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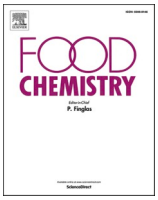

Physical and oxidative stability of 5 % fish oil-in-water emulsions stabilized with lesser mealworm (*Alphitobius diaperinus* larva) protein hydrolysates pretreated with ultrasound and pulsed electric fields

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ABSTRACT

Lesser mealworm (*Alphitobius diaperinus* larva) meal was pretreated with ultrasound (US) or pulsed electric fields (PEF) and hydrolyzed using Alcalase or Trypsin enzymes. The resulting hydrolysates were evaluated for their ability to maintain physical and oxidative stability of 5 % fish oil-in-water emulsions. The effects of the pretreatment on enzymatic hydrolysis were assessed by measuring the degree of hydrolysis (DH), protein yield, and molecular weight distribution. Hydrolysates with 19–28 % DH were produced. Physical stability was evaluated in terms of creaming index, Turbiscan stability index, ζ-potential, and droplet size. Emulsions stabilized with US-pretreated Trypsin hydrolysates presented the smallest droplet sizes (0.626 μm). Primary and volatile secondary oxidation products were measured during storage. However, none of the hydrolysate-stabilized emulsions exhibited greater oxidative stability than sodium caseinate, the reference protein. These results suggest that although US-pretreated Trypsin hydrolysates exhibit potential as emulsifiers, additional antioxidants are needed to effectively control lipid oxidation.

1. Introduction

Diets rich in omega-3 polyunsaturated fatty acids (PUFAs), particularly in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been shown to have beneficial effects on human health. However, declining consumption of food items naturally rich in omega-3 PUFAs has created a demand for omega-3-fortified food (Patel et al., 2022). Oils rich in omega-3 PUFAs, such as fish oil, are particularly prone to oxidation which can lead to the degradation of both nutritional and sensory quality of the product (Frankel, 2005). Emulsion-based delivery systems were shown to be efficient to protect and incorporate oil rich in PUFAs in liquid food (McClements et al., 2007). While emulsions have a large oil-water interface which can accelerate oxidation, the encapsulation provides a physical barrier protecting the oil from prooxidants and oxygen (Aslam & Schroën, 2023; Berton-Carabin et al., 2014). Hence, the characteristics of the interface (e.g., thickness, charge, and compounds present) play a crucial role on the lipid oxidation in emulsions (Hennebelle et al., 2024).

In recent years there has been a growing demand for natural and sustainable food ingredients, driven by the growing environmental and health awareness, and increasing worldwide population (McClements et al., 2017). In particular, insect proteins are being explored as sustainable alternatives to conventional emulsifiers (e.g., synthetic emulsifiers and milk proteins) due to their nutritional and environmental benefits (Gravel & Doyen, 2020). By 2024, 4 insects (*Tenebrio molitor* larva, *Locusta migratoria*, *Acheta domesticus*, and *Alphitobius diaperinus* larva) have been authorized as novel food in Europe (European Commission, 2017). Despite this regulatory progress, insect consumption in Western countries remains limited compared to other novel foods such as algae, largely due to negative consumer perceptions (Onwezen et al., 2021). However, research suggests that incorporating insect-derived ingredients, such as protein and fat, into familiar food products can enhance their acceptability (Baiano, 2020). Thus, efforts are focused on producing insect-based ingredients that can be integrated in a variety of

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food products. Specifically, lesser mealworm proteins have already demonstrated potential as a substitute to conventional dairy proteins, such as whey protein, in food emulsions (Ballon et al., 2024; Jayakumar et al., 2023; Wang et al., 2021).

Enzymatic hydrolysis is widely used to modify proteins from various protein sources (e.g., wheat gluten, pea protein, whey protein), with the aim of enhancing their techno-functional and bioactive properties (Liceaga, 2019). Studies have shown that partial hydrolysis not only improved the solubility of insect proteins but also affected the functional and bioactive properties. The functionality of the hydrolysates varied significantly in function of the substrate, enzyme, and hydrolysis conditions (Liceaga, 2019; Nongonierma & FitzGerald, 2017). Green processing technologies, such as microwave, pulsed electric fields (PEF), and ultrasound (US), have been used to treat the substrate before enzymatic hydrolysis (i.e., pretreatment) or during hydrolysis (i.e., assisted hydrolysis) to improve protein extraction and the functionality and/or bioactivity of the hydrolysates produced (Habinshuti et al., 2023; Nasrabadi et al., 2021). Among these, PEF treatment is reported to induce structural changes and to affect techno-functional and bioactive properties of various proteins (Mannozzi et al., 2023; Taha et al., 2022). To the best of our knowledge, no literature is available on the techno-functional properties of insect protein hydrolysates obtained after PEF treatment of the substrate. Similarly, US treatment of insect proteins have been shown to cause conformational changes and modified techno-functional properties (Mannozzi et al., 2023). In particular, sonication pretreatment before enzymatic hydrolysis of black soldier fly (*Hermetia illucens*) meal improved both the emulsifying and antioxidant capacity, compared to the hydrolysates obtained without pretreatment (Mintah et al., 2019).

Thus, this study aims to study the impact of US or PEF pretreatment on the enzymatic hydrolysis of lesser mealworm defatted meal, and on the physical and oxidative stability of 5 % fish oil-in-water (O/W) emulsions stabilized by the hydrolysates. Two enzymes were used to perform hydrolysis, Alcalase, a commonly used enzyme, and Trypsin. The performance of the hydrolysates was compared to a conventional dairy protein (sodium caseinate, NaCas).

2. Materials and methods

2.1. Materials

Edible lesser mealworm (*Alphitobius diaperinus* larva) powder with 45 % protein content (Ballon et al., 2024) was purchased from Kreca Ento-Food BV (Wageningen, The Netherlands). Sodium caseinate (NaCas, Miprodan 30) with a reported 93.5 % protein content was kindly provided by Arla Foods Ingredients Group P/S (Viby J, Denmark). Commercial cod liver oil was provided by Vesteraalens AS (Sortland, Norway) and stored at −40 °C until further use. The peroxide value (PV) of the fish oil, determined using the method described in Section 2.6.2.2., was found to be 0.51 ± 0.04 mEq O_2/kg oil. The fatty acid composition (%, *w*/w) of the fish oil was determined by Yesiltas, Soria Caindec, et al. (2023) by gas chromatography (GC) analysis of fatty acid methyl esters to be: C14:0 (3.7), C16:0 (9.0), C16:1n-7 (9.6), C18:0 (1.9), C18:1n-9 (15.8), C18:1n-7 (4.5), C18:2n-6 (2.4), C18:3n-3 (1.0), C20:1n-9 (13.7), C20:5n-3 (8.4), C22:1n-11 (5.6), and C22:6n-3 (10.7). The fish oil tocopherol content was determined, as described in Section 2.6.2.3., as follows: α-tocopherol, 192 ± 4 μg/g oil, β-tocopherol, 0 ± 0 μg/g oil, γ-tocopherol, 111 ± 4 μg/g oil, and δ-tocopherol, 42 ± 3 μg/g oil. Alcalase (2.4 L FG) and a recombinant Trypsin (Formea Prime) (Novozymes A/S, Bagsvaerd, Denmark) were used for the enzymatic hydrolysis. All chemicals and solvents used were of analytical grade. Deionized water was used for the preparation of all the reagents and samples.

2.2. Determination of protein content

The protein content of samples was determined by measuring the nitrogen content using the Dumas method for the defatted meal and hydrolysates, and the Kjeldahl method for the whole meal. L-aspartic acid was used as a standard for the Dumas analysis. The measurements of nitrogen content were performed in duplicates. Different nitrogen-to-protein conversion (Kp) factors were used, to take into account the presence of non-protein nitrogen compounds, such as chitin, in insect meal. The total amino acid contents of the lesser mealworm meal and protein concentrate were measured (Ballon et al., 2024). Kp factors were calculated from the ratio of the sum of amino acid residue weights to nitrogen content (Janssen et al., 2017). A Kp factor of 4.87 was used for the whole meal and defatted meal, and a Kp factor of 5.67 was applied for the hydrolysates.

2.3. Production of hydrolysates

2.3.1. Defatting of insect meal

The removal of lipids from the insect meal was performed according to the method described in previous work (Ballon et al., 2024). Briefly, lesser mealworm powder was mixed with 2-methyltetrahydrofuran (1:5, *w/v*) and stirred for 1 h at room temperature. After decanting, the solid and liquid fractions were separated. This process was repeated a total of 3 times. Finally, the sample was placed under the fume hood overnight to evaporate the remaining solvent. The defatted meal was ground using a sample mill (Knifetec 1095, FOSS TECATOR, Hillerød, Denmark) for 10 s. The resulting powder was stored at −20 °C until further use.

2.3.2. Pretreatment of insect meal

The defatted meal powder was dispersed in distilled water (5 %, *w*/w) in a bottle and stirred for 2 h. The samples were stored in the fridge overnight. The day after, pH was adjusted to 8.0 and the dispersions were subjected to US and PEF treatment.

2.3.2.1. US treatment. Around 30 mL of the dispersion was placed in a jacketed beaker linked to a water bath (10 °C) to avoid a large increase in temperature during the treatment. The conditions of the US pretreatment were chosen according to Queiroz et al. (2021), using a higher percentage of amplitude and shorter time of treatment. The treatment was performed using a Branson sonifier (SFX550 Model, Branson Ultrasonics Corp., Danbury, CT, USA) in pulse mode (3 s ON and 5 s OFF) during 10 min at 20 kHz and 90 % amplitude.

2.3.2.2. PEF treatment. The PEF treatment was applied using a commercial PEF generator for laboratory use (OmniPEF, VITAVE, Prague, Czech Republic). Around 40 g of the dispersion was treated in a batch chamber with a 1-cm electrode gap. The conductivity of the sample was 1.68 ± 0.07 mS/cm. The following conditions were chosen based on equipment and sample limitations, to mitigate the effects of temperature and current rise in the system: square bipolar pulses (800 reps), 10 μs duration, 3 kV/cm, applied at a frequency of 10 Hz.

2.3.3. Enzymatic hydrolysis

The defatted meal solution (pretreated or untreated) was placed in a water bath at 50 °C while being shaken at 50 rpm. The pH was adjusted to pH 8.0, optima for Alcalase and Trypsin, according to Novozymes' recommended conditions. The hydrolysis was initiated by adding Alcalase or Trypsin to reach 1 % E/S ratio. The hydrolysis was run for 1 h under free-fall pH conditions, before inactivation of the enzyme in a separate water bath at 90 °C for 15 min. Samples were cooled down to room temperature, using an ice bath, and the pH was adjusted to 7.0. The samples were centrifuged at 6700 ×*g* for 20 min at 20 °C to separate the soluble hydrolysates from the insoluble matter. The supernatant retrieved was weighted to measure the yield of hydrolysis. An aliquot

was taken to analyze the protein content and degree of hydrolysis. The remaining supernatant was freeze-dried, ground into a fine powder, and stored at 4 °C until further use. The hydrolysis was performed in triplicate for each condition. Hydrolysates produced without pretreatment using Trypsin and Alcalase without prior pretreatment were named Try and Alc, respectively. Hydrolysates produced using US or PEF pretreatment will be referred to as US_Alc and US_Try, or PEF_Alc and PEF_Try, respectively. DM refers to the water-soluble proteins of the defatted meal. The protein yield was calculated based on the mass of protein in the supernatant and the initial mass of protein before hydrolysis (see Eq. 1).

$$Yield\ (\%) = \frac{m_{protein,supernatant}}{m_{protein,initial}} \bullet 100 \quad (1)$$

where $m_{protein,supernatant}$ is the the total weight of protein in the supernatant (g), and $m_{protein,initial}$ is the weight of protein in the defatted meal dispersion initially (g).

2.4. Characterization of hydrolysates

2.4.1. Degree of hydrolysis

The degree of hydrolysis (DH) is defined as the proportion of the peptide bonds cleaved during hydrolysis (Rutherfurd, 2010). The DH was measured by the o-phthalaldehyde (OPA) assay. The OPA reagent (100 mL) was prepared by combining 10 mL of 0.15 M $Na_2CO_3{\cdot}10\ H_2O$, 10 mL of 0.6 M $NaHCO_3$, 88 mg of DTT, 80 mg of OPA dissolved in 2 mL of ethanol (96 %) and 10 mL of 1 % SDS. The reagent was covered with aluminum foil to protect it from light and was freshly prepared daily. The protein hydrolysates samples were diluted to a protein concentration of around 0.15 % using deionized water. Four dilutions were prepared with dilution factor of 1, 2, 4, and 8. The OPA assay was carried out by mixing 20 μL of each dilution with 200 μL of OPA reagent in an Eppendorf 96-well microplates. The microplate was shaken for 20 s and immediately measured at 340 nm. An eight-point standard curve was prepared using L-leucine (0–0.5 mg/mL). The sample equivalent serine was calculated according to Eq. 2.

$$sample\ (mg\ serine/mL) = \frac{\left(\left(Abs_{sample} - Abs_{blank}\right) - intercept\right)}{slope} \bullet DF \quad (2)$$

where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank, *DF* is the dilution factor, and *slope* and *intercept* are the slope and intercept of the L-leucine standard curve.

The DH was calculated as follows:

$$DH\ (\%) = \frac{Sample\ (mg\ serine/mL)}{P \bullet 10} \bullet 100 \quad (3)$$

where *P* is the protein content (%).

2.4.2. SDS-PAGE

Samples were dissolved in distilled water to a protein concentration of 1 mg/mL and mixed with Laemmli buffer. They were then boiled for 3 min at 100 °C before being centrifuged for 3 min at 12000 rpm. Ten microliters of samples were loaded onto a 10 % Bis-Tris gel (NuPAGE™ 10 % Bis-Tris Gel, Invitrogen). The electrophoresis was carried out under reducing conditions, using a NuPAGE® MOPS (3-(N-morpholino)propanesulfonic acid) SDS running buffer system, according to manufacturer guidelines. The gel was run at 200 *V* for approximately 1 h and stained (GelCode™ Blue Stain Reagent). SeeBlue™ Plus2 prestained standard was used to estimate the molecular weight of the samples.

2.4.3. Fourier transform mid-infrared (FT-MIR) spectroscopy analysis

MIR spectra were collected using an Agilent Cary 630 portable unit equipped with a single bounce diamond attenuated total reflectance (ATR) crystal and a deuterated triglycine sulphate (DTGS) detector (Agilent Technologies Inc., Danbury, CT, USA). To perform the analysis, the samples were homogenized, and around 0.1 g were placed onto the ATR sampling window. A pressure clamp was used to ensure the contact between the sample and the diamond crystal. Each spectrum was the average of 128 scans of a 4 cm^{-1} resolution, co-added to improve the signal to noise ratio over the 4000–650 cm^{-1} range. The spectra acquisition was repeated four times under the same conditions for each sample using MicroLab PC software (Agilent Technologies Inc., Danbury, CT, USA). The relative percentage content of each secondary structure element was determined by curve fitting of the second derivative of the amide I region (1700–1600 cm^{-1}) following the procedure of Hackshaw et al. (2023) using OriginPro 2023 (Origin Lab, MA, Northampton, USA). The raw spectra (in the region 1700–1600 cm^{-1}) was first normalized, and then smoothed (Savitsky-Golay function, 7 points). The second derivative was calculated using a Savitsky-Golay function (9 points), multiplied by (−1) for visualization purposes and baseline-corrected. Finally, the spectra were fitted using the "Multiple Peak Fit" option (Chi-square tolerance value of 1×10^{-9}) and the area of the different peaks fitted was divided by the total area to obtain the relative area of each band. The wavelength of the bands was correlated with the secondary structures as follows: β-sheet (antiparallel: 1618–1637 cm^{-1} and parallel: 1682–1695 cm^{-1}), random coil (1637–1645 cm^{-1}), α-helix (1645–1662 cm^{-1}), and β-turn (1662–1682 cm^{-1}) (Gkinali et al., 2022).

2.4.4. Intrinsic fluorescence

The intrinsic fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA, USA) according to the method described by Ballon et al. (2024). Emission spectra of solutions of hydrolysates (1 mg/mL in 10 mM phosphate buffer pH 7.0) was measured in the range 270–500 nm using an exciting wavelength set to 280 nm. Each sample was measured in duplicate.

2.4.5. Surface hydrophobicity

Surface hydrophobicity (H_0) was determined using 8-anilino-1-naphthalenesulfonic acid (ANS) as fluorescence probe as described by Ballon et al. (2024). Briefly, 4 mL sample solution (0.005–0.1 mg/mL in 5 mM phosphate buffer pH 7.0) was mixed with 20 μL of ANS solution (8 mM). The fluorescence intensity was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 390 and 470 nm, respectively. The protein surface hydrophobicity was determined as the slope of the linear regression of the fluorescence intensity against the protein concentration (mg/mL). The determination was performed in duplicate for each sample.

2.5. Emulsion production

Fish O/W emulsions (5 wt%) were produced using the hydrolysates obtained as emulsifiers with NaCas as a reference, in 250 g batches. The aqueous phases (237.5 g) were prepared by dissolving the proteins in 10 mM sodium acetate and 10 mM imizadole buffer. The amount of hydrolysate powder added was calculated to have a final protein concentration in the emulsion of 0.2 %. The solutions were stirred for 2 h at room temperature and left overnight stirring at 4 °C for complete hydration. The pH of the solutions was measured and adjusted to pH 7 if needed. To produce the emulsion, first, a coarse emulsion was produced by doing a pre-homogenization of the aqueous phase and fish oil using a Ystral homogeniser (X10/25, Ystral GmbH, Ballrechten-Dottingen, Germany) at 16000 rpm for 3 min. All the oil (12.5 g) was slowly added during the first minute. The coarse emulsion was then homogenized 3 times using a microfluidizer (Model M-110 L, Microfluidics, Newton, MA, USA) at 9 kpsi pressure. Sodium azide solution (10 %) was then added to the emulsion to inhibit microbial growth to reach a final concentration of 0.05 wt%. The physical and oxidative stabilities of the emulsions were assessed under accelerated conditions. Freshly prepared $FeSO_4$ solution was added to a final concentration of 50 μM in the emulsion to catalyze oxidation. The pH of the emulsion was adjusted to

7.0 ± 0.1, if necessary. The emulsions were then stored in amber bottles (one bottle per measurement day) in the dark at room temperature up to 9 days.

2.6. Characterization of emulsions

2.6.1. Physical stability

2.6.1.1. Creaming index. Aliquots of the emulsions were placed in a 5 mL measuring cylinder and the creaming index (CI) was determined on days 0, 1, 3, 6, and 9, according to Eq. 4 (do Prado Silva et al., 2021):

$$CI = \frac{h_u}{h_t} \bullet 100 \quad (4)$$

where h_u is the upper phase layer (lowest density) height (cm) and h_t is the total height (cm).

2.6.1.2. Turbiscan analysis. After production, aliquots of emulsions (20 mL) were placed in a Turbiscan tower (Formulaction, Toulouse, France) and analyzed for one day. The Turbiscan stability index (TSI) and changes in the backscattering data was followed as a measure of physical stability. Samples having TSI values above 3 were considered to be unstable and stable if the TSI value stayed below 1.

2.6.1.3. Droplet size. The droplet size distribution of the emulsions was measured on day 1 and day 9 by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Emulsions were diluted in recirculating water (3000 rpm), until it reached an obscuration of 12–15 %. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given as surface-weighted ($d_{3,2}$) mean diameter. Measurements were carried out in duplicate.

2.6.1.4. ζ-Potential. The surface charge of the emulsion droplets was assessed by measuring the ζ-potential of the emulsions on day 1 and day 9 in a Zetasizer Nano ZS (Malvern instruments Ltd., Worcestershire, UK) at 20 °C. The emulsions were diluted in buffer (40 μL emulsion in 20 mL buffer). The ζ-potential range was set to −100/ +50 mV and the samples were analyzed with 100 runs. Measurements were performed in duplicate.

2.6.2. Oxidative stability

The oxidative stability of the emulsions was determined by analyzing the peroxide value, tocopherol consumption and the formation of volatile compounds on samples of emulsions collected on day 0, 1, 3, 6, and 9 of storage. On the sampling day, aliquots of emulsions were divided into brown bottles, flushed with nitrogen, and stored at −40 °C until analysis.

2.6.2.1. Lipid extraction. Lipids were extracted from the emulsion using the method described by Bligh and Dyer (1959) with modifications. First, 10 g of emulsions were mixed with 5 mL of deionized water, 30 mL of methanol and 15 mL of chloroform at 15000 rpm for 20 s (T25 digital Ultra-Turrax, IKA-Werke GmbH & Co. KG, Staufen, Germany). Then, 15 mL of chloroform was added, and the mixture was mixed again at 15000 rpm for 20 s. Finally, 15 mL of deionized water was added, and the mixture was mixed at 15000 rpm for 20 s. Samples were centrifuged at 2800 rpm at 18 °C for 10 min to separate the chloroform phase containing the lipids from the aqueous phase which was discarded. Two extractions were done from each emulsion sample.

The oil content of the extract was determined by weighing around 10 g of the extract in a dry beaker whose dry weight was known. The beaker was left overnight in the hood, put in an oven at 105 °C for 2 h, and weighed after cooling down in a desiccator.

2.6.2.2. Primary oxidation products - peroxide value. Peroxide value (PV) was determined on lipid extracts obtained in Section 2.6.2.1 using the colorimetric ferric thiocyanate method at 500 nm according to Shantha & Decker (1994). Measurements were carried out in duplicate. Results were expressed as milliequivalent (mEq) peroxides per kg oil using Fe(III) as standard.

2.6.2.3. Tocopherols. The tocopherol content was determined using lipid extracts obtained in Section 2.6.2.1 according to the American Oil Chemists' Society (1998) by HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA, USA) equipped with a Spherisorb S5W column (250 × 4.6 mm) (Phase Separation Ltd., Deeside, UK) and fluorescence detector. Elution was performed with an isocratic mixture of n-heptane/2-propanol (100,0.4, *v*/v) at a flow of 1 mL/min. Measurements were performed in duplicate, and results were expressed in μg tocopherol per kg oil using a tocopherol standard.

2.6.2.4. Volatile secondary oxidation products. Volatiles compounds formed in the emulsion during storage were measured according to García-Moreno et al. (2016) using dynamic headspace GC–MS. Four grams of emulsion, 5 mL of distilled water and 30 mg of an internal standard (4-methyl-1-pentanol) were placed in pear shape bottles. The bottles were placed in a water bath at 45 °C and the mixtures were bubbled with nitrogen (150 mL/min) for 30 min. The volatile compounds released were trapped on Tenax GR tubes. Afterwards, the tubes were placed in an Automatic Thermal Desorber (TurboMatrix 350 ATD, Perkin Elmer, Norwalk, CN) connected to gas chromatography (GC Agilent 6890 N, Palo Alto, CA, USA; Column: DB-1701, L: 30 m, I.D. 0.25 mm, df:1.0 μm; J&W GC Columns, Palo Alto, CA, USA). To separate and identify individual volatile compounds a mass spectrometer (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA; 70 eV; mass to charge ratio scan between 30 and 250) and MS library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard, New York, NY, USA) were used, respectively. The following volatile compounds were selected for quantification: butanal, 3-methylbutanal, 2-ethylfuran,1-penten-3-one, pentanal, 1-penten-3-ol, 2,3-pentanedione, hexanal, heptanal, (Z)-4-heptenal, 1-octen-3-ol, octanal, benzaldehyde, (E,E)-2,4-heptadienal, (E,E)-2,4-decadienal. To prepare the standard curve, a stock standard solution was prepared in ethanol with all the volatile compounds mentioned previously, apart from heptanal and 3,5-octadien-2-one, diluted to seven levels (0.5–100 μg/mL). The diluted standard solution (30 mg) was added into an emulsion similar to one of the emulsions analyzed and the emulsion was analyzed as previously described, without addition of internal standard. The analysis was performed in triplicate, and the results were expressed in ng/g of emulsion. The volatile concentration of heptanal and 3,5-octadien-2-one, whose standards could not be added to the calibration curve, were expressed in area/g of emulsion.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was performed to determine significant differences ($p < 0.05$) using Minitab 19 statistical software (State College, PA, USA). Tukey post hoc test was used when the assumption of homogeneity of variance was met, otherwise Games-Howell post hoc test was performed. The normality of residuals was checked using the Anderson-Darling test. Principal component analysis (PCA) was carried out on the data of lipid oxidation using Origin 2024 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Characterization of the hydrolysates

3.1.1. Protein content, yield, and degree of hydrolysis

The protein content, degree of hydrolysis and the yield of hydrolysis of the protein hydrolysates produced with Alcalase or Trypsin, without or with pretreatment of the insect meal (US or PEF) are presented in Table 1. All the hydrolysates presented a protein content above 68 %, significantly higher than the original lesser mealworm meal (45 %). Previous research reported protein content of lesser mealworm hydrolysates between 59 and 69 % (Leni, Soetemans, Jacobs, et al., 2020; Sousa et al., 2020). The protein yields obtained after hydrolysis ranged from 43 to 45 %, with no significant differences between samples. However, they were significantly higher than the protein yield of the defatted meal obtained after solubilization in water (protein yield: 14.2 ± 0.6). These results are in line with a previous study in which lesser mealworm meal was hydrolyzed with Alcalase, at large scale, achieving a protein yield of 42 % (Leni, Soetemans, Jacobs, et al., 2020). US and PEF treatments have been shown to enhance protein extraction yields from insect substrates by facilitating enzyme-substrate interactions, due to increased membrane permeability and cellular fragmentation induced by cavitation, respectively (Mannozzi et al., 2023). Since no differences in protein yield were observed between the samples with and without pretreatment, we can hypothesize that the operating conditions of US and PEF pretreatment may not be optimal to enhance the protein yield. It is also possible that the extraction efficiency may already be close to its maximum under the applied conditions of hydrolysis.

The hydrolysates obtained had DH values ranging from 19 to 28 % (Table 1). These values are in the range of DH values obtained after enzymatic hydrolysis of different insects. DH values from 26 to 52 % were measured after enzymatic hydrolysis of whole crickets by Alcalase (0.5 to 3 % E/S, 30 to 90 min of hydrolysis) (Hall et al., 2017). Dion-Poulin et al. (2020) studied the enzymatic hydrolysis of mealworm and cricket meal by Alcalase and reported values ranging from 28 to 34 % (3 % E/S, 120 min of hydrolysis). However, some studies reported lower DH after hydrolysis of lesser mealworm meal. Sousa et al. (2020) measured DH values of 9–10 % after 1 h of hydrolysis at 0.5–1.5 % E/S while Leni, Soetemans, Caligiani, et al. (2020) obtained hydrolysates with a DH of 5.7 % after 1 h of hydrolysis. The method to measure the DH, substrate (e.g., insect type, presence of fat), and specific conditions of hydrolysis (e.g., temperature, pH, E/S, and enzyme) might account for these differences. The Alcalase hydrolysis of the lesser mealworm meal yielded hydrolysates with higher DH than the Trypsin hydrolysis with DH in the range 24–28 % and 19–23 % after hydrolysis with Alcalase and Trypsin, respectively. This is likely due to the difference in specificity of the enzymes. Both enzymes are *endo*-proteases, but Alcalase has a broad specificity while Trypsin favors basic residues such as lysine and arginine (Ma et al., 2005). Similar results have been observed when lesser mealworm protein concentrate (unpublished results) or mealworm (Dai et al., 2013) were hydrolyzed with Alcalase or Trypsin.

The hydrolysates obtained after PEF pretreatment of the insect meal both presented significantly lower DH than their counterparts. However, various authors reported that PEF pretreatment prior to enzymatic hydrolysis led to an increase in the degree of hydrolysis by increasing the cell permeability promoting the accessibility of enzymes to the proteins (Akaberi et al., 2019; Li et al., 2016). López-Gámez et al. (2024) stated that proteins may aggregate and denature after absorbing the electrical energy which may affect the enzymatic hydrolysis and lead to lower DH (López-Gámez et al., 2024). US treatment may cause the protein to unfold which can enhance the enzyme-substrate interaction (Mintah et al., 2019). Nevertheless, no significant difference in DH was observed for the US pretreated samples. Rivero-Pino et al. (2020) also reported that US pretreatment of *Tenebrio molitor* meal did not modify the kinetics of hydrolysis of the proteins by Alcalase, Trypsin or their combination. The ultrasound treatment may also lead to the aggregation and denaturation of the proteins affecting the resulting degree of hydrolysis (Cropotova et al., 2024). The DH of hydrolysates is a critical parameter for its functionality. Prolonged enzymatic hydrolysis is often linked with decreased emulsifying properties, because the smaller peptides form a weaker film around the oil droplets (Wouters et al., 2016). Yet, it has been observed in some studies that hydrolysates with higher DH have higher emulsifying activities than hydrolysates with lower DH (Hall et al., 2017; Wouters et al., 2016).

Table 1
Protein content, protein yield, degree of hydrolysis (DH), and surface hydrophobicity (H_0) of lesser mealworm protein hydrolysates.

Sample	Protein content (%)	Protein yield (%)	DH (%)	H_0 (−)
Alc	68.2 ± 0.8 ^{a}	42.5 ± 1.7 ^{a}	27.2 ± 1.8 ^{d}	47.4 ± 5.3 ^{b,c}
US_Alc	69.8 ± 1.3 ^{a,b}	43.3 ± 0.9 ^{a}	27.8 ± 2.0 ^{d}	42.8 ± 3.4 ^{a,b}
PEF_Alc	70.0 ± 0.6 ^{b}	43.1 ± 1.4 ^{a}	24.2 ± 1.8 ^{c}	35.1 ± 2.4 ^{a}
Try	70.6 ± 0.7 ^{b}	44.1 ± 1.1 ^{a}	21.9 ± 1.3 ^{b}	48.9 ± 2.0 ^{b,c}
US_Try	69.9 ± 1.1 ^{a,b}	44.9 ± 1.4 ^{a}	22.7 ± 1.2 ^{b}	53.0 ± 7.0 ^{c}
PEF_Try	70.4 ± 4.4 ^{a,b}	43.8 ± 2.8 ^{a}	18.9 ± 1.2 ^{a}	47.2 ± 6.4 ^{b,c}

Different letters indicate significant difference within each column ($p < 0.05$). Notations: DM, soluble proteins from defatted meal; Alc, non-pretreated Alcalase hydrolysates; US_Alc, ultrasound pretreated Alcalase hydrolysates; PEF_Alc, pulsed electric field Alcalase hydrolysates; Try, non-pretreated Trypsin hydrolysates; US_Try, ultrasound pretreated Trypsin hydrolysates; PEF_Try, pulsed electric field Trypsin hydrolysates.

3.1.2. Molecular weight distribution – SDS-PAGE

The defatted meal presented bands at approximately 90, 50, 38, 28, and below 17 kDa (Fig. S1, Supplementary material). The hydrolysates showed bands corresponding to low molecular weight peptides (< 17 kDa), indicating the successful hydrolysis of insect proteins. Compared to Alcalase hydrolysates, weak bands of higher molecular weight peptides were observed for Trypsin hydrolysates, which is in accordance with the lower DH observed for these hydrolysates. No clear differences were observed due to the pretreatment used.

3.1.3. Fourier transform mid-infrared (FT-MIR) spectroscopy

The ATR-FTMIR raw spectra of the lesser mealworm defatted meal and the hydrolysates are presented in Fig. 1A. Different spectral regions could be identified. In all the samples, a broad band was observed at around 3500–3000 cm^{-1}, attributed to intermolecular H-bonded NH and OH-stretching characteristic of hydrophilic materials, polysaccharides including chitin and residual water (Gkinali et al., 2022). The band at 2950 cm^{-1} was assigned to the vibration of -CH_2 groups of lipids and chitin (Gkinali et al., 2022; Mellado-Carretero et al., 2020). Bands characteristics of proteins and chitin were observed at 1700–1600 cm^{-1} (amide I; mainly C═O stretching), 1600–1500 cm^{-1} (amide II; in-plane N—H bending and C—N stretching), and 1300–1200 cm^{-1} (amide III) (Yang et al., 2015). Finally, the IR band around 1200–900 cm^{-1} was attributed to carbohydrates (Mellado-Carretero et al., 2020). Similar spectral mid regions have been identified by authors who studied lesser mealworm meal (Mellado-Carretero et al., 2020), mealworm defatted meal (Gkinali et al., 2022) and black soldier fly hydrolysates (Mintah et al., 2020). Differences in amide I and amide II bands were expected after enzymatic hydrolysis as the N—H, C—N, and C═O vibrations will be directly impacted by the hydrolysis of the peptide bonds.

Shifts of bands and differences in intensity were observed between DM and its hydrolysates. The band at around 3250 cm^{-1} was slightly broader in the hydrolysate spectra compared to DM, which could be ascribed to higher amount of free -OH groups (Kuptsov & Zhizhin, 1998). The amide I and II bands are usually used to study the secondary structure of the proteins, as they are directly sensitive to changes in the protein backbone conformation (Yang et al., 2015). A shift of the amide I and amide II bands towards higher wavenumber (9–11 cm^{-1} and 15–34

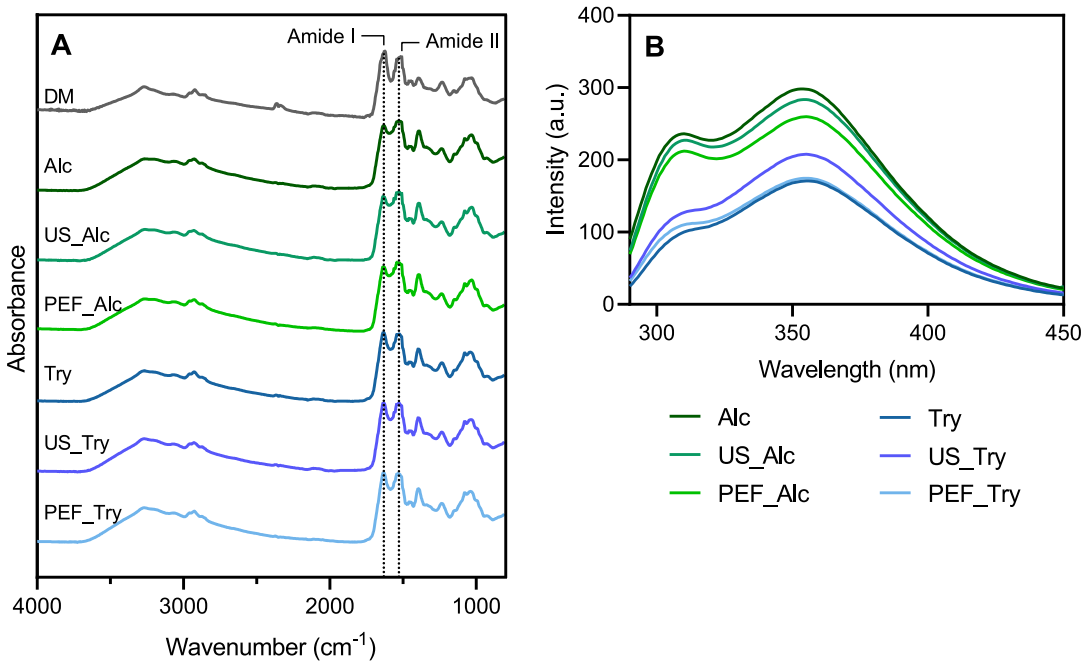

Fig. 1. Structural changes in lesser mealworm protein due to US and PEF pretreatments before enzymatic hydrolysis. A) FT-MIR spectra of lesser mealworm defatted meal and its hydrolysates and B) intrinsic fluorescence spectra of lesser mealworm hydrolysates.

cm^{-1}, for amide I and amide II, respectively) was observed when comparing the hydrolysates to DM. In addition, the amide II band observed in the DM spectra had lower intensity than the amide I band (ratio amide I/amide II: 1.08). However, for the Alcalase hydrolysates, the amide II band presented higher intensity than the amide I band (ratio amide I/amide II: 0.93–0.94). The amide I and amide II bands had similar intensity for the Trypsin hydrolysates (ratio amide I/amide II: 1.03, 1.00 and 1.01 for Try, US_Try, and PEF_Try, respectively). Overall, the pretreatment used before hydrolysis did not seem to impact the ratio amide I/amide II. The increase in absorbance intensity in the amide II region with extent of hydrolysis have been linked to the N—H bending vibrations, becoming stronger after enzymatic hydrolysis (Poulsen et al., 2016).

In order to study more precisely the secondary structure of the hydrolysates and defatted meal, a deconvolution of the secondary derivative of the amide I, the most sensitive region to protein secondary structure composition, was performed. The protein secondary structure contents are shown in Table 2. The major secondary structure of DM were β-sheet (55.4 %) and α-helix (25.2 %). Random coil and β-turn accounted for 12.1 and 7.3 % of the secondary structure, respectively. The hydrolysis of the protein induced a significant reduction in β-sheet and α-helix structure content and a significant increase in random coil and β-turn structure content. These differences were more pronounced for Alcalase hydrolysates compared to Trypsin hydrolysates. There was no significant effect of the PEF pretreatment. However, the US pretreatment before Alcalase hydrolysis led to significant higher content of random coil. The random coil content also increased for US_Try but the difference between US_Try and Try was not significant. The ultrasound treatment might have led to denaturation of the proteins, increasing the random coil contents (Cropotova et al., 2024). Higher contents of random coil have been linked to increased flexibility which could facilitate the unfolding of the protein at the oil-water interface (Chen et al., 2011).

Table 2
Secondary structure content of the lesser mealworm defatted meal and hydrolysates.

Sample	Secondary structure content (%)			
	β-sheet	Random coil	α-helix	β-turn
DM	55.4 ± 3.3 ^{a}	12.1 ± 0.9 ^{d}	25.2 ± 0.9 ^{a}	7.3 ± 3.1 ^{c}
Alc	36.4 ± 0.9 ^{c}	28.6 ± 2.2 ^{b}	20.1 ± 0.8 ^{d}	14.9 ± 1.3 ^{a}
US_ Alc	34.0 ± 2.1 ^{c}	33.1 ± 2.0 ^{a}	19.1 ± 1.2 ^{d}	13.8 ± 1.8 ^{a,b}
PEF_ Alc	36.3 ± 1.2 ^{c}	28.2 ± 1.6 ^{b}	20.2 ± 0.4 ^{d}	15.3 ± 0.7 ^{a}
Try	41.2 ± 2.4 ^{b}	24.0 ± 2.0 ^{c}	21.7 ± 0.4 ^{b}	13.1 ± 1.1 ^{a,b}
US_ Try	42.8 ± 2.7 ^{b}	25.8 ± 3.6 ^{b,c}	20.4 ± 0.8 ^{c,d}	10.8 ± 1.7 ^{b}
PEF_ Try	43.6 ± 2.4 ^{b}	23.7 ± 1.5 ^{c}	21.8 ± 0.9 ^{b,c}	10.9 ± 1.3 ^{b}

Different letters indicate significant difference within each column ($p < 0.05$). Notations are as in Table 1.

3.1.4. *Intrinsic fluorescence*

Both US and PEF technologies are reported to induce conformational changes in proteins (Mannozzi et al., 2023). These changes were determined by intrinsic fluorescence spectroscopy. Indeed, fluorescent amino acids such as tryptophan have a high sensitivity to its local environment. Thus, from the characteristics of the fluorescence spectra (i.e., position of the maximum emission, and intensity) we can obtain information about the environment of tryptophan and changes in protein conformation (Hellmann & Schneider, 2019). The fluorescence spectra of the hydrolysates are presented in Fig. 1B. All spectra presented a peak at around 310 nm and 355 nm, which could possibly be attributed to Raman scattering of water and tryptophan, respectively (Hellmann & Schneider, 2019). The maximum emission wavelength (λ_{max}) of tryptophan around 355 nm indicates that the tryptophan residues are exposed to a relatively hydrophilic environment (Hellmann & Schneider, 2019). The values of λ_{max} were as follows: Alc (353.4 nm) < US_Alc (354.7 nm) < PEF_Alc = PEF_Try (354.8 nm) < US_Try (355.3 nm) < Try (356.0 nm). This indicates that the local environment of tryptophan in Alcalase hydrolysates was slightly more hydrophobic than the Trypsin hydrolysates. The fluorescence intensity usually depends on i) the environment, intensity is higher in apolar environment, ii) quenching process from other amino groups, iii) energy transfer between tryptophan and tyrosine residues (Hellmann & Schneider, 2019). Thus, the decreased fluorescence intensity along with the slight red shift of the maximum peak may be an indication of a more unfolded structure of Trypsin hydrolysates, compared to Alcalase hydrolysates. Alc, US_Alc and PEF_Alc had different fluorescence intensity indicating that the

pretreatment impacted the tertiary structure of the hydrolysates. In the case of Trypsin, only the US pretreatment induced changes in the fluorescence spectra, with higher intensity obtained. Alcalase and Trypsin exhibit different specificities, the selective cleavage by Trypsin might have resulted in longer peptides that are more susceptible to unfolding. The pretreatment applied, US or PEF, before hydrolysis, might have modified the structure of the proteins, exposing different residues, therefore contributing to variations in fluorescence intensities.

3.1.5. Surface hydrophobicity

The surface hydrophobicity (H_0) of protein is an important parameter to take into account when emulsifying properties of proteins. Indeed, the solubility and the ability of proteins to adsorb at the oil-water interface is highly dependent on the balance of exposed hydrophobic and hydrophilic patches of the proteins (Wouters et al., 2016). The surface hydrophobicity of the hydrolysates is presented in Table 1. Alc and Try presented similar surface hydrophobicity (47.4 and 48.9, respectively). The surface hydrophobicity of Alcalase hydrolysates decreased when the defatted meal was pretreated with either US or PEF. Although US has been reported to cause the exposure of hydrophobic patches (Mintah et al., 2019), Alcalase which is nonselective may have cleaved part of the patches exposed. In contrast, US pretreatment before Trypsin hydrolysis led to an increase in surface hydrophobicity, reaching 53.0. The hydrophobic patches might have been less disrupted by Trypsin hydrolysis, as Trypsin cleaves preferentially next to lysine and arginine which are both positively charged.

3.2. Stability of fish oil emulsions during storage

The physical and oxidative stability of fish O/W emulsions prepared with lesser mealworm DM and its hydrolysates were compared to emulsions stabilized with a protein of reference, NaCas, a commonly used dairy protein.

3.2.1. Physical stability of emulsions

3.2.1.1. Creaming and turbiscan analysis. The stability of the emulsions against destabilization phenomena, such as creaming, sedimentation, flocculation, and coalescence was analyzed visually and by determining the Turbiscan Stability Index (TSI) and creaming index (CI) (Fig. 2). NaCas-stabilized emulsions were highly stable under the storage conditions used. They did not present any creaming during storage and reached a TSI of 2.6 after 1 day. According to the equipment company, systems having a TSI below 3 are considered visually stable and in the early stage of destabilization. On the contrary, the emulsions stabilized with DM were the least stable. After 9 days of storage, the serum layer of the emulsion was almost clear, and they had a CI of around 15 %. The Trypsin hydrolysates seemed to provide better stabilization of the emulsions against creaming in contrast to the Alcalase hydrolysates, as evidenced by the whitish color of the serum layers at the end of storage. This is in accordance with the TSI results, ranking the emulsions from the most physically stable to the least as following: Try > US_Try > PEF_Try > US_Alc > Alc > PEF_Alc > DM.

3.2.1.2. Droplet size and ζ-potential. The ζ-potential and droplet size, measured after 1 and 9 days of storage, are reported in Fig. 3. Throughout the storage the absolute values of ζ-potential of all the emulsions remained lower than 30 mV (Fig. 3A). This value is considered to be enough to provide sufficient repulsion to avoid aggregation and coalescence of droplets. The DM emulsions presented the lowest absolute value of ζ-potential (−32 mV). The emulsions stabilized with the hydrolysates had higher ζ-potential values (between −43 and − 48 mV), similar or higher than the one of the emulsions stabilized with NaCas (−44 mV). Few changes in ζ-potential occurred during storage, only the emulsions stabilized with US_Alc and Try showed significant differences with ζ-potential going from −45 to −48 mV, and − 47 to

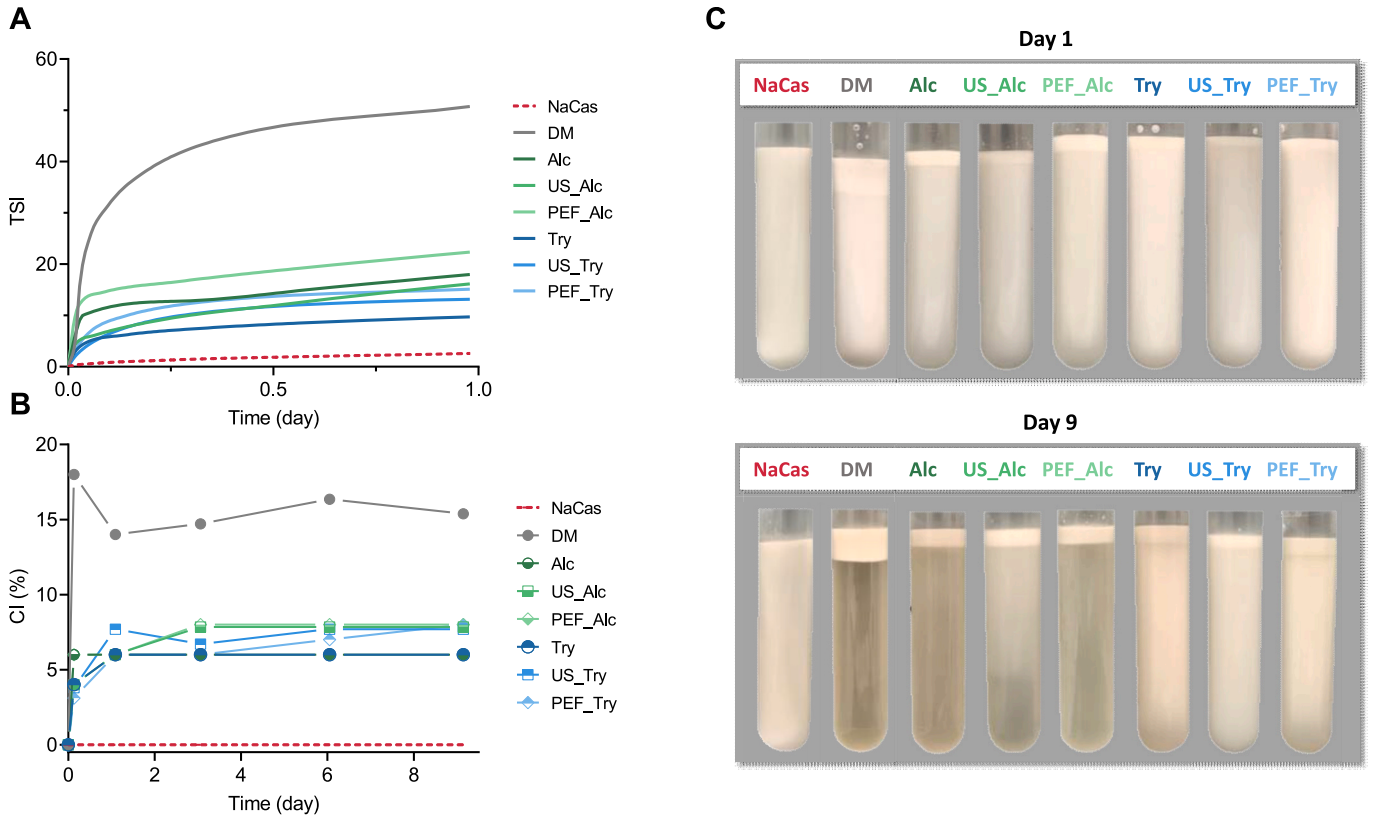

Fig. 2. Physical stability of emulsions produced with NaCas, DM and lesser mealworm protein hydrolysates: A) turbiscan stability index (TSI), B) creaming index (CI), and C) visual appearance of the emulsion at day 1 and day 9.

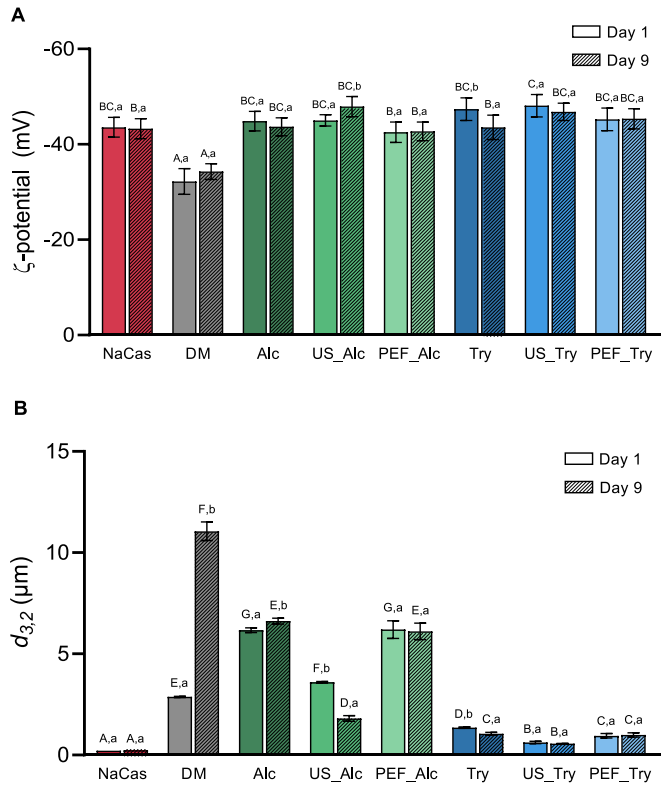

Fig. 3. A) Particle size and B) ζ-potential of emulsions stabilized with NaCas, DM, and lesser mealworm protein hydrolysates after 1 and 9 days of storage. Different uppercase letters indicate significant differences between different emulsions at the same storage time and different lowercase letters indicate significant differences between the same emulsion at different storage time ($p < 0.05$).

−44 mV, respectively. During storage, the proteins might rearrange themselves at the oil-water interface exposing different groups. Moreover, the compounds released during the oxidation of the oil phase adsorb at the interface promoting chemical changes. Queiroz et al. (2021) reported similar values (between −39 and − 58 mV) for fish O/W emulsions stabilized with black soldier fly proteins during 10-day storage.

NaCas-stabilized emulsions had the smallest droplet size throughout storage (0.218–0.240 μm) (Fig. 3B). Emulsions stabilized with insect protein or hydrolysates presented initial droplet size values ranging from 0.626 to 6.199 μm, with Trypsin hydrolysates emulsions having the smallest droplet size (0.626–1.35 μm). Among them, the US pretreatment led to a reduction in droplet size compared to Try and PEF_Try emulsions. The emulsions produced with DM were the least stable in terms of droplet size over storage, which corroborate the TSI and visual results. At the end of the storage, the droplet size slightly increased for Alc and did not change for PEF_Alc, US_Try and PEF_Try. The mean droplet size of the emulsions stabilized with US_Alc and Try reduced from day 1 to day 9 as a small population of droplets was detected in the measurements performed on day 9 (data not shown). Even though care was taken to homogenize properly the sample before sampling, as the emulsions started to cream between production and sampling, heterogeneity within the samples might account for these differences. Initial droplet size of the emulsions obtained in this study, apart from the emulsions stabilized with US_Try, was larger ($d_{3,2}$: 0.951–6.199 μm) than other emulsions produced using the same method and stabilized either by insect proteins ($d_{3,2}$: 0.57–0.90 μm) or potato protein hydrolysates ($d_{3,2}$: 0.134–0.334 μm) (Queiroz et al., 2021; Yesiltas, García-Moreno, et al., 2023). Overall, emulsions stabilized with Trypsin hydrolysates exhibited smaller droplet sizes and enhanced physical stability throughout storage. Moreover, for each enzyme, US pretreatment before hydrolysis resulted in emulsions with smaller droplet size, compared to their counterparts.

The characteristics of the hydrolysates, such as the DH, secondary and tertiary structure, flexibility, and surface hydrophobicity play a crucial role in their ability to adsorb and stabilize the oil-water interface. Leni, Soetemans, Caligiani, et al. (2020) reported that the emulsifying activity of lesser mealworm hydrolysates decreased with increasing DH, as smaller peptides may lack the ability to form a strong film at the oil-water interface (Wouters et al., 2016). In addition, unfolded structures can enhance the functional properties of hydrolysates, such as foaming and emulsifying properties (Mintah et al., 2019). Finally, an optimal number of hydrophobic patches is necessary for surface activity and emulsion stabilization (Wouters et al., 2016; Zhu et al., 2017). Thus, the smaller droplet sizes observed in emulsions stabilized with Trypsin hydrolysates could be attributed to their relatively bigger peptide size compared to Alcalase hydrolysates (Section 3.1.1 and 3.1.2). Moreover, US pretreatment before Trypsin hydrolysis resulted in a reduction of the droplet size which could indicate better stability in long term studies. The enhanced emulsifying properties of US_Try hydrolysates could be linked to their highest surface hydrophobicity. Similarly, US pretreatment before Alcalase hydrolysis led to smaller droplet sizes in emulsions compared to both Alc and PEF_Alc, resulting in an improved TSI index at the beginning of storage. This improvement can be attributed to the higher random coil content observed in US_Alc (Section 3.1.3), which may have enhanced their flexibility and ability to adsorb at the interface.

3.2.2. *Oxidative stability of emulsions*

Oils rich in PUFAs, such as fish oil, are prone to undergoing lipid oxidation, which impair the nutritional and sensorial characteristics of the oil and fortified food products (Patel et al., 2022). Lipid oxidation is a free-radical chain reaction. First oxidation products formed are hydroperoxides, which then degrade into volatile or non-volatile secondary oxidation products (Frankel, 2005). Marine fish oil presents natural chain-breaking antioxidants such as tocopherols (McClements, 2015; Yesiltas, Soria Caindec, et al., 2023). The main mechanisms behind their antioxidant activity include the donation of hydrogen to lipid radicals, thus preventing the formation of hydroperoxides, and the stabilization of the peroxides already formed, inhibiting the production of secondary products of oxidation (Kamal-Eldin & Budilarto, 2015). In this study, the oxidative stability of the fish O/W emulsions was studied during 9 days of storage based on the formation of primary oxidation products (peroxide value, PV), the consumption of tocopherols initially present in the oil and the formation of volatile secondary oxidation products.

3.2.2.1. Primary oxidation products – peroxide value. The formation of primary oxidation products was analyzed based on the formation of lipid hydroperoxides estimated by the PV during 9 days of storage (Fig. 4A). Initially, all the emulsions presented similar values (around 3 to 6 mEq O_2/kg oil) significantly higher than the original PV of the oil before emulsification (0.51 ± 0.04 mEq O_2/kg oil). This is in accordance with Queiroz et al. (2021) and Yesiltas, García-Moreno, et al. (2023) who also observed higher PV after production of emulsions using a microfluidizer. Results indicate that the oxidation is already happening during the emulsification process, probably due to high shear stress and incorporation of air in the system (Yesiltas, Soria Caindec, et al., 2023). During storage, the PV of the emulsions stabilized with insect proteins increased steadily, without lag phase, and faster than for the emulsions stabilized with NaCas. At day 9, the emulsions stabilized with DM and US_Try presented the highest PV (85.1 ± 1.6 mEq O_2/kg oil, and 84.6 ± 2.8 mEq O_2/kg oil, respectively). PEF_Alc provided the highest protection against the formation of hydroperoxides of all the insect proteins with a PV reaching 61.6 ± 0.4 mEq O_2/kg oil at day 9. Nevertheless, this value remained substantially higher than the PV obtained for NaCas-stabilized

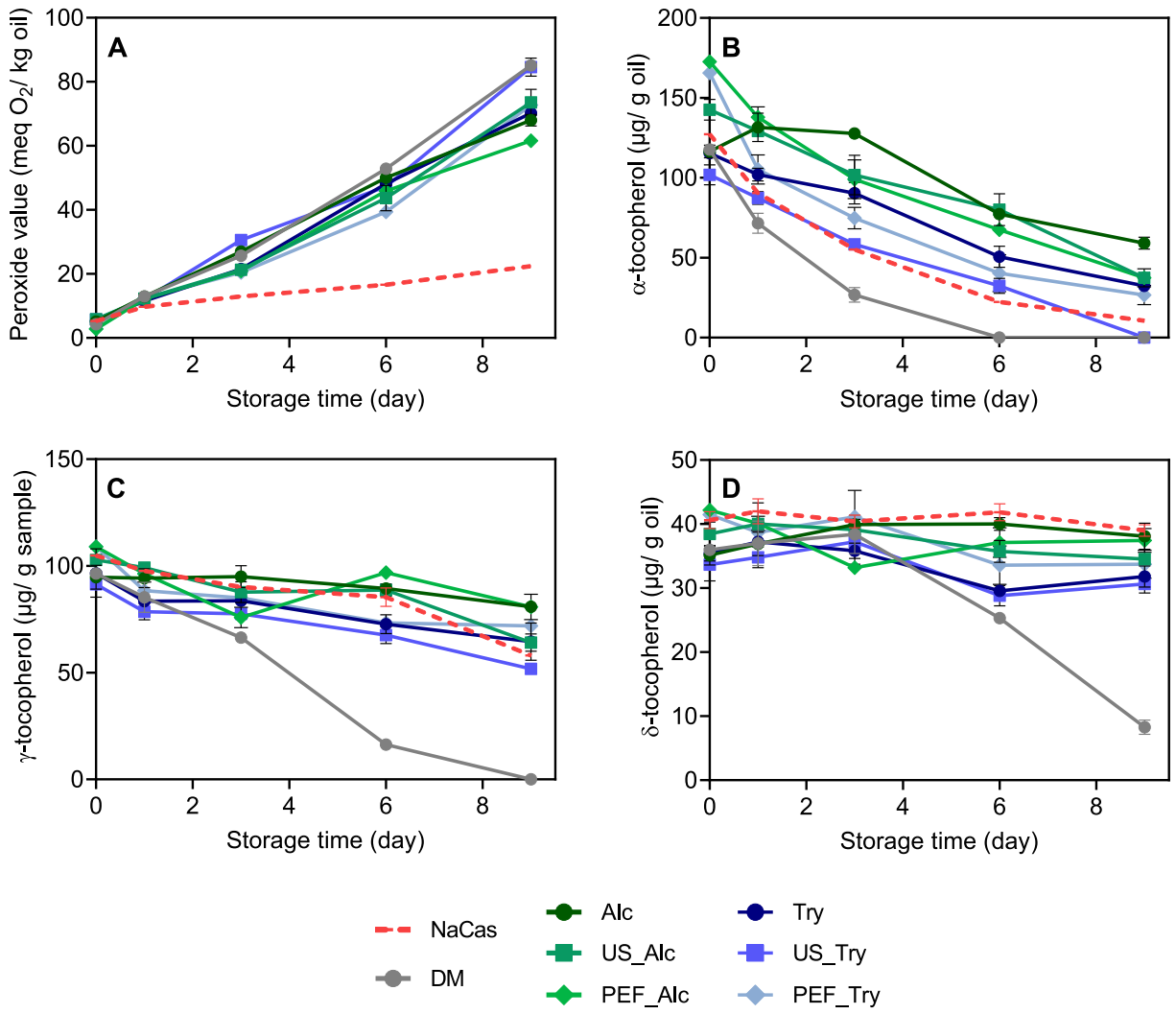

Fig. 4. A) Formation of hydroperoxides and consumption of B) α-tocopherols, C) γ-tocopherols, and D) δ-tocopherols in fish oil-in-water emulsions stabilized with NaCas, DM or lesser mealworm protein hydrolysates during 9 days of storage at room temperature in presence of $FeSO_4$.

emulsions on the last day of storage (22.4 ± 0.3 mEq O_2/kg oil). Lipid oxidation in O/W emulsions stabilized with proteins depends on several factors, including the interfacial area, the thickness and viscosity of the interfacial layer, the quantity and type of free proteins in the aqueous phase, the surface charge of the droplets, and the properties of the proteins (Frankel, 2005; Gumus et al., 2017). All emulsions presented negative charged droplets, so they are prone to oxidation as prooxidant ions are attracted to the droplets. Compared to NaCas, the insect protein hydrolysates might have lower metal chelating and radical scavenging properties, which could result in limited ability to inhibit lipid hydroperoxide formation. Results are in accordance with a previous study in which black soldier fly proteins were used to stabilized fish O/W emulsions (Queiroz et al., 2021). After 10 days of storage in presence of $FeSO_4$, the PV of the emulsions reached 41 to 81 mEq O_2/kg oil, which was significantly higher than the NaCas-stabilized emulsions (27 mEq O_2/kg oil).

3.2.2.2. Tocopherol consumption. Fig. 4B-D show the evolution of α-, γ-, and δ-tocopherol during the 9-day storage. Initial tocopherols contents were in the range of 101.8–172.6 µg/g oil for α-tocopherol, 91.4–109.0 µg/g oil for γ-tocopherol, and 33.7–42.1 µg/g oil for δ-tocopherol. From day 0, the tocopherol contents differed between each emulsion, with lower levels than originally found in the fish oil, indicating that they were consumed when donating hydrogen to scavenge free radicals. This is consistent with the PV results showing lipid oxidation happening during the emulsification process. The α-tocopherol content in all emulsions significantly decreased during storage, with consumption ranging from 50 to 100 % (Table S1, Supplementary material). α-tocopherols were entirely consumed between day 3 and day 6 for DM and between day 6 and day 9 for US_Try, which correlate well with the PV results, where DM and US_Try stabilized emulsions presented the highest oxidation. α-tocopherols contributed the most to preventing lipid oxidation compared to δ- and γ-tocopherols. The consumption of these tocopherols did not exceed 45 % during storage, apart from the DM-stabilized emulsions where the consumption of δ-tocopherols and γ-tocopherols reached 100 and 77 %, respectively, at the end of the storage. Overall, the emulsions stabilized with Alcalase hydrolysates presented lower consumption of α-tocopherols throughout storage than the ones stabilized with Trypsin hydrolysates (50–78 % consumption compared to 72–100 %). Finally, the emulsions stabilized with the pretreated hydrolysates presented higher consumption of α- and δ-tocopherols than the untreated ones. The results indicate that Alcalase hydrolysate might have higher radical scavenging activity than Trypsin hydrolysate, which may explain the lower PV and lower consumption of tocopherols during storage (Hennebelle et al., 2024). The pretreatment prior to hydrolysis altered the structure and hydrophobicity of the hydrolysates (Section 3.1.3, 3.1.4, and 3.1.5) which could potentially impact their radical scavenging activity and consequently, their ability

to protect lipid against oxidation.

3.2.2.3. Volatile secondary oxidation products. The oxidative stability of the emulsions was studied at room temperature in the presence of $FeSO_4$ which facilitate the decomposition of hydroperoxides into volatile secondary oxidation products (Frankel, 2005). A total of 16 volatiles were followed during storage, chosen based on their formation during storage and origin. Out of the volatiles measured during the 9-day storage period, 4 of them (1-penten-3-ol, 2,3-pentanedione, hexanal, and 2,4-heptadienal), selected based on their abundance, are presented in Fig. 5**.** The other volatiles measured are shown in the Supplementary materials (Fig. S2 and Fig. S3). Both (*E*-E)-2,4-heptadienal and 1-penten-3-ol are derived from omega-3 fatty acids, while hexanal comes from the oxidation of omega-6 fatty acids. 1-Penten-3-ol, (E,E)-2,4-heptadienal, and hexanal presented a similar trend: volatile formation had a 3-day lag phase, after which it increased significantly, especially between days 6 and 9. In contrast, contents of 2,3-pentanedione increased without a lag-phase.

Overall, the emulsions stabilized with NaCas presented the highest oxidative stability during storage which is in accordance with PV results. NaCas-stabilized emulsions had the lowest volatile contents (apart from (Z)-4-heptenal, (E,E)-2,4-heptadienal, and 3,5-octadien-2-one) (Fig. S2 and Fig. S3, Supplementary material, and Fig. 5), and 3-methylbutanal was not detected throughout storage (Fig. S2B, Supplementary material). NaCas is a protein known for its ability to slow down or inhibit lipid oxidation, which can be attributed to its metal chelating activity (Berton-Carabin et al., 2014; Hennebelle et al., 2024). However, no clear conclusion could be drawn about which lesser mealworm protein hydrolysate has the lowest or the highest protection effect against lipid oxidation because different patterns were observed for the different volatiles.

3.2.2.4. Principal component analysis (PCA). As no clear trend was observed on the impact of the emulsifier on the lipid oxidation, principal component analysis (PCA) was performed on the volatile data, quantified with the calibration curve, to determine the similarities in volatile profiles at the end of the storage period. In particular, we evaluated the impact of the pretreatment, and the enzyme used on the efficacy of the hydrolysates to maintain the oxidative stability of the emulsion. The PCA was performed using the volatile data obtained at the end of the storage. The first principal component (PC1) explained 61.3 % of the differences and the second principal component (PC2) accounted for 15.1 % (Fig. 6). The volatiles having the most impact on PC1 were butanal, 2-ethyfuran, pentanal, 1-penten-3-ol, hexanal, 4-heptenal, 1-octen-3-ol, octanal, and 2,4-decadienal on the positive side. While 1-penten-3-one, 2,3-pentanedione, and benzaldehyde were the major components contributing to PC2. NaCas was located in the lower left quadrant indicating low level of lipid oxidation. This is in agreement with the conclusions drawn from the analysis of the 4 volatiles. DM and Alc were located in the lower right quadrant indicating that they

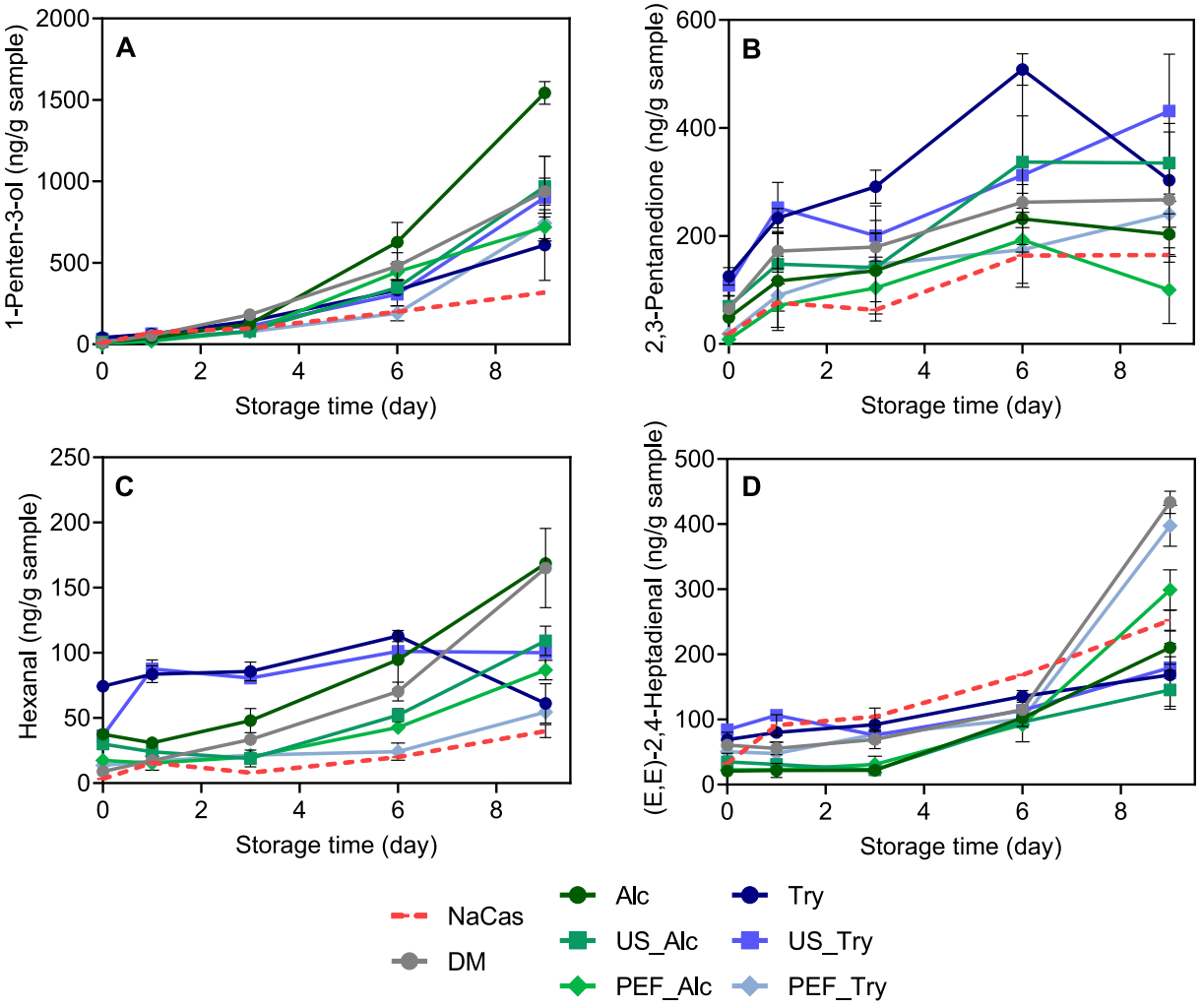

Fig. 5. Development of volatile secondary oxidation products in emulsions during 9-day storage at room temperature in presence of $FeSO_4$: A) 1-penten-3-ol, B) 2,3-pentanedione, C) hexanal, and D) (E,E)-2,4-heptadienal.

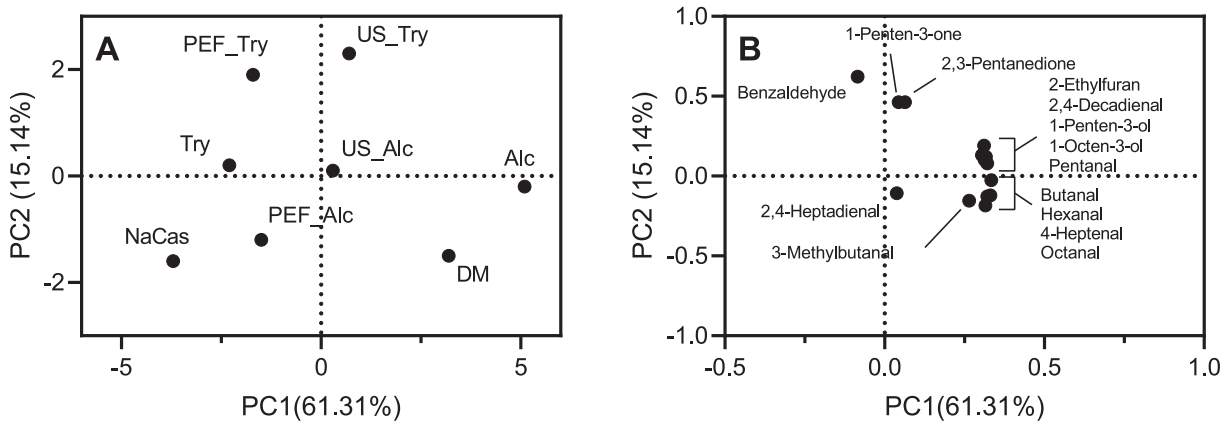

Fig. 6. A) PCA scores and B) PCA loadings of means of volatile secondary oxidation products measured in the emulsions stabilized with NaCas, DM and lesser mealworm hydrolysates.

presented the highest content of the volatiles contributing to the primary component. PC1 allowed separation of the hydrolysates in function of the pretreatment, US_Try and US_Alc, and PEF_Try and PEF_Alc both have similar score on PC1. From the PCA we can observe that the pretreatments performed before the enzymatic hydrolysis impacted the antioxidant activity of the hydrolysates as the volatile secondary oxidation product contents were different from the untreated hydrolysates. Emulsions stabilized with Trypsin hydrolysates exhibited similar or lower levels of volatile secondary products of oxidation, despite having higher PV (Section 3.2.2.1). While an increase in PV is an indicator of lipid oxidation, it may also be due to the metal-chelating activity of proteins, inhibiting the decomposition of peroxides (Frankel, 2005). Thus, Trypsin hydrolysates might have higher metal-chelating activity than Alcalase hydrolysates.

Among the Trypsin hydrolysates, Try performed the best followed by PEF_Try. Try-stabilized emulsions have higher droplet size than US_Try and PEF_Try (Section 3.2.1), meaning that a smaller interface area is available for the lipid oxidation to take place (Berton-Carabin et al., 2014). But differences in structures and hydrophobicity between Try and its counterparts could explain the differences in formation of volatile secondary products of oxidation observed. In Section 3.1.4, Try was found to have a more unfolded structure than US_Try and PEF_Try which could affect adsorption of the protein at the oil-water interface, thus changing the interface barrier properties or exposing different groups having higher antioxidant activity.

The US and PEF pretreatment before enzymatic hydrolysis with Alcalase was beneficial for the oxidative stability of the emulsions. US_Alc was located close to the center point and PEF_Alc, in the lower left quadrant indicating that higher protection against lipid oxidation was provided compared to Alc, located on the far-right side. The US_Alc emulsions had smaller droplet size compared to Alc emulsions, resulting in a larger interface area. Thus, the enhanced oxidative stability provided by US_Alc hydrolysates, which are more flexible due to the higher content of random coil, could be due to the formation of a stronger barrier at the oil-water interface (Gkinali et al., 2023). In addition, PEF_Alc exhibited lower degree of hydrolysis than Alc, the presence of larger peptides could help forming a thicker interfacial layer around the droplets, potentially providing additional protection against lipid oxidation (Leni, Soetemans, Caligiani, et al., 2020).

4. Conclusion

In this study, the impact of US and PEF pretreatment on the enzymatic hydrolysis of lesser mealworm protein and the techno-functionality of the hydrolysates in O/W system was investigated. The protein yield of the hydrolysis did not significantly change with the use of pretreatment. However, the degree of hydrolysis, and secondary and tertiary structures of the hydrolysate were impacted by the enzyme and pretreatment used. Emulsions stabilized with ultrasound pretreated Trypsin hydrolysates were the most physically stable, showing less creaming, no apparition of clear aqueous water phase and low droplet size. Yet, they showed lower physical and oxidative stability compared to NaCas-stabilized emulsions, the protein chosen as reference. These results showed that lesser mealworm hydrolysates, especially the ones obtained from insect meal pretreated with ultrasound and hydrolyzed with Trypsin, have potential to be used as emulsifiers in fish O/W emulsions. Nevertheless, additional antioxidants are needed to effectively prevent oil against oxidation. This is the first study reporting the use of PEF pretreatment before enzymatic hydrolysis of insect meal. Research is needed to further investigate and optimize the treatment to improve the functionality of insect protein hydrolysates.

CRediT authorship contribution statement

Aurélie Ballon: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Lucas Sales Queiroz:** Methodology, Conceptualization. **Sílvia de Lamo-Castellví:** Resources, Methodology. **Carme Güell:** Supervision, Funding acquisition, Conceptualization. **Montse Ferrando:** Supervision, Funding acquisition, Conceptualization. **Charlotte Jacobsen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Betül Yesiltas:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143339.

Data availability

Data will be made available on request.

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