating levels significantly higher than those in LG flour. The use of ultrafiltration marginally separated chlorophylls in LG-US-UF. The strong green pigmentation may come from chlorophyll a/b binding proteins as determined in the "Protein Profile Identification" section. They are classified as insoluble proteins located in the stroma of chloroplasts. Soluble proteins, on the other hand, found in the cytoplasm, typically appear white or yellowish in color (Chiesa & Gnansounou, 2011; Tamayo Tenorio et al., 2016; Kobbi et al., 2017; Muller et al., 2023). The increase in chlorophyll extraction is in line with Inguanez et al. (2023), who worked with the same amplitude and ultrasound processor on *L. minor*. This confirms the effectiveness of ultrasound waves in extracting colorants (bioactive compounds) from plant cells specifically working with green plants (algae) and other duckweed species (Kong et al., 2014; Vernès, Vian and Chemat 2020; Bozdemir et al., 2022; Mirón-Mérida et al., 2024).

The enhanced chlorophyll extraction was also confirmed by measurements in the CIE LAB color space (Table 11). Negative *a*-values recorded for LG-US-C and LG-US-UF signified a distinctly visible green hue, whereas LG-C and LG-UF have low positive a-values, indicating a light reddish color. The positive *b*-values across all samples indicate a predominantly light green to yellow color, where the highest values have been measured in the ultrasound-treated concentrates (LG-US-C and LG-US-UF). The increased "yellowness" in LG-US-C and LG-US-UF suggests a stronger yellow component, possibly due to the release of carotenoids. The protein concentrate LG-UF achieved the highest *L*-value, making it the lightest powdered LG protein concentrate. LG-C followed by showing a significant difference. LG-US-C and LG-US-UF had notably lower *L*-values, confirming the enhanced pigment extraction by ultrasound. In addition, ΔE was used for

the numerical evaluation of the color difference between protein concentrate and duckweed flour. In order to be able to make statements about the partial absolute color difference, ΔE is generally divided into different grades (Limbo & Piergiovanni, 2006). With ΔE values, exceeding 12 for all four protein concentrates, they are considered to have different colors relative to LG flour.

Antioxidant Capacity and Phenolic Content

The phenolic content using the Folin-Ciocalteu method and antioxidant capacity were measured using two complementary methods, FRAP and DPPH. The data values are expressed in mg/g dry weight (DW). The FRAP method represents predominantly hydrophilic antioxidants (Munteanu & Apetrei, 2021) and shows a significant increase in the antioxidant capacity of the protein concentrates LG-US-C and LG-US-UF that have undergone ultrasound-assisted extraction (Fig. 5A), compared to the respective samples LG-C and mild protein extraction LG-UF. This indicates the release of antioxidants by the UAE.

The antioxidant capacities of the protein concentrates measured by the DPPH method (Fig. 5B), representing predominantly hydrophobic antioxidants (Munteanu & Apetrei, 2021), show a contrary behavior. In both extraction processes (LG-US-C and LG-US-UF), UAE resulted in a lower antioxidant capacity than LG-C and LG-UF respectively. This may be explained by a reduced unfolding and dissolution of agglomerates and the exposure of hydrophobic groups by UAE, resulting in less interaction between proteins, bioactive substances, and water molecules (Jiang et al., 2014). Another explanation reveals that cavitation in aqueous suspensions can break down water molecules to form reactive radicals such as H^+ and OH^- ions (Rahman & Lamsal, 2020). These free radicals in turn lead to protein oxidation and the degradation of antioxidants, which had been investigated for hydrophobic flavonoids (Vilkhu et al., 2008). Duckweeds contain a remarkable amount of hydrophobic antioxidants, e.g., carotenoids, and of this β-carotene, lutein as published by Appenroth et al. (2017) on *W. microscopica* and flavonoids were analyzed for *L. gibba* and *L. minor* (J. Veen, 1975).

Using the Folin-Ciocalteu test, the highest phenolic contents were found for LG-UF, followed by LG-US-UF (Fig. 5C). Conventionally extracted protein concentrate (LG-C) has a significantly lower phenolic content, and there is no significant difference to LG-US-C. It is also

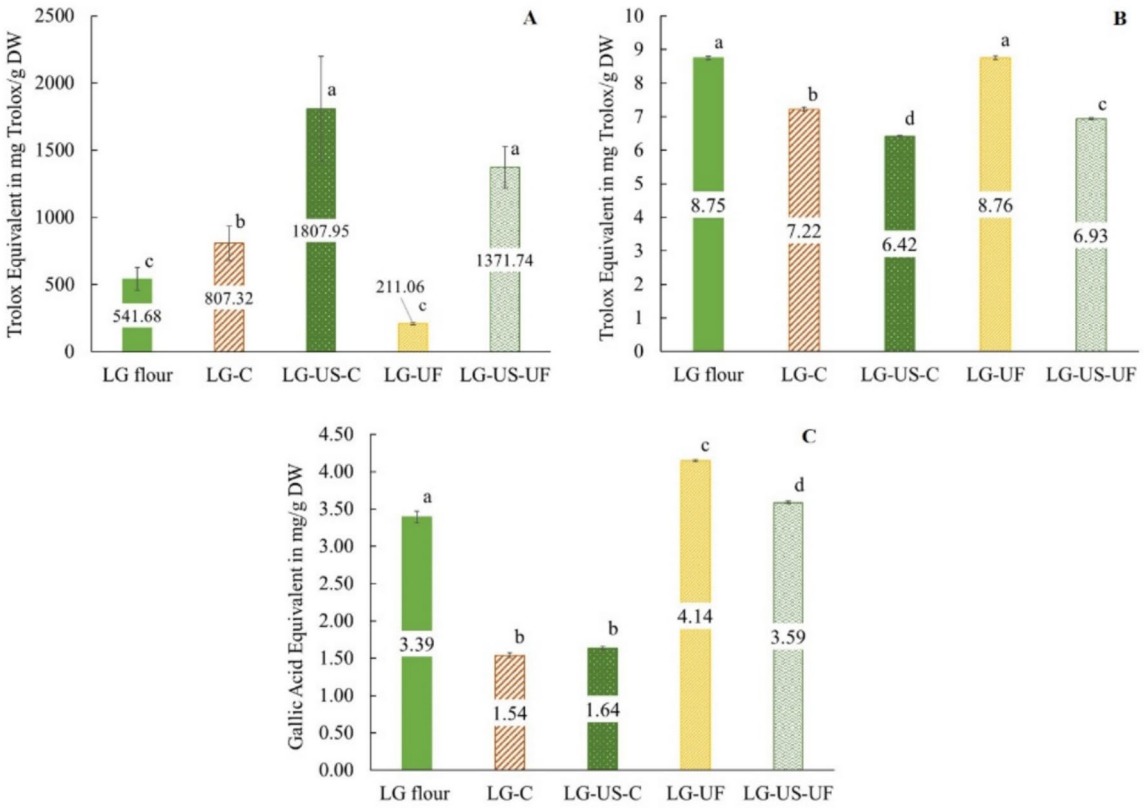


Fig. 5 The antioxidant capacity methods FRAP (**A**), DPPH (**B**), and Folin-Ciocalteu (**C**) method for phenolic compounds. Chart columns with different lowercase letters show a significant difference at $\rho < 0.05$, according to Tukey-Post-hoc

significantly lower than LG flour. The protein concentrates LG-C and LG-US-C were subjected to acidic precipitation during the extraction process in order to isolate the proteins. Since phenolic compounds such as flavonoids, which are abundant in duckweed (J. Pagliuso et al., 2020; Veen, 1975), can be hydrolyzed under acidic conditions (Martins et al., 2023; Pasquet et al., 2024), it can be assumed that flavonoids (phenols) were already separated during the precipitation and centrifugation step, which might lead to lower phenol content in LG-C and LG-US-C. In addition, complexes with proteins, carbohydrates, or other elements may hinder the complete extraction of some phenolics (Khoddami, Wilkes and Roberts 2013). Although the strong coloration of the protein extracts (mainly resulting from antioxidants) may be challenging for food applications with plant-based proteins, their beneficial health effects, such as anti-inflammatory, antithrombotic, or cardioprotective (phenols) properties, can offer an advantage for human health in nutrition. In this study, UAE enhanced the release of antioxidants in some samples to a certain extent. The improved extraction of antioxidants is caused by cavitation bubbles of ultrasound waves that disrupt the cell membrane and make them easier accessible toward digestive enzymes which improve the absorption in the gastrointestinal tract (Meena et al., 2024). Pointing this out, more research is needed on this topic to better understand the effects of the respective extraction methods and applied technologies.

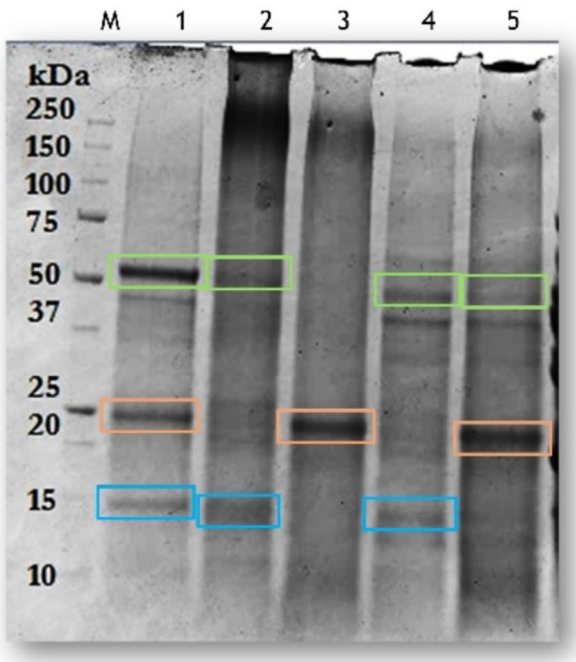


Fig. 6 SDS-PAGE analysis of duckweed samples: (1) LG flour, (2) LG-C, (3) LG-US-C, (4) LG-UF, (5) LG-US-UF, (M) protein marker

Protein Profile Identification

The SDS-PAGE analysis revealed the presence of 3 major bands. The first one is higher than 50 kDa (in green), the second one is around 25 kDa (in pink), and the third one is above 15 kDa (in blue) as shown in Fig. 6. To identify the proteins, two studies were taken as references. The first one was carried out by Mirón-Mérida et al. (2024) on the protein profile of duckweed (*Lemna minor*), and the second one was carried out on the RuBisCo from spinach (Martin et al., 2014). From the comparison with the literature, the 56 and 15 kDa bands were attributed to the subunits of RuBisCO. The band corresponding to 25 kDa was instead attributed to the LHCB1, a major chlorophyll a/b binding protein.

A difference in the protein profile was observed between samples. In particular, raw 1 (control sample) showed all three bands. When moving to the different extraction procedures, a different efficiency in extracting the different proteins was noted. In detail, samples extracted with conventional IEP precipitation and ultrafiltration (2 and 4 respectively) showed no bands around 25 kDa, indicating that LHCB1 is not extracted with this methodology. While the same samples pre-treated with ultrasound (3 and 5) appear to be more efficient in extracting the LHCB1, with a loss in the extractability of RuBisCo.

Amino Acid Profile

The amino acid profile was determined on all samples and compared to highlight possible differences due to the methodology of extraction applied. Due to the different protein contents of the extracts, to better highlight the difference in the amino acid profile, data were expressed as relative % of amino acid (AA) on total protein. Results are shown in Fig. 7.

Looking at the profile, some differences were observed in the content of aspartic acid, cysteine, glutamic acid, histidine, leucine, methionine, proline, tryptophan, and tyrosine. The other AAs showed no statistical difference. Anyhow, despite the differences observed, no particular trend ascribable to the methodology of extraction was observed.

The AA content was slightly higher than what was reported in the literature (Appenroth et al., 2017) for the AA composition of *Lemna gibba*, with a slightly lower content only in lysine. However, the relative distribution of amino acids, and therefore the AA profile, resulted to be comparable.

When comparing the AA profile with the one reported by Turck et al. (2023) for the whole plant material of a mixture of *Lemna gibba* and *Lemna minor*, some differences appear. In particular, in that study, a higher content of leucine, lysine, tryptophan, and tyrosine was reported. These differences can be ascribed to the use of the combination of two different species of duckweed (*Lemna gibba* and *Lemna*

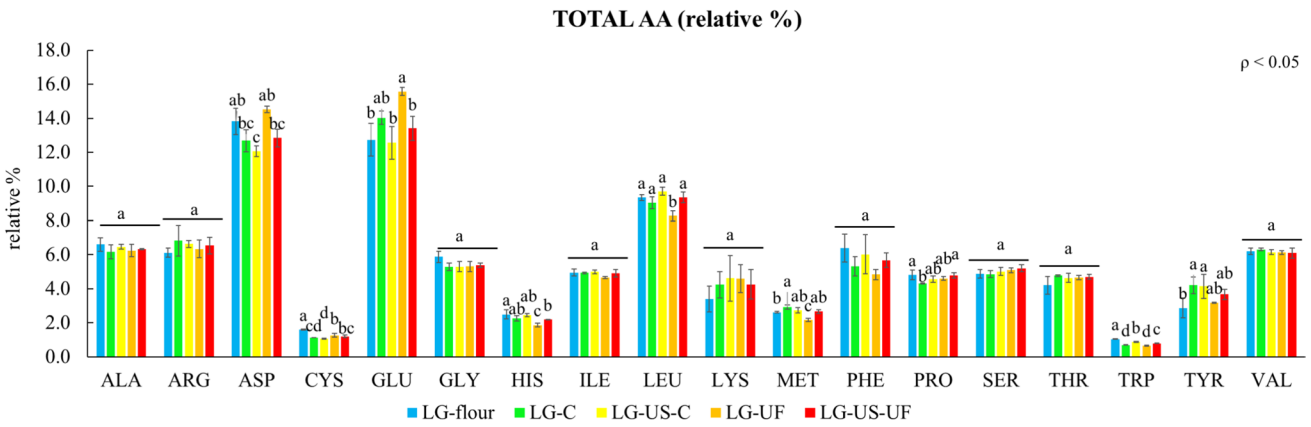


Fig. 7 Total amino acidic profile expressed as relative % of amino acid on total protein. Statistical analysis was performed using Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM Corp., Chicago, IL) and performed at $\rho < 0.05$ of the significance level. Letters on the bars refer to the difference between samples, for which a one-way ANOVA with Tukey's *b* multiple pairwise comparisons test was used ($\rho < 0.05$)

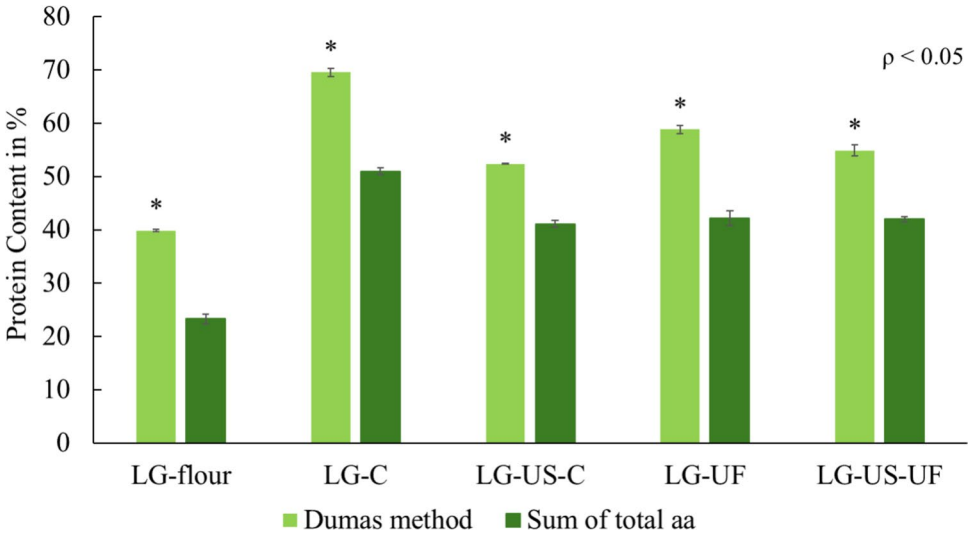


Fig. 8 Protein content determined with Dumas method (conversion factor 6.25) and as the sum of total AA (corrected conversion factor ~ 5.8). Statistical analysis was performed using Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM Corp., Chicago, IL) and performed at $\rho < 0.05$ of the significance level. The asterisks on the bars refer to the difference between the two methodologies for each sample, for which a paired *t*-test was used ($\rho < 0.05$)

minor), which are known to have a different AA content (Appenroth et al., 2017).

Specific Nitrogen Conversion Factor

From the comparison between the protein content obtained with the Dumas method (conversion factor 6.25) and the protein content determined as the sum of total AA (Fig. 8), the same trend was observed. However, an important difference in the content was observed. This can be attributed to an overestimation of the protein content from the use of the general 6.25 conversion factor.

Therefore, the conversion factor for nitrogen to protein was estimated from the determined total amino acids. The actual conversion factor of duckweed protein appeared to be 5.78 ± 0.02. This value is in accordance with what was estimated by Nieuwland et al. (2021). In this study, particularly, the authors have assumed the 5.8 conversion factor for duckweed based on the conversion factor of RuBisCo in spinach, assuming the homology of proteins. To the best of our knowledge, this is the first work estimating an accurate conversion factor for duckweed. Moreover, this result is in accordance with what was observed in our SDS-PAGE analysis. Comparing, indeed, the protein profile of our samples with a SDS-PAGE of RuBisCo from spinach (Martin et al., 2014), the similarity of this protein profile was noted.

Essential Amino Acid Profile

To investigate the nutritional value of the duckweed-extracted proteins deeper, the essential amino acid (EAA)

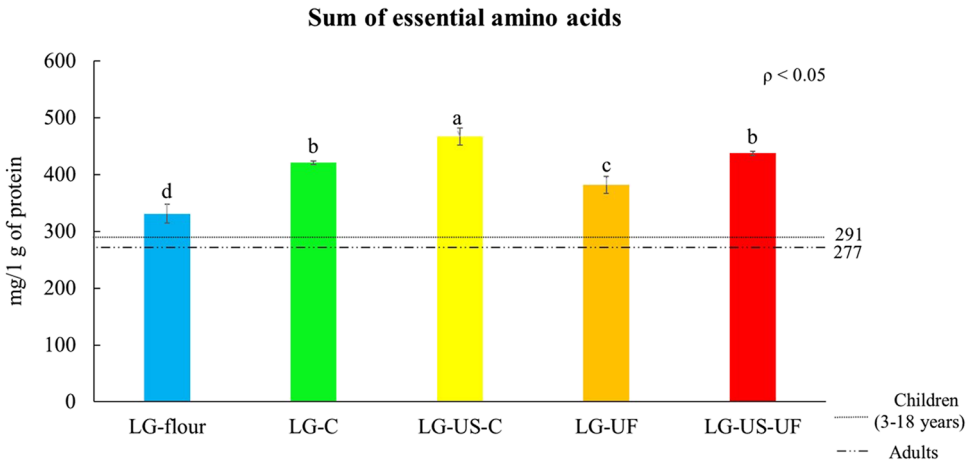


Fig. 9 Sum of essential amino acids (EAA) reported for samples and compared with the requirements (reference lines) set by FAO, 2011 for children (from 3 to 18 years) and adults. Statistical analysis was performed using Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM Corp., Chicago, IL) and performed at $\rho < 0.05$ of the significance level. Letters on the bars refer to the difference between samples, for which a one-way ANOVA with Tukey's *b* multiple pairwise comparisons test was used ($\rho < 0.05$)

profile was examined. In particular, the sum in EAA (Fig. 9) was found to be adequate to fulfill the requirements set by FAO, 2011 for two different segments of the population, children (from 3 to 18 years) and adults. The lower value was observed, as expected, for the control sample (LG flour), while the higher values were recorded in samples with conventional IEP precipitation and ultrafiltration when combined with ultrasound treatment (LG-US-C and LG-US-UF respectively). These results suggest the potentiality of ultrasound pre-treatment to enhance the EAA content.

To understand the differences highlighted in the amino acidic profile, the amino acid score (AAS) was also estimated. Results, displayed in Table 12, showed generalized good scores. The limiting AA for all samples was lysine. The results are not in accordance with what is found in the literature for a product (whole plant material) made with a mixture of *Lemna gibba* and *Lemna minor* (Turck et al., 2023). Indeed, sulfur-containing AA (Cys), in particular, resulted from the limiting one, despite all EAA overtaking the 1 values. This, in accordance with the results observed for the AA profile, confirms that the combination of different species of duckweed can improve the EAA profile.

Looking at the differences between samples, all methodologies of extraction tend to improve the AAS, with higher values for tryptophan and lysine. However, values near 1 or higher (for adults) were only achieved with conventional IEP precipitation and ultrafiltration when combined with ultrasound treatment (LG-US-C and LG-US-UF respectively). This result confirms what was previously stated for the sum of EAA indicating the potentiality of ultrasound pre-treatment to enhance the EAA profile. On the other hand, the sole use of ultrafiltration showed worsened AAS, indicating that this methodology is not recommended for the extraction of proteins from this matrix.

Table 12 Amino acid score estimated for duckweed samples compared to the requirements set by FAO, 2011 for children (from 3 to 18 years) and adults

Children (3–18 years)	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
LG flour	1.15	1.21	1.12	0.52	1.34	1.66	1.24	1.16	1.14
LG-C	1.30	1.51	1.36	0.81	1.62	2.13	1.76	0.97	1.45
LG-US-C	1.51	1.64	1.57	0.94	1.63	2.45	1.83	1.30	1.51
LG-UF	1.05	1.40	1.22	0.87	1.34	1.76	1.68	0.90	1.38
LG-US-UF	1.31	1.57	1.47	0.85	1.62	2.19	1.81	1.16	1.47
Adults	**His**	**Ile**	**Leu**	**Lys**	**SAA**	**AAA**	**Thr**	**Trp**	**Val**
LG flour	1.22	1.21	1.16	0.55	1.40	1.79	1.34	1.28	1.17
LG-C	1.38	1.51	1.41	0.87	1.69	2.30	1.91	1.06	1.48
LG-US-C	1.61	1.64	1.62	1.01	1.70	2.64	1.98	1.43	1.55
LG-UF	1.12	1.40	1.26	0.93	1.40	1.90	1.83	0.99	1.42
LG-US-UF	1.40	1.57	1.52	0.91	1.69	2.36	1.97	1.28	1.50

Conclusion

Results of the current study emphasize the pros and cons between conventional and UAE protein extraction with the potential of UAE as a powerful technique for improving the protein yield and nutritional profile of duckweed (amino acid profile), although with some trade-offs in purity. UAE significantly boosted chlorophyll and pigment extraction and exceeded the values of LG flour, the raw material for extraction. This indicates a more intense green hue and suggests ultrasound enhances the release of antioxidants. The essential amino acid content in all samples generally surpassed the values reported in the literature for *Lemna gibba*, with lysine identified as the limiting amino acid in all the samples, except LG-US-C. However, when ultrasound methods were applied, tryptophan levels improved, exceeding the recommended amino acid score (AAS) threshold of 1. The EAA profile across all samples met the FAO nutritional requirements for both children and adults. The study determined a nitrogen-to-protein conversion factor for duckweed that provides a more precise tool for future nutritional assessments of duckweed proteins and to the best of our knowledge; this is the first study to accurately estimate a conversion factor for duckweed species (*L. gibba*). Ultimately, careful consideration of the extraction method is essential to balance the desired outcomes in yield, purity, and nutritional value. The findings of this study provide a basis for advancing extraction and purification processes to improve product and protein yields. Further research focusing on optimizing these parameters could enable more efficient extraction and protein recovery. Additionally, the resulting extracts should be thoroughly analyzed for their functional properties to assess the potential applications of duckweed protein in the food and feed industries.

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Author Contribution Patricia Maag: formal analysis, investigation, data curation, writing—original draft. Özlem Özmutlu: formal analysis, investigation, data curation, writing—review and editing, supervision, project administration, funding acquisition. Sara Cutroneo: formal analysis, investigation, data curation, writing—review and editing. Tullia Tedeschi: conceptualization, data curation, writing—review and editing. Cornelia Rauh: conceptualization, writing—review and editing, supervision. Sabine Grüner-Lempart: conceptualization, writing—review and editing, supervision. All authors reviewed the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Competing Interests The authors declare no competing interests.

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