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Non-thermal technologies modify protein structure and enhance functional properties of cricket protein concentrate

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ABSTRACT

This study aimed to investigate the potential of ultrasound (US), pulsed electric fields (PEF), and high pressure (HP) to produce cricket protein concentrates with functional properties suitable for the food industry. Protein concentrates were produced from *Gryllus assimilis* using these non-thermal technologies. The impacts of these technologies on the protein structure and functional properties of the protein concentrates were evaluated. US treatment reduced particle size by 33 %, increased negative surface charge by 59 %, and enhanced hydrophobicity by 29 %, leading to improved solubility, water retention (40 %), foam capacity (31 %) and stability. HP partially unfolded proteins and increased surface hydrophobicity by 10 %, improving oil-holding capacity (10 %) and gelation properties (17 %). PEF increased particle size by 26 %, which enhanced foam capacity (29 %) and stability.

Industrial relevance: This study demonstrate that non-thermal technologies—US, PEF, and HP—alter the protein structure and functionality of cricket protein concentrates. These findings suggest that non-thermal technologies, particularly US, can assist in protein extraction to produce cricket protein concentrates with enhanced functional properties, making them suitable for novel food applications.

1. Introduction

The United Nations' Agenda 2030 promotes sustainable development through seventeen goals, including zero hunger, good health and well-being, responsible consumption and production, climate action, and life on land (United Nations, 2020). Edible insects are promising in advancing these goals due to their high nutritional value, sustainable production, and economic viability (Mariutti et al., 2021).

Despite their advantages, edible insects are not yet widely accepted in most countries. Strategies to increase their acceptance include using cricket species, which are more appreciated by the consumers than other insects and processing them into forms such as powders or protein concentrates. These forms not only facilitate incorporation into conventional food products but also reduce the visual recognition of the whole insect, which can mitigate the 'disgust effect' while maintaining transparency through appropriate labeling (Bisconsin-Junior, Rodrigues, Behrens, Silva, & Mariutti, 2022; Rovai et al., 2021; Tzompa-Sosa et al., 2023). Crickets, such as *Acheta domesticus*, *Gryllus assimilis*, and *Gryllus bimaculatus*, offer high protein content (55–65 % dry weight) and are rich in lipids, minerals, and vitamins (Magara et al., 2021). They are farmed commercially in various regions, with small-scale and automated large-scale operations (Caparros Megido, Haubruge, & Francis, 2017; Tanga et al., 2021).

The most adopted treatments in insect processing for food applications are blanching and drying. These traditional thermal processing are highly effective in reducing the microbiota that can lead to the development of foodborne diseases or product deterioration, as well as extending the shelf life of the food product (Melgar-Lalanne, Hernández-Álvares, & Salinas-Castro, 2019).

However, it is worth noting that traditional processing technologies

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using elevated temperatures can adversely affect the final product's quality, leading to color browning, lipid oxidation, and other changes. For example, Khatun et al. (2021) observed browning and lipid oxidation after blanching and oven-drying adult crickets of the *A. domesticus* and *G. assimilis* species. Similarly, Mishyna, Haber Benjamin, Martinez, and Chen (2020) examined the impact of different drying methods on the volatile and sensory characteristics of edible locusts and silkworms. They found that locusts and silkworms subjected to microwave-drying exhibited Maillard reactions and lipid oxidation, resulting in low sensory scores from the panelists due to undesirable appearance, flavor, and aroma development.

In addition to these harmful effects, thermal technologies can significantly impact proteins' structure and functional properties, often causing protein denaturation. Additionally, active compounds such as reducing sugars and carbonyl compounds can react with proteins during heating, causing further structural changes (Zhang et al., 2021). Azagoh et al. (2016) studied the effects of blanching (90 °C for 10 min) followed by oven drying (75 °C for 6 h) on *T. molitor* proteins. Their results showed a significant reduction in solubility and surface hydrophobicity due to thermal treatments. Similarly, Bußler et al. (2016) observed that heating *T. molitor* above 100 °C caused a significant decrease in protein solubility, water binding capacity, and intrinsic fluorescence intensity. Kröncke, Böschen, Woyzichovski, Demtröder, and Benning (2018) also found that high temperatures during drying significantly reduced *T. molitor* protein solubility. These changes in protein solubility, surface hydrophobicity, water binding capacity, and fluorescence intensity are likely due to protein denaturation caused by heat. Denaturation causes proteins to unfold, exposing hydrophobic regions that were previously buried within the structure. This exposure makes the proteins prone to aggregation through ion-exchange and hydrophobic interactions, leading to the formation of larger particles.

Non-thermal technologies such as ultrasound (US), pulsed electric fields (PEF), and high pressure (HP) offer alternatives to avoid the adverse effects of heat on proteins. These methods provide shorter treatment times and eco-friendly processing with minimal environmental impact (Mohd Zaini et al., 2023). A comprehensive review by Ojha, Bußler, Psarianos, Rossi, and Schlüter (2021) investigated processing pathways for edible insects and the application of non-thermal technologies. For instance, US can enhance protein extraction and modify its functional properties. Mokaya, Mudalungu, Tchouassi, and Tanga (2024) showed that using US on *Gynanisa belina* and *Gonimbrasia maja* increased the protein extraction yield. Mishyna, Martinez, Chen, and Benjamin (2019) found that US-assisted extraction increased the solubility, coagulability, and foam stability of *Schistocerca gregaria* protein extracts while it reduced solubility and coagulability in *Apis mellifera* extracts. Regarding PEF, Psarianos et al. (2022) applied it directly to ground *A. domesticus* and evaluated the freeze-dried samples. Their results showed that more intense PEF treatments improved oil binding capacity, protein solubility, and emulsification capacity. For HP, Boukil, Marciniak, Mezdour, Pouliot, and Doyen (2022) applied treatments ranging from 70 to 600 MPa for 7 min on *T. molitor* soluble proteins. They confirmed protein unfolding and the formation of large aggregates by observing decreases in fluorescence intensity, shifts in maximum emission wavelength, increases in surface hydrophobicity, and changes in molecular weight.

This study aims to investigate the effects of US, PEF, and HP on the production of cricket protein concentrates, with a focus on their influence on protein structure and key functional properties, including solubility, water and oil holding capacities, foaming, emulsification, and gelation.

2. Material and methods

2.1. Raw material

Frozen adult Jamaican field crickets (*Gryllus assimilis*) were purchased from Fauna Topics GmbH (Marbach am Neckar, Germany) and stored frozen at −40 °C. Before producing protein concentrates, the crickets were washed with cold water and drained to remove impurities. All chemicals were purchased from Carl Roth Gmbh & Co. Kg (Karlsruhe, Germany).

2.2. Cricket protein concentrates preparation

To ensure experimental consistency and focus on the direct impact of the non-thermal treatments (US, PEF, and HP), a single-step extraction protocol was used to produce the cricket protein concentrates. Although additional procedures, such as defatting with hexane, can enhance protein concentration, they also introduce variables that could confound the interpretation of how each non-thermal technology influences protein structure and the functional properties of the resulting concentrates (Feyzi, Varidi, Zare, & Varidi, 2017; Mohtashami et al., 2019; Kim et al., 2020; Kim et al., 2022).

2.2.1. Non-thermal assisted protein extraction

First, frozen crickets were homogenized using a T-25 Ultra Turrax (IKA, Staufen, Germany) with a 0.025 M NaOH solution at a cricket-to-solution ratio of 1:6 (*w/v*) at 10,000 rpm for 10 s. The obtained slurry, with a pH of 9.6 ± 0.1, was then subjected to one of the non-thermal treatments. The US, PEF, and HP treatment conditions were selected based on a previous study (Bisconsin-Junior et al., *in press*), which optimized cricket protein extraction. The conditions used in this study were designed to enhance the proportion of soluble proteins and ensure efficient production of cricket protein concentrates. The temperature, which was monitored with a digital thermocouple (General Tools & Instruments, NJ), did not exceed 30 °C during the treatments.

2.2.1.1. US-assisted protein extraction. For US treatment, 200 mL of cricket slurry was placed in a 250 mL beaker and positioned in an ice water bath to prevent overheating. The US treatment was applied using a UIP1000hdT ultrasonic device (Hielscher, Germany) equipped with a BS4d22 sonotrode (tip diameter of 22 mm). The probe was immersed to half the slurry height. The duty cycle was set at 100 %, with a frequency of 20 kHz and an output power of 200 W (power density of 1 W/mL). Sonication was conducted in cycles of 30 s on and 30 s off, for a total sonication time of 14 min.

2.2.1.2. PEF-assisted protein extraction. For PEF treatment, the cricket slurry was processed using the ELCRACK HVP-5 PEF system (DIL, Quackenbrück, Germany) in a batch chamber with an electrode gap of 80 mm. The pulses were monitored using a using a Voltcraft DSO-1062D oscilloscope (Conrad electronics, Germany) with two channels: one displaying the applied peak voltage and the other showing the current. The oscilloscope displayed the values as peak-to-peak readings. The pulse was nearly rectangular. Treatments were conducted at 2.15 kV/cm, with a constant nominal pulse width of 18 μs and a frequency of 10 Hz. A total of 800 pulses were applied.

2.2.1.3. HP-assisted protein extraction. For HP treatment, the cricket slurry was deaerated and sealed in sterile plastic bags. The bags were then placed in a 100 mL hydrostatic pressure chamber (U33, Unipress, Warsaw, Poland) filled with distilled water as the pressure-transmitting medium. The HP treatment was conducted at 200 MPa for 9 min. The compression rate was 8 MPa/s, and decompression occurred in less than 8 s.

2.2.2. Standard alkaline protein extraction (control)

A control concentrate was produced using a standard alkaline protein extraction method (Zielińska, Karaś, & Baraniak, 2018) for comparison. The crickets were homogenized with distilled water at a ratio of 1:6 using a T-25 Ultra Turrax (IKA, Staufen, Germany) at 10,000 rpm for

10 s. The pH of the slurry was adjusted to 10 using 6 M or 1 M NaOH. The slurry was stirred at 400 rpm for 1 h at room temperature, with the pH monitored and maintained at 10 throughout the extraction period.

2.2.3. Post-extraction processing

After the control alkaline extraction or extraction assisted by a non-thermal technology, the resulting slurry was centrifuged at 3240 *g* for 30 min at 4 °C. The supernatant was collected, and its pH was adjusted to 7.0 using 6 M or 1 M HCl. The neutralized supernatant was frozen overnight at −40 °C and freeze-dried for 48 h at −20 °C using a Christ Alpha 1–4 LD Plus freeze-dryer (Osterode, Germany).

Fig. 1 shows the produced cricket protein concentrates. Their crude protein contents were determined by the Kjeldahl method (Bradstreet, 1954), using a nitrogen-to-protein factor of 5.6, as suggested by Janssen, Vincken, van den Broek, Fogliano, and Lakemond (2017) and Boulos, Tännler, and Nyström (2020). The protein content (dry matter basis) was 59.8 % for control, 55.3 % for US, 58.0 % for PEF, and 61.7 % for HP. A previous study (Bisconsin-Junior et al., 2024) has discussed the effects of these non-thermal technologies on protein extraction.

2.3. Protein structural characteristics of cricket protein concentrates

2.3.1. Molecular weight distribution (SDS-PAGE)

The extraction was performed according to Sagu, Huschek, Homann, and Rawel (2021). First, 100 mg of each protein concentrate was mixed with 1.5 mL of polyvinylpolypyrrolidone (PVPP) extraction buffer (0.2 M 3-(N-morpholino) propane sulfonic acid (MOPS) pH 7.0, 5 % PVPP, 1 % Triton X-100, 10 % glycerol, and 2 mM DTT) at 95 rpm for 60 min. The mixtures were centrifuged at 10,000 *g*, 4 °C for 5 min. The supernatants were collected, placed at −20 °C for 20 min, thawed at 4 °C for 20 min, briefly vortexed, and centrifuged at 10,000 *g*, 4 °C for 5 min. The clear supernatants were used in SDS-PAGE.

The molecular weight distribution of protein concentrates was determined using polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE), according to Laemmli (1970), with some modifications. The protein concentrate extracts were mixed in a 1:1 (*v/v*) ratio with sample buffer (125 mM Tris buffer, pH 6.8, 20 % (v/v) glycerol, 2 % (*w/v*) SDS, 0.1 % (w/v) bromophenol blue). For reduced proteins, 2 % (v/v) 2-mercaptoethanol was added. The mixtures were then heated for 5 min at 95 °C. After cooling to room temperature, 10 μL of each sample was loaded onto gels (Invitrogen NuPAGE 10 % Bis-Tris protein gel, 12 wells, Thermo Fisher Scientific, Carlsbad, CA, USA). A Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) was used for calibration, containing proteins with molecular weights of 10, 15, 25, 35, 55, 70, 100, 130, and 250 kDa.

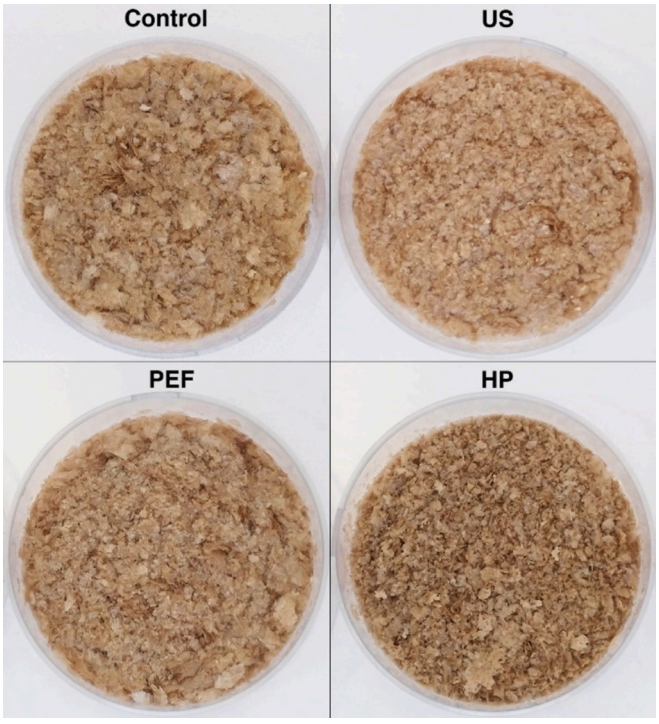

Fig. 1. Protein concentrate extracted from cricket: control, US-assisted, PEF-assisted, and HP-assisted.

The gels were initially run at 20 mA for 30 min, then increased to 50 mA until complete separation. They were stained overnight with a Coomassie blue solution and destained with a 10 % acetic acid, with the destaining solution changed regularly for about 3 h. Finally, the gels were scanned using a Bio-5000 Professional VIS Gel Scanner (SERVA Electrophoresis GmbH, Heidelberg, Germany) and analyzed using Image Lab software (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom).

2.3.2. Intrinsic fluorescence

Each protein concentrate was dissolved in 0.1 M phosphate buffer (pH 7.0) to achieve a 0.5 mg protein/mL concentration. Fluorescence emission spectra were measured using a PerkinElmer LS55 fluorescence spectrometer (Rodgau-Jügesheim, Germany) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928) at an excitation wavelength of 280 nm. The spectra were scanned from 300 to 400 nm, applying a scan speed of 500 nm/min and excitation/emission slits width of 5 nm. A 290 nm cut-off filter was placed in front of the emission monochromator. The same phosphate buffer used for protein concentrate dissolution was used as blank (Bußler, Steins, Ehlbeck, & Schlüter, 2015).

2.3.3. Surface hydrophobicity

The surface hydrophobicity of cricket protein concentrates was determined using the hydrophobic fluorescence probe ANS, according to Mintah et al. (2019), with some modifications. Protein concentrate suspensions (0.03–0.12 mg protein/mL) in phosphate buffer (0.01 M, pH 7.0) were prepared. The suspensions were vortexed and allowed to stand for 10 min at 25 °C, then centrifuged at 4000 *g* for 10 min. The supernatant protein solution (4 mL) was mixed with 20 μL of 8.0 mM ANS in 0.01 M phosphate buffer (pH 7.0), kept in the dark for 14 min, and measured for fluorescence (relative intensity). Fluorescence was measured using a Shimadzu RF-1501 spectrofluorometer (Kyoto, Japan) with excitation at 390 nm and emission from 400 to 600 nm (slit width of 5 nm and scan speed of 120 nm/min). Surface hydrophobicity was calculated as the ratio between the initial slope (Is) of the relative fluorescence intensity and the concentration of protein (mg/mL), applying a linear regression.

2.3.4. Particle size, polydispersity index and zeta potential

Particle size, polydispersity index (PDI) and zeta potential were measured using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK). Protein concentrate dispersions were diluted to 0.1 % (*w/v*) in 10 mM phosphate buffer (pH 7).

For particle size and PDI measurements, dispersions were filtered through 0.45 μm syringe filters (Pall, New York, USA). Measurements were taken at 25 °C, with a wavelength of 633 nm and a 90° scattering angle, using a refractive index of 1.45 for the particles and 1.33 for the solvent.

For zeta potential measurement, dispersions were placed in a folded capillary cell and measured at 25 °C after 1 min of equilibration. The dispersant had a refractive index of 1.33, a dielectric constant of 78.5, and a viscosity of 0.887 mPa·s.

2.4. Functional properties of cricket protein concentrates

2.4.1. Effect of pH on protein solubility

To assess protein solubility, 50 mg of protein concentrate was dispersed in 50 mL of distilled water. The pH of the mixture was adjusted to values ranging from 2 to 10 using 1 M or 6 M HCl or NaOH. After stirring for 90 min, the mixture was centrifuged at 3240 *g* for 30 min at 4 °C. The protein content in the supernatant was determined using the Bradford method (Bradford, 1976). The protein content of the concentrate was measured using the Kjeldahl method with a nitrogen-to-protein conversion factor of 5.60 (Boulos et al., 2020; Janssen et al., 2017).

Protein solubility was calculated using the formula:

$$\text{Protein solubility (\%)} = (P_s/P_c) \times 100 \quad (1)$$

where P_s is the protein content in the supernatant and P_c is the total protein content in the concentrate.

2.4.2. Water holding capacity (WHC) and oil holding capacity (OHC)

WHC and OHC were determined following the methods described by Bußler et al. (2015). Briefly, 0.5 g of protein concentrate was added to pre-weighted 15 mL centrifuge tubes and mixed with 2.5 mL of either water (for WHC) or canola oil (for OHC). The mixtures were vortexed for 60 s and centrifuged at 4000 *g* for 20 min. After discarding the supernatant, the pellet was weighed.

WHC and OHC were calculated using the following formulas:

$$\text{Water holding capacity (WHC) (g water/g d.m.)} = (P–M)/M_{DM} \quad (2)$$

$$\text{Oil holding capacity (OHC) (g oil/g d.m.)} = (P - M)/M_{DM} \quad (3)$$

where P is the pellet weight, M is the sample weight, and M_{DM} is the sample weight on a dry matter basis.

2.4.3. Foaming capacity (FC) and foam stability (FS)

The protein concentrate was dispersed in distilled water at 1 % (*w/v*), and the pH was adjusted to 7.0 to evaluate FC and FS. The mixture was stirred on a shaker for 1 h at 25 °C and then transferred to a 50 mL graduated cylinder. The suspension was homogenized using a T-25 Ultra Turrax (IKA, Staufen, Germany) at 12,000 rpm for 2 min. The volume of the foam layer was measured at 10 s (for FC) and 5, 10, 30, 60, 120, and 180 min (for FS) (Mishyna et al., 2019).

FC and FS were calculated using the following formulas:

$$\text{Foaming capacity (FC) (\%)} = [(V_t - V_0)/V_0)] \times 100 \quad (4)$$

$$\text{Foam stability (FS) (\%)} = (FC/FC_0) \times 100 \quad (5)$$

where V_t is the volume after homogenization at different times, V_0 is the volume before homogenization, and FC_0 is the foaming capacity at 10 s.

2.4.4. Emulsion capacity (EC) and emulsion stability index (ESI)

The evaluation of EC and ESI followed the method of Zielińska et al. (2018). Protein concentrates were dispersed in distilled water (1 % *w/v*) and the pH was adjusted to 7.0. Then, 15 mL of each dispersion was homogenized with 15 mL of canola oil using a T-25 Ultra Turrax (IKA, Staufen, Germany) at 20,000 rpm for 1 min. For EC, the emulsions were centrifuged at 3000 *g* for 5 min, and the volume of each layer was recorded. For ESI, the emulsions were heated for 30 min at 80 °C and centrifuged at 3000 *g* for 5 min. The volume of each layer was recorded.

EC and ESI were calculated using the following formulas:

$$\text{Emulsion capacity (EC) (\%)} = (V_e/V) \times 100 \quad (6)$$

$$\text{Emulsion stability index (ESI) (\%)} = (V_{30}/V_e) \times 100 \quad (7)$$

where V_e is the volume of the emulsified layer, V is the total volume of tube contents, and V_{30} is the volume of the emulsified layer after heating.

2.4.5. Least gelling concentration (LGC)

The LGC was determined following the method described by Santiago, Fadel, and Tavares (2021). Protein concentrates were suspended in distilled water at varying concentrations (4–9 % *w*/w), then heated in a water bath at 90 °C for 15 min. The tubes containing the dispersions were allowed to cool at room temperature for 2 h. Gelation was evaluated by inverting the tubes and recording the concentration at which the gel remained intact without sliding or falling.

2.5. Data analysis

All measurements were performed at least in triplicate, and the results were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate the significance of the data and mean values were compared using Tukey's post hoc test ($p < 0.05$). OriginPro software, version 9 (OriginLab Corporation, Northhampton, MA, USA) was used for statistical analysis and graph plotting.

3. Results and discussion

3.1. Protein structural characteristics of cricket protein concentrates

3.1.1. Molecular weight distribution (SDS-PAGE)

The impact of non-thermal technologies on the molecular weight profile of proteins in cricket protein concentrates is presented in Fig. 2. Under non-reducing conditions, control, PEF, and HP protein concentrates exhibited similar distribution of bands and molecular weight profiles. About 41 % of the proteins had a molecular weight above 100 kDa, 32 % were between 35 and 100 kDa, 17 % ranged from 15 to 35 kDa, and 9 % were below 15 kDa. Notably, the US treatment altered this profile, reducing the intensity of protein aggregates visible at the top of the gel, with molecular weight above 250 kDa and the band strength at 110 kDa. This treatment decreased the relative volume of proteins with molecular weight higher than 100 kDa from 41 % (control) to 34 %. Additionally, the bands between 35 and 55 kDa were more intense, increasing the relative volume of proteins in the 35–100 kDa range from 32 % to 39 %.

Under reducing conditions, the high molecular weight aggregates visible at the top of the non-reducing gel disappeared, indicating that disulfide bonds were responsible for forming these aggregates in cricket protein concentrates. Like the non-reducing conditions, the US concentrate was the only one displaying a different molecular weight distribution profile. There was an intensity reduction in bands above 100 kDa and an increase in bands between 35 and 55 kDa.

The US treatment appeared to disrupt the disulfide interactions that form stable protein aggregates, likely due to the cavitation effects of ultrasound, as suggested by Ding et al. (2022). This disruption reduced the number of high molecular weight molecules, concentrating the affected molecules within the 35–55 kDa range. Zhang et al. (2023) observed a similar trend. They found that US treatment of *T. molitor* larvae decreased the molecular weight of proteins, significantly weakening bands between 48 and 75 kDa while increasing band strength in the 25–35 kDa range.

3.1.2. Intrinsic fluorescence

Some hydrophobic amino acids, such as tryptophan and tyrosine, emit fluorescence upon excitation. Among these, tryptophan residues' fluorescence is commonly employed as an indicator of conformational changes in the tertiary structure of proteins, as they are sensitive to the polarity of their microenvironments (Wang, Sun, Pu, & Wei, 2017). The use of non-thermal technologies during extraction induced changes in the tertiary structure of cricket protein, as evidenced by the decrease in relative fluorescence intensity (Fig. 3a) and the shift in the maximum

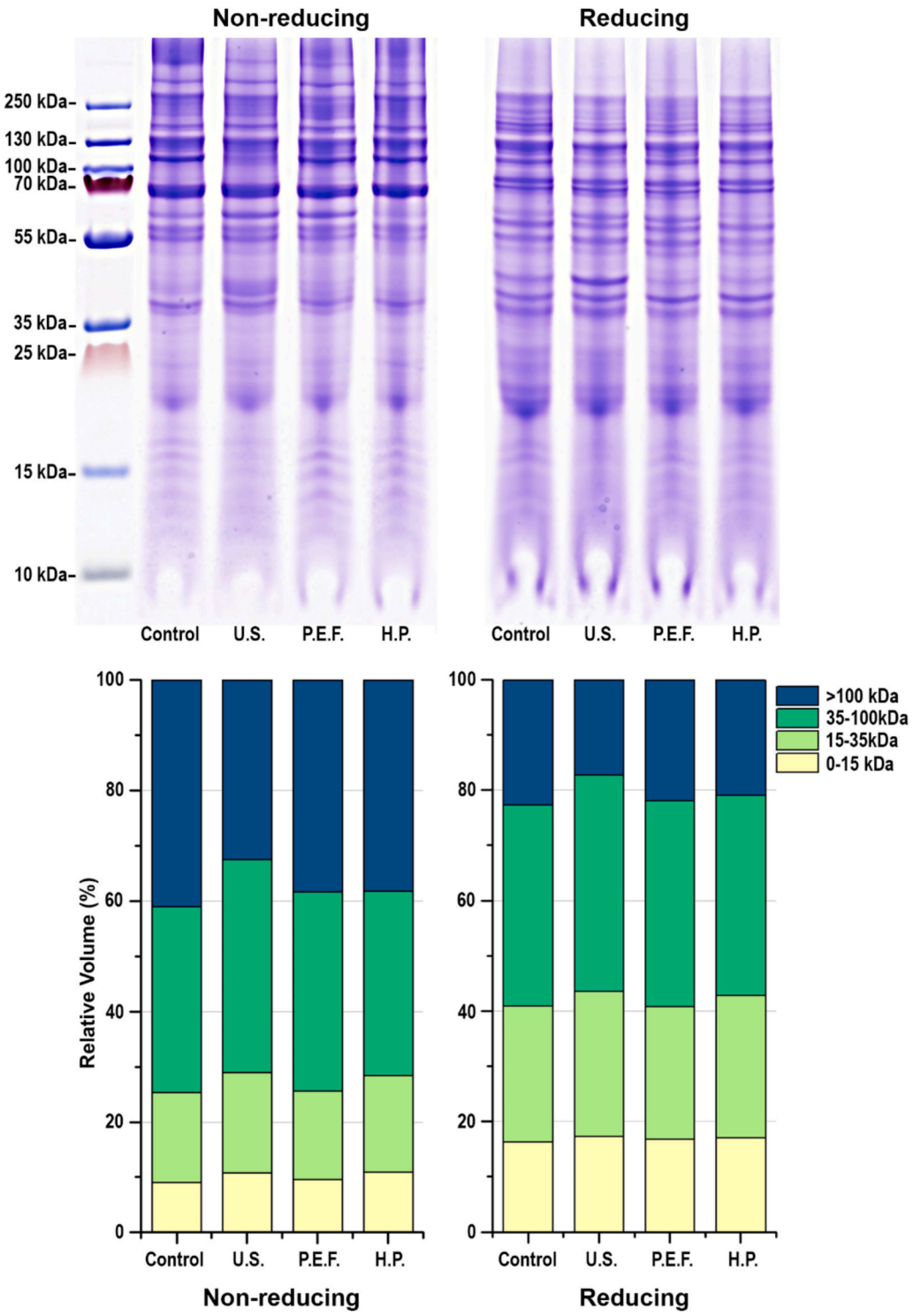

Fig. 2. SDS-Page gel profiles and molecular weight relative volume from control, US, PEF, and HP protein concentrates in non-reducing and reducing conditions (with β-mercaptoethanol).

emission wavelength (λ_{max}) compared to the control (Fig. 3b).

Among the evaluated non-thermal technologies, the US caused the most significant reduction in fluorescence intensity, followed by HP and PEF. This reduction may be attributed to the different efficiency of energy transfer between tryptophan and tyrosine or to the partial unfolding of the protein structure, exposing tryptophan residues to the aqueous environment, resulting in quenching of tryptophan fluorescence (Wang et al., 2017; Zhang et al., 2023). Other studies also reported a reduction in fluorescence intensity after treating protein samples with PEF (Li et al., 2022), HP (Boukil et al., 2022), and US (Tian, Roos, & Miao, 2024).

The maximum emission wavelength (λ_{max}) followed a similar pattern to fluorescence intensity. US and HP caused a shift in λmax toward longer wavelengths (redshift), indicating that tryptophan residues moved to a more polar environment (Malik & Saini, 2018). This likely resulted from the disruption of hydrophobic interactions during the treatments, causing hydrophobic groups to move from the core toward the surface of the protein and become exposed to the aqueous solvent.

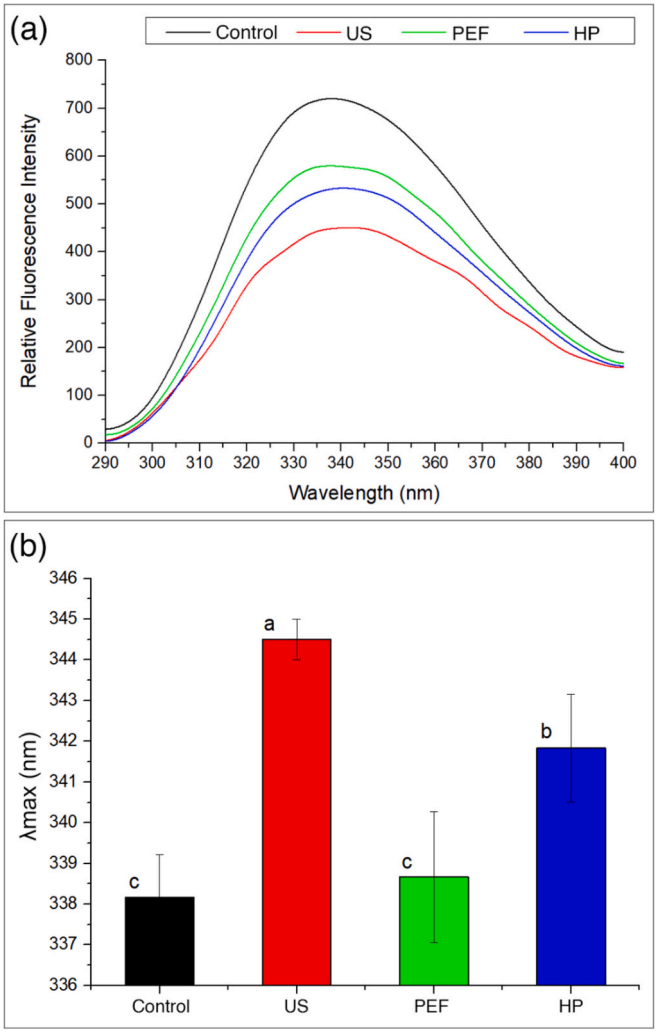

Fig. 3. (a) Intrinsic fluorescence spectrum, and (b) maximum emission wavelength (λ_{max}) of control, US, PEF, and HP protein concentrates. Values are mean ± SD from triplicate determinations. Different letters indicate significant differences ($p < 0.05$).

Similarly, proteins from *T. molitor* exhibited a redshift in wavelength after being subjected to treatments with HP (Boukil et al., 2022) and US (Zhang et al., 2023).

3.1.3. Surface hydrophobicity

Surface hydrophobicity is a crucial characteristic of proteins, revealing the content of hydrophobic groups exposed on their surface. It is often used to assess conformational changes that may affect protein functional properties (Liu, Wang, Xue, & Adhikari, 2022). Table 1 presents the changes in surface hydrophobicity of proteins in cricket protein concentrates. The control and PEF-treated concentrates had similar surface hydrophobicity. However, HP and US concentrates exhibited higher surface hydrophobicity than the control, with the US showing a more significant increase (+29 %). This increase correlates with the decrease in fluorescence intensity (Fig. 3a), confirming the exposure of internal hydrophobic regions, such as tyrosine and tryptophan residues, to the external environment.

The increase in surface hydrophobicity following HP treatment can be attributed to the disruption of non-covalent interactions, exposing interior hydrophobic regions to the molecule surface (Braspaiboon & Laokuldilok, 2024). Proteins subjected to HP treatment up to 400 MPa gradually expose hydrophobic regions buried inside the molecules, increasing surface hydrophobicity (Mula, Subramanian, & Dar, 2022).

Table 1
Average particle size, polydispersity index (PDI), zeta potential, and surface hydrophobicity of control, US, PEF, and HP protein concentrates.

Protein concentrates	Avg. particle size (nm)	PDI	Zeta potential (mV)	Surface hydrophobicity (slope x 10^3)
Control	45.09^{b} ± 0.47	0.41^{b} ± 0.01	-16.04^{b} ± 1.54	4.41^{c} ± 0.23
US	30.02^{c} ± 0.48	0.41^{b} ± 0.01	-25.48^{a} ± 1.63	5.70^{a} ± 0.12
PEF	56.83^{a} ± 0.77	0.43^{a} ± 0.01	-18.11^{b} ± 0.81	4.59^{c} ± 0.09
HP	44.67^{b} ± 0.12	0.41^{b} ± 0.01	-18.87^{b} ± 1.08	4.83^{b} ± 0.07

Values are mean ± SD from triplicate determinations.
Different letters in the same column indicate significant differences ($p < 0.05$).

Similarly, the increase in surface hydrophobicity following US treatment is due to cavitation, which induces a degree of molecular unfolding of proteins, leading to the exposure of hydrophobic groups initially buried inside the protein molecules (Bezerra, Sanches, Lamarão, & Campelo, 2022). Additionally, US treatment reduced particle size (Table 1) and decreased protein aggregates in the molecular weight profile (Fig. 2). This reduction in particle size and aggregates further increased the exposure of hydrophobic regions. Thus, the pronounced increase in surface hydrophobicity observed in US concentrate can be attributed to cavitation-induced unfolding and larger protein aggregates dissociation.

3.1.4. Particle size and polydispersity index (PDI)

Protein particle size and PDI are important factors that significantly influence functional properties such as solubility, foamability, and emulsification (Rahman & Lamsal, 2021). The effects of US, PEF, and HP treatments on these parameters are presented in Table 1. The treatments exhibited varying effects on particle size. The control and HP concentrates had similar average particle sizes (approximately 45 nm). In contrast, PEF increased particle size by 28 %, while the US reduced it by 33 % compared to the control.

PDI reflects the uniformity of particle size distribution, with higher values indicating greater size heterogeneity, which can negatively impact protein functionality and applicability (Danaei et al., 2018). Across all samples, PDI values showed minimal variation, ranging from 0.41 to 0.43, indicating a moderately dispersed particle size distribution. US and HP treatments did not significantly affect PDI, while PEF caused only a slight increase. These findings suggest the high stability of the protein fraction obtained from *G. assimilis*. The consistent particle size distribution and stability highlight the potential of treated insect proteins for applications in the food sector.

Perez and Pilosof (2004) explored the mechanism by which PEF increases protein particle size in β-lactoglobulin and egg white. They found that PEF induces polarization of the protein molecule, disrupts the quaternary structure by breaking noncovalent bonds between protein subunits, and alters protein conformation by exposing internal hydrophobic and sulfhydryl groups. In solutions with high protein concentration, PEF promotes protein aggregation through hydrophobic interactions and the formation of disulfide bonds. However, in our study, despite the increase in the average particle size and PDI (Table 1), we did not observe an increase in protein aggregates in the non-reduced SDS-PAGE gel (Fig. 2), indicating the absence of covalent bonds, such as disulfide bonds, in the formation of these aggregates.

On the other hand, US treatment dissociated high molecular weight protein (Fig. 2), breaking possible aggregates and reducing the average particle size. This observation aligns with previous studies reporting a reduction in protein size following US treatment (Mintah et al., 2020; Huang et al., 2023; Zhang et al., 2024;). Such size reduction is attributed to cavitation and micro-streaming, which generate strong collisions and result in smaller particles (Rahman & Lamsal, 2021).

3.1.5. Zeta potential

Zeta potential is a crucial parameter for understanding the surface charge characteristics of proteins and their electrostatic interactions with the surrounding solvent. This feature affects proteins' solubility and functional properties, such as emulsification and foaming (Amiri et al., 2021). In this study, we observed negative zeta potential values for all proteins in cricket protein concentrates (Table 1), indicating that the number of negative charges on the protein surface was greater than the number of positive charges. This is attributed to evaluating the protein at pH 7.0, higher than its isoelectric point (pH 5.0), as indicated in Fig. 4.

The zeta potential of control, PEF, and HP concentrates, which were not significantly different from each other, ranged from −10 to −20 mV, indicating that their dispersions were relatively stable (Cano-Sarmiento et al., 2018). In contrast, US treatment increased the absolute value of the zeta potential, resulting in negative charges ranging from −20 to −30 mV, which indicates a moderately stable dispersion. The increase in negative charge on the protein surface may be due to the reduced particle size (Table 1), which can facilitate the exposure of internal amino acid groups to the surrounding solvent, thereby enhancing the number of amino acids with negative charges on the protein surface (Kumar et al., 2022; Zhao et al., 2022).

3.2. Functional properties of cricket protein concentrates

3.2.1. Protein solubility

The interactions between proteins and water largely influence the functionality of protein-based ingredients in food production. High solubility is a desirable property for these ingredients. Fig. 4 displays the protein solubility of the cricket protein concentrates across different pH values. The concentrates displayed a characteristic U-shaped solubility profile, with higher solubility (>70 %) observed at more acidic (≤3) and alkaline (≥9) pH values. The pH range between 4.5 and 5.5 showed low solubility (<10 %), with the lowest solubility at pH 5.0, which could be attributed to the isoelectric point of the cricket proteins.

At the isoelectric point, electrostatic repulsion between protein molecules is minimized, promoting aggregation and precipitation, which results in reduced solubility (Verfaillie, Janssen, Van Royen, & Wouters, 2023). These findings are consistent with previous studies indicating the isoelectric point of cricket proteins falls within this pH range (Brogan, Park, Matak, & Jaczynski, 2021; Kim, Setyabrata, Lee, Jones, & Kim, 2017; Santiago et al., 2021). At neutral pH (7.0), the cricket protein concentrates demonstrated better solubility compared to most commercial soy, wheat, and pea protein powders. However, their solubility was similar to or slightly lower than that of commercial animal proteins such as egg, whey, and caseinate (Day, Cakebread, & Loveday, 2022).

Among the protein concentrates, the one treated with US exhibited higher solubility at almost all evaluated pH values. Various intrinsic and extrinsic factors influence protein solubility. Although surface hydrophobicity generally negatively impacts protein solubility, the US concentrate demonstrated higher solubility due to the smaller particle size and higher zeta potential value of its proteins (Table 1). An improved pH-solubility profile has also been observed in other insect proteins treated with US, such as those from *H. illucens* (Mintah et al., 2019), *T. molitor* (Huang et al., 2023), and *S. purpurascens* (Cruz-López, Escalona-Buendía, Román-Guerrero, Domínguez-Soberanes, & Alvarez-Cisneros, 2022).

3.2.2. Water holding capacity (WHC) and oil holding capacity (OHC)

WHC refers to a protein's ability to absorb and retain water within its three-dimensional structure. OHC indicates the capacity of proteins to absorb and retain oil and interact with lipid molecules. These properties are vital for developing desirable food texture and affect other functional properties such as solubility, emulsification, foamability, and gelation (Zhang et al., 2021). The application of non-thermal technologies caused significant changes in the WHC and OHC of protein concentrates (Fig. 5).

WHC values ranged from 4.0 to 6.3 g/g of protein concentrate. Despite statistical differences, the control, PEF, and HP concentrates had adjacent WHC values (4.0–5.0 g/g). In contrast, the US-treated concentrate showed a notable 40 % increase in WHC compared to the control. The increase in the WHC of protein concentrates caused by US treatment can be attributed to various factors. One potential mechanism is the reduction of particle size (Table 1) caused by cavitation and microstreaming, which enhances the surface area for interaction between proteins and water molecules. Another contributing factor is the forming of a sponge-like structure within the protein's peptide backbone and some ionized polar groups, which can further increase the contact area and interaction forces between proteins and water (Zou et al., 2017).

Regarding OHC, the values ranged from 4.5 to 5.4 g/g, with HP showing a 10 % increase compared to the control. Hydrophobic interactions primarily determine the stability of lipid-protein complexes and proteins with more hydrophobic regions tend to exhibit better OHC. Bolat, Ugur, Oztop, and Alpas (2021) subjected powders of *T. molitor* and *A. domesticus* to HP treatment at 500 MPa. They reported a 13 % reduction in OHC for mealworms, whereas crickets exhibited a 9 %

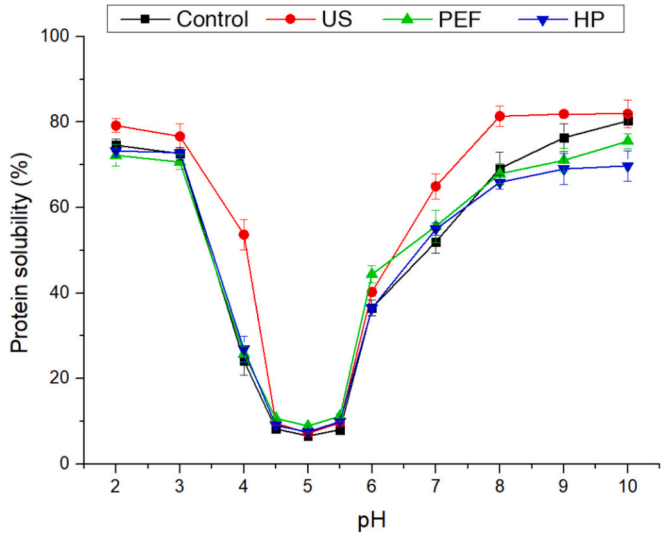

Fig. 4. Protein solubility of control, US, PEF, and HP protein concentrates. Values are mean ± SD from triplicate determinations.

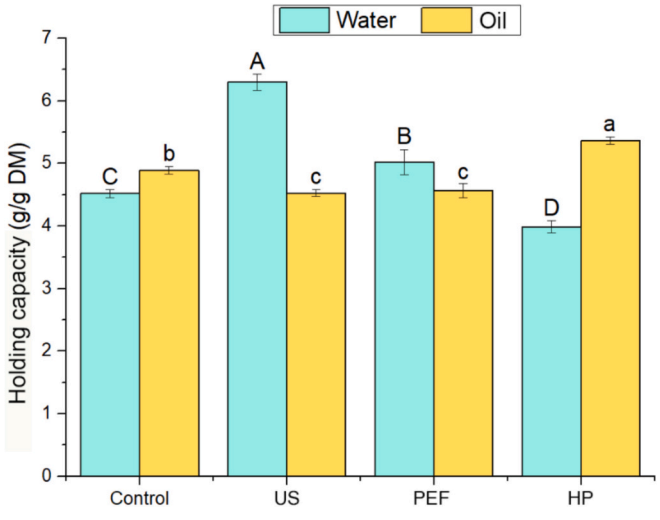

Fig. 5. Water and oil holding capacities of control, US, PEF, and HP protein concentrates. Values are means ± SD from triplicate determinations. Different letters indicate significant differences ($p < 0.05$).

increase in OHC, consistent with our findings. Thus, different insects may yield varying outcomes following similar HP treatments, as functionality depends not only on the applied treatment but also on the protein profile, which varies according to insect species, life stage, and diet, among other factors (Queiroz, Silva, de Carvalho, & Casanova, 2023).

Cricket protein concentrates demonstrated superior WHC and OHC compared to most plant-based and animal-based proteins reported by Gravel and Doyen (2020) and Day et al. (2022). However, their WHC was slightly lower than certain chickpea, soy, and gelatin proteins, while their OHC was surpassed by some plant-based proteins, including chickpea, lentil, mung bean, quinoa, and soy.

High WHC is critical for maintaining juiciness and softness in intermediate and high moisture foods, whereas high OHC is essential for proteins used as binders in emulsion (Huamaní-Perales, Vidaurre-Ruiz, Salas-Valerio, Cabezas, & Repo-Carrasco-Valencia, 2024). The intermediate to high WHC and OHC of cricket protein concentrates make them promising candidates for inclusion in meat products. Previous studies have shown the benefits of insect proteins in meat applications. For example, incorporating mealworm protein improved the cooking yield and textural properties of meat emulsion sausages (Choi et al., 2017; Kim, Setyabrata, Lee, Jones, & Kim, 2016). Similarly, adding silkworm pupae as an ingredient in pork meat batter enhanced viscous properties, hardness, gumminess, and chewiness, while reducing cooking loss (Park et al., 2017).

3.2.3. Foaming capacity (FC) and foam stability (FS)

A foam is a dispersion system in which a gaseous phase is surrounded by a continuous phase (liquid or solid). It is an unstable system and will readily collapse unless stabilized. The process of protein foam formation occurs in three stages. First, the protein must rapidly adsorb at the air-water interface. Next, the proteins unfold and rearrange at the interface, with hydrophobic groups oriented toward the air phase. Finally, the proteins interact to form a viscous and continuous film (Queiroz et al., 2023).

FC is related to the amount of interfacial area a protein can create, while FS indicates the protein's capacity to prevent foam collapse against opposing forces like gravity. Non-thermal treatments used during the production of protein concentrates significantly altered their FC (Fig. 6a) and FS (Fig. 6b). US, PEF, and HP protein concentrates had higher FC than control, with US and PEF showing the most significant increases reaching approximately 220 %. Similarly, US and PEF showed superior performance in FS, producing the most stable foams. Notably, US maintained a foam with over 50 % of its initial volume even after 180 min.

Protein solubility, surface flexibility, and hydrophobicity are the main determinants of an efficient FC. Once the foam is formed, FS depends on the protein film's physical properties, protein-protein interactions, and environmental factors (Zhang, Sharan, Rinnan, & Orlien, 2021). The observed structural changes can explain the superior performance of US and PEF in FC and FS. As indicated in the intrinsic fluorescence (Fig. 3), partial protein unfolding may have contributed to the rapid adsorption at the air-water interface and increased protein flexibility, allowing for rapid structural changes and adaptation to the interface. Additionally, the disruption of protein aggregates (Fig. 2), smaller particle size, higher hydrophobicity (Table 1), and increased solubility (Fig. 4) favored the foam properties of the US concentrate.

Compared to other protein sources, cricket concentrates exhibited superior FC and FS, particularly those treated with US and PEF, outperforming most plant-based proteins reported by Ma et al. (2022). Furthermore, their foaming properties were comparable to those of milk and egg proteins (Gravel & Doyen, 2020). Foaming properties are critical for a variety of food products, such as whipped cream, meringue, ice cream, and leavened baked goods. The intermediate to high FC and FS of cricket protein concentrates highlight their potential as functional ingredients in baked goods. For instance, gluten-free bread enriched with cricket powder showed better functional values than formulations using lentil or buckwheat (Rosa Machado & Thys, 2019). Additionally, bread supplemented with mealworm powder exhibited superior characteristics, including increased specific volume and softer texture, compared to standard bread formulations (Roncolini et al., 2019).

3.2.4. Emulsion capacity (EC) and emulsion stability index (ESI)

Emulsions are homogeneous mixtures of two immiscible liquids, with one liquid dispersed in small droplets within the other continuous phase. In the food industry, emulsions are commonly classified as oil-in-water or water-in-oil. Emulsions are inherently unstable due to the interfacial tension between the two liquids, and this instability increases with a larger contact area. Amphiphilic molecules reduce interfacial tension and stabilize the emulsion. Among the various emulsifiers, proteins are extensively used in food systems due to their ability to migrate to the interface and orient their polar and nonpolar amino acid

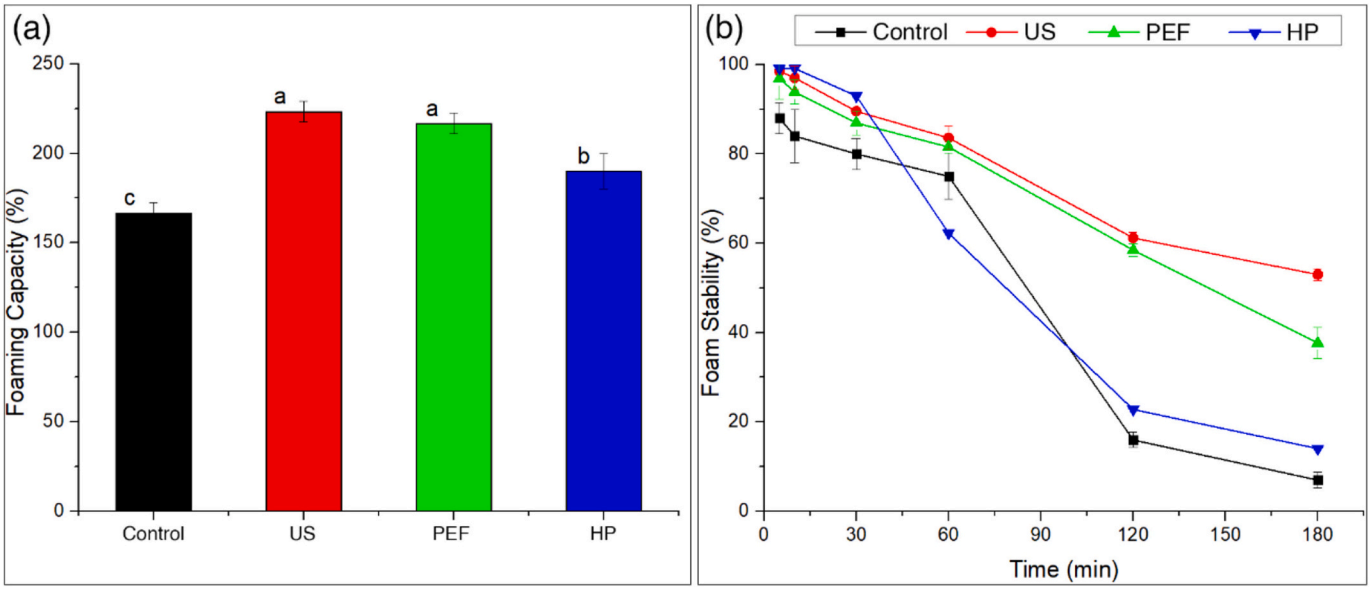

Fig. 6. (a) Foaming capacity, and (b) foaming stability of control, US, PEF, and HP protein concentrates. Values are means ± SD from triplicate determinations. Different letters indicate significant differences ($p < 0.05$).

residues toward the aqueous and lipid phases, respectively. This orientation forms a stable coating around the droplets and enhances the stability of the emulsion (Queiroz, Silva, Jessen, et al., 2023; Zhang, Sun, et al., 2021).

EC measures the amount of oil that can be emulsified per gram of protein, while ESI relates to the resistance of the emulsion under specific conditions. Overall, the applied treatments caused minimal changes in EC (Table 2). Specifically, HP treatment resulted in a slight increase in EC compared to the control, while the other treatments did not show significant differences, with an average EC of 52 %. The slight rise in EC with HP treatment can be attributed to increased exposure of the hydrophobic sites without reducing particle size (Table 1). During HP treatment, water molecules can permeate protein molecules and interact with hydrophobic residues, exposing hydrophobic groups on the surface, as indicated by the intrinsic fluorescence (Fig. 3). This structural modification decreases surface tension and promotes interactions between proteins and lipids (Xue, Qian, Kim, Xu, & Zhou, 2018). Similarly, Bai et al. (2021) observed a modest enhancement in the EC of myosin after exposure to HP at 150 MPa for 2 min.

In terms of ESI (Table 2), all cricket protein concentrates exhibited similar results, with an average ESI of 58 %. While the EC and ESI of the cricket protein concentrates were adequate, they were lower than those reported for legumes proteins (EC: 59.9–243.7 %; ESI: 66.0–99.9 %) and significantly lower than those of whey (EC: 210 %; ESI: 100 %), egg (EC: 198 %; ESI: 95 %), and soy proteins (EC: 173 %; ESI: 100 %), as summarized by Huamaní-Perales et al. (2024).

3.2.5. Least gelling concentration (LGC)

Gelation capacity measures a protein's ability to aggregate and form a gel, primarily due to disulfide bonds and hydrophobic interactions between protein molecules. LGC is used as an index of gelation capacity and is defined as the lowest concentration at which a protein solution does not flow after inversion. Most studies on the gelation of food proteins describe heat-induced gel formation, which involves protein denaturation with subsequent conformational changes, followed by interactions, aggregation, and eventual gelation (Villaseñor, Enriquez-Vara, Urías-Silva, & Mojica, 2022).

As shown in Fig. 7, solutions prepared from the control and PEF concentrates had the same LGC value (6.0 %), while US exhibited a higher value (7.0 %) and HP a lower one (5.0 %). These results are consistent with Santiago et al. (2021), where the LGC of a protein isolate from the same cricket species (*G. assimilis*) was reported to be 6.5 %. The gelation capacity of proteins is a complex phenomenon influenced by several factors, including protein concentration, pH, ionic strength, processing conditions, and protein denaturation (Queiroz, Silva, Jessen, et al., 2023).

Although US induced protein structural changes typically associated with improved gel formation - such as reducing high molecular weight aggregates (Fig. 2), partially unfolding proteins (Fig. 3), increasing hydrophobicity, and decreasing average particle size (Table 1) - the treatment resulted in a higher LGC. This seemingly counterintuitive result can be attributed to differences in protein content among the concentrates. HP, which exhibited the lowest LGC, had the highest protein content (61.7 %), providing more protein molecules to form a cohesive gel network. In contrast, US concentrate, with the lowest protein content (55.3 %), required a higher concentration to achieve gelation.

Cricket protein concentrates displayed superior gelation capacities compared to most plant-based proteins, as summarized by Ma et al. (2022) and Webb, Li, and Alavi (2023). This highlights their potential for use in foods where gelation is critical to texture and juiciness, including sausages, cheese, yogurt, surimi, desserts, and tofu (Yang et al., 2025). For example, incorporating 1.5 % locust protein into cheddar cheese significantly improved sensory attributes, such as color, flavor, and overall acceptability, without adversely affecting texture-related properties like firmness, elasticity, and cohesiveness (Singh et al., 2023). Similarly, the addition of cricket protein to mackerel surimi enhanced its gel properties, leading to improved textural characteristics, including hardness, springiness, chewiness, and gumminess (Somjid et al., 2022).

Table 2
Emulsion capacity (EC) and emulsion stability index (ESI) of control, US, PEF, and HP protein concentrates.

Protein concentrates	EC (%)	ESI (%)
Control	$52.5^{b} \pm 1.1$	$60.0^{a} \pm 0.0$
US	$51.3^{b} \pm 0.8$	$55.8^{a} \pm 1.4$
PEF	$52.3^{b} \pm 0.6$	$58.3^{a} \pm 2.9$
HP	$54.4^{a} \pm 0.5$	$59.2^{a} \pm 1.4$

Values are mean ± SD from triplicate determinations.
Different letters in the same column indicate significant differences ($p < 0.05$).

4. Conclusions

This study examined the effects of non-thermal technologies - specifically ultrasound (US), pulsed electric fields (PEF), and high pressure (HP)- on the protein structure and functional properties of cricket (*Gryllus assimilis*) protein concentrates. Each technology caused distinct structural changes in the cricket proteins, leading to alterations in their functional properties.

US demonstrated the most pronounced effects, breaking larger protein aggregates into smaller fragments, partially unfolding proteins, reducing particle size, intensifying negative surface charge, and increasing surface hydrophobicity. As a result, US-treated protein concentrate exhibited higher solubility, water retention capacity, foam formation, and stability but lower gel-forming capacity compared to the other treatments.

HP and PEF treatments caused subtle structural changes. HP improved oil holding capacity, emulsion, and gel formation by promoting partial protein unfolding and increasing surface hydrophobicity. PEF enhanced foam capacity and stability, alongside a slight increase in particle size.

These findings highlight the potential of non-thermal technologies, particularly US, to enhance the functional properties of cricket protein concentrates. By assisting in the extraction process and tailoring functionality, these technologies support the incorporation of cricket protein concentrates into novel food formulations, such as baked goods, meat products, and gel-based foods.

Insects are a sustainable protein source, and non-thermal technologies offer eco-friendly processing options. However, the potential environmental impact of multiple processing steps highlights the need for further research to quantify their sustainability. Additionally, the microbial safety of non-thermal-treated protein concentrates also requires investigation to ensure their suitability for food applications. Finally, exploring the performance of cricket protein concentrates produced with non-thermal technologies in food systems will provide practical insights into their potential as sustainable and functional food ingredients.

CRediT authorship contribution statement

Antonio Rocha Bisconsin-Junior: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giacomo Rossi:** Writing – review & editing, Methodology, Formal analysis. **Sorel Tchewonpi Sagu:** Writing – review & editing, Methodology, Formal analysis. **Harshadrai M. Rawel:** Writing – review & editing, Resources, Methodology. **Lilian Regina B. Mariutti:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Oliver K. Schlüter:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition,

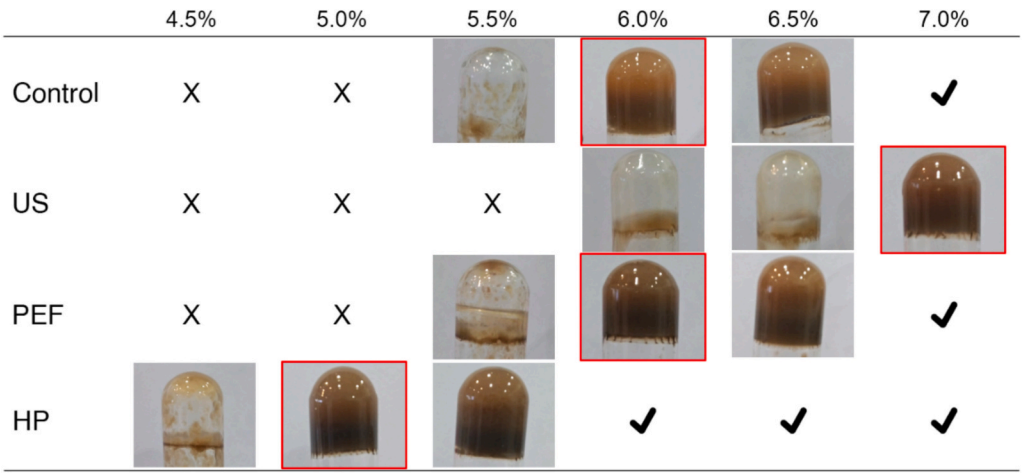

Fig. 7. Least gelling concentration of control, US, PEF, and HP protein concentrte.

Conceptualization.

Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that could have influenced the work presented in this paper.

Data availability

Data will be made available on request.

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