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Conjugation of lesser mealworm (*Alphitobius diaperinus*) larvae protein with polyphenols for the development of innovative antioxidant emulsifiers

Aurélie Ballon [a], Maria-Paz Romero [b], Luis E. Rodriguez-Saona [c], Sílvia de Lamo-Castellví [a], Carme Güell [a], Montserrat Ferrando [a,*]

[a] *Department d'Enginyeria Química, Escola Tècnica Superior d'Enginyeria Química, Universitat Rovira i Virgili, Avda. Països Catalans, 26, 43007 Tarragona, Spain*
[b] *Food Technology Department, Escuela Técnica Superior de Ingeniería Agraria, Universidad de Lleida, Avda. Alcalde Rovira Roure, 191, 25198 Lleida, Spain*
[c] *Department of Food Science and Technology, The Ohio State University, 110 Parker Food Science and Technology Building, 2015 Fyffe Road, Columbus, OH 43210, United States*

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ABSTRACT

Lesser mealworm protein concentrate (LMPC) was conjugated with chlorogenic acid (CA) or tannic acid (TA) using an alkaline method. The impact of polyphenol type and concentration on the physicochemical and structural characteristics, antioxidant, interfacial, and emulsifying properties of the LMPC-polyphenol conjugates were investigated. Under the conditions tested, TA demonstrated higher affinity for LMPC compared to CA. The conjugation of LMPC induced conformational changes as showed by intrinsic fluorescence and FT-MIR raw spectra analysis. The surface hydrophobicity of the conjugates was reduced, leading to increased interfacial tension values for LMPC-TA conjugates without impairment of the emulsifying activity. The antioxidant properties were significantly improved by the conjugation. Flaxseed oil-in-water (O/W) emulsions stabilized by the conjugates and LMPC remained physically stable for 12 days at 50 °C with a notable reduction of secondary oxidation products when conjugates were used. LMPC-TA and LMPC-CA exhibited potential to be used as novel antioxidant emulsifiers in O/W emulsions.

1. Introduction

The consumption of edible insects is gaining increasing attention as an alternative to conventional proteins due to the growing food demand. Edible insects are a good source of high-quality proteins, vitamins, and minerals, as well as unsaturated fats. In addition, insect farming has environmental advantages compared to conventional livestock production (van Huis & Oonincx, 2017). As of 2023, four insects have been approved by the European Food Safety Authority (EFSA) as novel foods to be consumed as snacks or incorporated as food ingredients. The approved insects include mealworm (*Tenebrio molitor* larva), migratory locust (*Locusta migratoria*), house cricket (*Acheta domesticus*), and lesser mealworm (*Alphitobius diaperinus* larva) (EU Regulation, 2017/2470, 2017). Although insects present health and environmental benefits, their utilization as sustainable protein sources in western countries is limited by consumers' aversion to visible whole insects in food products (Kröger et al., 2022). Therefore, current research is focused on processing whole insects into insect meal and insect-derived ingredients such as protein extracts, chitin, and fats.

Insects generally have a high true protein content ranging from 37 to 55 % which can vary based on factors such as the insect species, life stage, and diet (Boulos et al., 2020; Janssen et al., 2017). Several studies presented protein extraction methods tailored for insects, aiming to obtain protein isolate or concentrate. These methods include alkaline solubilization followed by isoelectric point precipitation, ultrasound-assisted extraction, and enzymatic extraction (Pan et al., 2022). Lesser mealworm (LM) contain approximately 49 % protein and 13–29 % fat (Janssen et al., 2017; Skotnicka et al., 2021). While there is not as much research conducted on LM compared to mealworm or cricket, studies assessing LM have shown the potential of lesser mealworm extracted proteins to replace conventional dairy proteins in food emulsions (Jayakumar et al., 2023; Wang et al., 2021a). Regarding the ability of insect proteins to maintain the oxidative stability of oil-in-water (O/W) emulsions compared to conventional proteins, only black soldier fly proteins have been investigated thus far (Queiroz et al., 2021). In comparison to conventional proteins, emulsions stabilized with black soldier fly proteins presented higher oxidation levels than those stabilized with sodium caseinate.

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[*] Corresponding author.

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To incorporate polyunsaturated fatty acids (PUFAs) as O/W emulsions has become a major trend to fortify liquid foods. This is a strategy to mitigate the negative effects on human health of not meeting the recommended daily intake for omega-3 fatty acids often observed in western societies (Patel et al., 2022). Nevertheless, including PUFAs as O/W emulsions is technologically challenging due to the high susceptibility of omega-3 PUFAs-rich oils to lipid oxidation, which is exacerbated by the large oil–water interface typical of emulsions. To overcome this challenge, recent research has focused on developing ingredients from proteins with dual functionality, mainly emulsifying and antioxidant (Sun et al., 2020b). The interaction between proteins and polyphenols has been explored as an approach to enhance the antioxidant capacity of proteins while maintaining their properties as emulsifiers. This approach aligns with consumer preferences for clean-label food products.

Polyphenols are potent antioxidants that can interact with proteins through non-covalent or covalent bonding. Covalent conjugation between proteins and polyphenols typically leads to stronger interactions and increased resistance to environmental stresses (Liu et al., 2023; Sui et al., 2018). Various methods can be used to produce protein-polyphenols conjugates including free-radical, alkaline, and enzymatic approaches. Among them, the alkaline method, performed at a pH of 9 in the presence of oxygen, is commonly employed due to its simplicity (Liu et al., 2019). Conjugating proteins with polyphenols has been shown to alter the structure and improve functional properties (e.g., antioxidant and emulsifying properties) of proteins derived from various sources, including animal proteins such as gelatin, myofibrillar proteins, collagen and whey protein, and plant proteins such as rice-protein hydrolysates, lentils, and zein (Liu et al., 2019; Yan et al., 2023). Several types of polyphenols have been successfully conjugated to proteins, including tannic acid (TA) - a hydrolysable tannin with antioxidant and antidiabetic properties (Lou et al., 2018) - and chlorogenic acid (CA) - a phenolic acid with antioxidant, hypotensive, and anti-inflammatory activities (Hwang et al., 2014; Liang & Kitts, 2015). The covalent interaction of both TA and CA with proteins has resulted in emulsifiers with enhanced antioxidant activity capable of providing emulsions with improved physical and oxidative stability (Pan et al., 2019; Zhao et al., 2021). To the best of our knowledge, there is no data available to date about insect protein–polyphenol conjugates and their use to stabilize O/W emulsions.

The objective of the present study was to produce antioxidant emulsifiers by conjugating lesser mealworm protein concentrate (LMPC), a protein obtained from a novel food source, with two different polyphenols (CA and TA) using the alkaline method with the aim to improve the oxidative stability of flaxseed O/W emulsions. We evaluated the impact of polyphenol type and concentration on the antioxidant, interfacial, emulsifying, and structural properties of the resulting conjugates. Additionally, the stability of flaxseed O/W emulsions stabilized by the LMPC-polyphenol conjugates was monitored over time and compared to emulsions stabilized with non-conjugated LMPC and a conventional dairy protein, WPI. This research provides valuable insights into the development of insect protein-based antioxidant emulsifiers for potential use in food applications.

2. Materials and methods

2.1. Materials

Edible lesser mealworm (*Alphitobius diaperinus*) larvae powder with a true protein content of 45 % (Kreca Ento-Food BV, Wageningen, The Netherlands) was used to produce LMPC. Whey protein isolate (WPI), with a reported protein content of 97.8 % on dry basis, was kindly provided by Davisco Foods International (Le Sueur, MN, USA). The detailed proximate composition of the lesser mealworm larvae powder and WPI is provided in Table S1. Sunflower oil (Borges, Tarragona, Spain) and flaxseed oil (Natursoy, Barcelona, Spain) were bought from local supermarkets. Hydrochloric acid (37 %, HCl), sodium hydroxide (NaOH), ethanol (96 %), sodium phosphate dibasic (Na_2HPO_4), sodium carbonate (Na_2CO_3), methanol, acetic acid, and 2-methyl tetrahydrofuran were purchased from Scharlab (Barcelona, Spain). 2-thiobarbituric acid (≥98 %, TBA), 1,1,3,3-tetraethoxypropane (≥96 %, TEP), 8-anilino-1-naphthalenesulfonic acid (≥97 %, ANS), 2,2-diphenyl-1-picrylhydrazyl (≥97 %, DPPH), isooctane, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (97 %, AAPH), fluorescein, sodium azide, and tannic acid were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Trichloroacetic acid (≥99 %, TCA), Trolox (97 %), Pierce^{TM} BCA protein assay kit, sodium phosphate monobasic (NaH_2PO4), and dialysis bags (MWCO 3500 Da) were supplied by Thermo-Fisher Scientific (Waltham, MA, USA). Folin-Ciocalteau's reagent and propan-2-ol were purchased from Chem-Lab NV (Zedelgem, Belgium). Chlorogenic acid was purchased from Biosynth (Staad, Switzerland). All reagents were analytical grade and deionized water was used for the preparation of the samples and reagents.

Glass microbeads used for microporous emulsification were purchased from Corpuscular LDT (Young, Canada) and had a diameter of 94 μm. The microbeads were supported by a nickel sieve having a pore size of 288 × 13 μm (length × width) with a thickness of 120 μm (Stork Verco, Erbeek, the Netherlands).

2.2. Defatting and extraction of protein

LMPC was obtained following the method described in previous work (Wang et al., 2021a) with some modifications. First, to prepare defatted LM powder, 50 g of LM powder was mixed with 250 mL of 2-methyltetrahydrofuran, a bio-based solvent, at 600 rpm for 1 h at room temperature. Subsequently, the mixture was left to decant until achieving complete phase separation. Following this, the solvent phase containing lipids was carefully removed. This process was repeated 3 times in total to ensure that all lipids were removed from the powder. Then, the sample was placed under a fume hood overnight to evaporate residual solvent. The protein extraction was performed by alkaline solubilization followed by acid precipitation at their isoelectric point. To do so, LM defatted powder was mixed with 0.25 M NaOH (ratio 1:5, w/v) and stirred at 400 rpm at 40 °C for 1 h. Afterward, the mixture was centrifuged (3358 × *g*, 15 min) and the supernatant was collected. The remaining pellet was used to repeat the process, which was performed a total of 3 times. The proteins from the supernatant were precipitated by adjusting the pH to 4.0–4.5 and the mixture was centrifuged (2343 × *g*, 15 min). The precipitates were freeze-dried (LYOQUEST-85 PLUS, Telstar, Barcelona, Spain), ground, and stored in plastic bags at 4 °C in a desiccator. The LMPC obtained had a true protein content of 73.4 %, determined as previously described by Wang et al. (2021b). Details about the determination of the true protein content are presented in supplementary file. All the proteins used in the present study were from the same batch.

2.3. Preparation of LMPC-polyphenol conjugates

The LMPC-polyphenol conjugates were prepared according to the method of Liu et al. (2016) with slight modifications. First, LMPC was dispersed in distilled water (3.2 wt %) and stirred at 400 rpm for 2 h. The pH of the mixture was adjusted to 9.0 every 30 min using 4 M NaOH. The mixture was then kept at 4 °C overnight to ensure complete hydration of proteins. Afterwards, the protein dispersion was centrifuged twice (2863 × *g*, 15 min, 25 °C) and the protein content in the supernatant was quantified by BCA analysis (Smith et al., 1985). The protein content was expressed in bovine serum albumin (BSA) equivalent value. TA and CA were dissolved in distilled water and the pH of the solution was adjusted to 9.0. TA or CA solution was then added to the protein solution while stirring, to reach a polyphenol content of 50, 100, and 150 μmol of polyphenol/g protein. The choice of the range of concentrations (50–150 μmol/g protein) was based on unpublished experiments, in which the appearance of visible precipitate was observed with higher concentration of TA. The final weight was adjusted with distilled water to obtain a final protein content of 1 %, and the pH of the solution was adjusted to 9.0, if necessary. Sodium azide (0.02 %) was added to prevent microbial growth. The solutions were stirred for 24 h at room temperature in the presence of oxygen. Unreacted polyphenols were removed through dialysis (MWCO 3500 Da) against distilled water for 44–48 h at 4 °C. Finally, the conjugates were freeze-dried and the samples were stored at 4 °C in a desiccator until further use. The resulting conjugates were named LMPC-CA50, LMPC-CA100, LMPC-CA150, LMPC-TA50, LMPC-TA100 and LMPC-TA150. A control was prepared under the same conditions without addition of polyphenol and will later be referred as LMPC-control.

2.4. Characterization of the conjugates

2.4.1. Determination of total polyphenol content

The polyphenol content was determined by the Folin–Ciocalteu colorimetric method, following the procedure of Wang et al. (2021a) with slight modification. 100 μL of sample (2 mg/mL) was mixed with 100 μL of Folin reagent, 2 mL of Na_2CO_3 solution (75 g/L), and 2.8 mL of distilled water. Mixtures were kept in the dark for 30 min and the absorbance was measured at 750 nm by a UV–VIS spectrophotometer (Hach Lange DR5000, Hach Lange SLU, Barcelona, Spain). The absorbance obtained for LMPC-control was used as a blank. Three measurements were performed for each sample. Calibration curves of CA (0–50 mg/L) and TA (0–400 mg/L) were used to determine the polyphenol content of the samples expressed in mg polyphenol/g of sample.

2.4.2. ζ-potential determination

The ζ-potential of LMPC-control and the conjugates was measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) at 25 °C. Samples were dissolved in distilled water (1.5 mg/mL of powder). The ζ-potential was calculated using the Smoluchowski model. Measurements were performed in triplicate.

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

Changes in secondary structures of proteins induced by the conjugation were measured by FT-MIR spectroscopy. Mid-infrared spectra were collected using an Agilent 4500 portable unit equipped with triple-reflection diamond ATR crystal and thermoelectrically-cooled DTGS detector (Agilent Technologies Inc., Danbury, CT, USA). The diamond crystal ATR system had a 2 mm diameter sampling surface with a 200 μm active area. The spectral resolution was 4 cm^{-1} and 64 spectra were co-added to improve the signal to noise ratio over the 4000–700 cm^{-1} range. The absorbance spectrum was obtained by ratioing the sample spectrum against background spectrum. The collected spectra were recorded using Agilent MicroLab PC software (Agilent Technologies Inc., Danbury, CT, USA). Spectra from samples were collected by placing 0.1 g of the sample onto the ATR crystal and using the pressure clamp. The spectra acquisition was repeated a minimum of 3 times under the same conditions. The spectra were normalized and smoothed out using a 13-point calculation window (Pirouette version 4.5, Infometrix Inc., Bothell, WA, USA). The second derivative was calculated using a Savitsky-Golay function (7 points) on OriginPro 2023 (Origin Lab, MA, 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 cm^{-1}-1600 cm^{-1}) following the procedure of Yang et al. (2015).

2.4.4. Intrinsic fluorescence spectroscopy

The intrinsic fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA, USA) according to the method described by Sun et al. (2020a) with modifications. Aqueous solutions of LMPC-control and LMPC-polyphenol conjugates (1 mg/mL) were prepared in 10 mM phosphate buffer at pH 7.0 and transferred to quartz cells. The exciting wavelength was set to 280 nm to target tryptophan and the emission spectra were measured in the range 260–500 nm. The exciting and emission slit widths were set to 5 nm. Samples were measured in triplicate.

2.4.5. Surface hydrophobicity

Surface hydrophobicity (H_0) was determined using the method of Mishyna et al. (2019) with slight modifications, using ANS as fluorescence probe. The conjugates and LMPC-control were dissolved in phosphate buffer (10 mM, pH 7.0) to a final concentration of 1 mg/mL, stirred for 2 h, and stored overnight at 4 °C. This stock solution was diluted to prepare sample solutions of 0.005, 0.025, 0.05, and 0.1 mg/mL. Then 20 μL of ANS solution (8 mM) was added to 4 mL of each sample solution, the mixture was vortexed and kept in the dark for 15 min. The fluorescence intensity was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 390 and 470 nm, respectively. The excitation and emission slit widths were set to 5 nm. The fluorescence intensity obtained for phosphate buffer was subtracted from all values. The protein surface hydrophobicity was determined as the slope of the linear regression of the fluorescence intensity against the protein concentration (mg/mL). All the samples were prepared in triplicate.

2.4.6. Interfacial tension

The interfacial tension between aqueous solution of LMPC-control and LMPC-polyphenol conjugates (1 mg/mL) prepared in phosphate buffer (10 mM, pH 7.0) and sunflower oil was determined using a force tensiometer (Sigma 702, Biolin Scientific, Espoo, Finland) by Du Noüy method at 25 °C. The aqueous phase was poured into the glass container and then the Du Noüy ring was lowered into the solution. Sunflower oil was carefully pipetted on the wall of the container to avoid mixing the two phases. The measurements were repeated three times for each sample. The ring was cleaned with distilled water and ethanol and flamed between samples.

2.4.7. Emulsifying activity

The emulsifying activity (EA) of LMPC and its conjugates was measured according to a previously described method (Wang et al., 2021b). Samples (10 mg/mL) were dissolved in phosphate buffer (pH 7, 0.2 M) and stirred for 2 h at room temperature at 150 rpm. The solutions were then put in the fridge overnight. The day after, 4 mL of the protein solution were homogenized with 4 mL of sunflower oil at 11 000 rpm for 30 s (Ultra Turrax T18 digital, IKA, Staufen, Germany). An aliquot (6 mL) was transferred into a centrifuge tube and centrifuged (3250 × g, 20 min). Duplicates were prepared for each sample. The EA was calculated as the volume of the emulsified layer after centrifugation divided by the total volume of the emulsion before centrifugation (see equation (1)).

$$EA(\%) = \frac{V_{EL}}{V_T} \bullet 100 \tag{1}$$

Where V_{EL} is the volume of the emulsified layer after centrifugation (mL) and V_T is the total volume of the emulsion (mL).

2.4.8. Antioxidant capacity

2.4.8.1. DPPH assay. The DPPH radical scavenging activity was measured according to a method previously reported with modifications (Xu et al., 2019). In brief, the samples were dissolved in deionized water and diluted to different concentrations. Seven points of concentration were used to build the inhibition curve. Concentrations were chosen to obtain a range of scavenging activity including 50 %. Freshly prepared DPPH solution (0.175 mM) prepared with methanol was mixed in equal proportion with the diluted sample. The samples were prepared in triplicate, vortexed and stored in the dark for 1 h at room temperature. The absorbance was measured at 517 nm. The DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity } (\%) = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \bullet 100 \tag{2}$$

Where A_{sample} is the absorbance of the sample, A_{blank} is the absorbance of the blank (diluted sample + methanol) and $A_{control}$ is the absorbance of the control (DPPH solution + deionized water).

A calibration curve prepared with Trolox (15–90 μM) was used to express the DPPH scavenging activity in μmol Trolox equivalent (TE)/g sample. The Trolox equivalent antioxidant capacity (TEAC) was calculated using equation (3) (Xiao et al., 2020).

$$\text{TEAC } (\mu molTE/gsample) = \frac{EC50_{Trolox}}{EC50_{sample} \bullet M_{Trolox}} \bullet 10^6 \tag{3}$$

Where $EC50_{Trolox}$ and $EC50_{sample}$ are the concentration of Trolox and sample, respectively, that scavenges 50 % of DPPH radicals (mg/mL), and M_{Trolox} is the molecular weight of Trolox (g/mol).

2.4.8.2. ORAC assay. The oxygen radical absorbance capacity (ORAC) assay was performed according to Huang et al. (2002) with some modifications. The assay was done in phosphate buffer (75 mM, pH 7.4) at 37 °C. 25 μL of sample or Trolox and 150 μL of fluorescein (68 nM) were added to a 96-well black microplate. After 5 min of incubation, 25 μL of AAPH (122 mM) was added. The decay in fluorescence was measured at 520 nm with excitation at 485 nm in a FLUO star OPTIMA fluorescence reader (BMG Labtech, Offenburg, Germany). The area under the curve was calculated and compared with a standard curve of Trolox (5–60 μM). Phosphate buffer (75 mM, pH 7.4) was used as blank. The ORAC activity was expressed as μmol TE/g of sample.

2.5. Characterization of the physical and oxidative stability of emulsions

2.5.1. Emulsion preparation and storage

Flaxseed O/W emulsions were prepared using LMPC-control, LMPC-CA150, LMPC-TA150 and WPI as emulsifier. Protein solutions were prepared by dissolving 1 g of sample in deionized water (99 g). Sodium azide (0.02 %) was added, and the solutions were stirred for 2 h. The protein solutions were kept in the fridge overnight for complete hydration. For emulsifying, first a coarse emulsion (20 % w/w flaxseed oil) was prepared using a rotor–stator homogenizer (Ultra Turrax T18 digital, IKA, Staufen, Germany) at 15 800 rpm for 3 min with 30 s of pause every 1 min to avoid temperature rising. Then, the coarse emulsion was refined using a dynamic membrane of tunable pore size (DMTS) system with an 8.3 mm layer of glass microbeads placed on top of a nickel sieve (Wang et al., 2021b). The DMTS module was connected to a vessel containing the coarse emulsion which was pressurized with nitrogen (450 kPa) to force the emulsion through the dynamic membrane. For each emulsion formulation, this process was repeated five consecutive times when no further reduction in droplet size was observed. The nickel sieve and glass beads were reused after being cleaned. Emulsions were prepared in duplicates.

The physical and oxidative stability of emulsions was studied under accelerated oxidation conditions, that is at 50 °C for 12 days. An aliquot of each emulsion (7 mL) was placed in 12 mL capped tubes and stored at 50 °C until further analysis. Samples of each tube were taken every 2 or 3 days to measure the droplet size, ζ-potential, and lipid oxidation.

2.5.2. Physical stability of emulsions

2.5.2.1. Droplet size distribution. The droplet size distribution of emulsions was analyzed by laser diffraction using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). Refractive index was set to 1.479 and 1.330 for flaxseed oil and water, respectively.

2.5.2.2. Droplet surface charge. The surface charge of the emulsion droplets was measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) at 25 °C. Emulsions were diluted (1:300) in deionized water. Refractive index was set to 1.479 and 1.330 for flaxseed oil and water, respectively. The ζ-potential was calculated using the Smoluchowski model.

2.5.2.3. Optical microscopy. The morphology of emulsions was visualized using an inverted microscope (Primovert, Carl Zeiss AG, Oberkochen, Germany) under a 40x objective, equipped with a camera (SpeedCam MacroVis EoSens, High Speed Vision GmbH, Ettlingen, Germany).

2.5.3. Oxidative stability

The oxidation of lipids in the emulsion during the storage at 50 °C was studied by measuring the formation of primary oxidation products (conjugated diene hydroperoxides) and secondary oxidation products (2-thiobarbituric acid reactive substances, TBARS).

2.5.3.1. Conjugated dienes hydroperoxides. Conjugated diene (CD) hydroperoxides were measured as previously described with slight modifications (Estévez et al., 2008). Aliquots (200 μL) of emulsion were mixed with 1 mL of isooctane/2-propanol (3:1 v/v) and vortexed for 1 min. After centrifugation (1000 × *g*, 5 min), the upper organic layer was diluted with isooctane and the absorbance was measured at 234 nm. The CD hydroperoxide concentration was calculated by Beer-Lambert law using 25200 $M^{-1}\cdot cm^{-1}$ as the molar extinction coefficient of lipid hydroperoxides and was expressed in mmol hydroperoxide (HP) per kg of oil.

2.5.3.2. TBARS. TBARS were measured according to the method described in Berendsen et al. (2014). Briefly, 250 or 500 μL (final volume adjusted to 500 μL with deionized water) of emulsion was mixed with 1 mL of TBA reagent (0.375 wt % TBA, 15 wt % TCA in 0.25 M HCl). The oil and aqueous phases were separated by centrifugation (1000 × *g*, 5 min). The aqueous phase was boiled in a water bath in capped test tubes for 15 min, cooled down to room temperature, and centrifuged (6250 × *g*, 5 min). The absorbance of the supernatant was measured at 532 nm. TBARS was calculated according to a calibration curve prepared with 1,1,3,3-TEP.

2.6. Statistical analysis

The control LMPC and conjugates were prepared in duplicate for each concentration. The emulsions were prepared in duplicate, and the analysis were performed in triplicate. 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. If the normality assumptions of residuals were not met, a Kruskall-Wallis test was used to determine significant differences.

3. Results and discussion

3.1. Properties of LMPC-polyphenol conjugates

3.1.1. Total polyphenol content

The amount of CA and TA bound to LMPC, characterized using the Folin-Ciocalteu method, are presented in Table 1. The extent of binding of both CA and TA varied with the initial polyphenol concentration and the polyphenol type. The total content of LMPC-polyphenol increased significantly with the initial concentration of TA and CA. This finding is in agreement with Sui et al. (2018) who also reported an increased

Table 1

Polyphenol content, ζ-potential, emulsifying activity, and antioxidant activity of LMPC-control and its conjugates. Different letters within a column indicate significant differences ($p < 0.05$).

Sample	Polyphenol content (μmol/g sample)	ζ-potential (mV)	Emulsifying activity (%)	TEAC (μmol TE/g sample)	ORAC (μmol TE/g sample)
LMPC-control	–	-28.3 ± 2.5^{a}	54.8 ± 1.4^{a}	19.3 ± 1.0^{a}	473.3 ± 33.4^{a}
LMPC-CA50	5.7 ± 3.8^{a}	-31.1 ± 1.6^{b}	54.1 ± 1.8^{a}	45.5 ± 1.3^{b}	$562.6 \pm 60.3^{a,b}$
LMPC-CA100	$18.1 \pm 3.8^{b,c}$	-30.6 ± 1.6^{b}	53.2 ± 0.9^{a}	71.2 ± 1.2^{c}	$573.6 \pm 37.2^{a,b}$
LMPC-CA150	24.1 ± 2.9^{c}	-30.5 ± 1.7^{b}	54.3 ± 2.0^{a}	91.6 ± 4.3^{d}	651.0 ± 58.6^{b}
LMPC-TA50	15.7 ± 1.3^{b}	-31.3 ± 1.9^{b}	54.1 ± 3.1^{a}	319.4 ± 4.7^{e}	700.6 ± 22.8^{b}
LMPC-TA100	31.5 ± 1.0^{d}	-32.0 ± 2.3^{b}	53.2 ± 0.8^{a}	614.1 ± 21.0^{f}	923.3 ± 64.1^{c}
LMPC-TA150	51.8 ± 1.9^{e}	-34.3 ± 1.6^{c}	53.9 ± 2.2^{a}	844.9 ± 50.2^{g}	1380.1 ± 270.0^{d}

amount of polyphenol bound to protein as the initial amount of polyphenols increased, when the conjugates were produced under alkaline conditions. Moreover, at the same initial concentration, the total content of TA in LMPC-TA conjugates was 2 to 3 times higher than CA in LMPC-CA conjugates. It indicates a higher affinity of TA toward bonding covalently with LMPC compared to CA when the conjugation was performed *via* the alkaline method. To explain these differences, we should consider that protein–polyphenol interactions are affected by different parameters, including the intrinsic properties of the protein and the polyphenol type (e.g., molecular weight [Ozdal et al., 2013; Sun et al., 2020b], number and position of hydroxyl groups (Parolia et al., 2022; Sun et al., 2022)). During the alkaline reaction, polyphenols are converted into their corresponding quinones which then react with nucleophile amino acids, such as glutamic acid, lysine and cysteine (Benjakul et al., 2021). In line with this, Chen et al. (2019) suggested that the oxidized TA is able to interact with more nucleophile sites than the oxidized CA what is consistent with the results obtained in our study. The predominant amino acids in LMPC include glutamic acid, aspartic acid, tyrosine, leucine and lysine (see Table S2) which are all nucleophile binding sites, apart from leucine.

3.1.2. *ζ-potential*

The ζ-potential value of protein is related to the surface charge which is determined by the distribution and orientation of the hydrophobic and hydrophilic amino acids towards the bulk aqueous phase. In this context, the hydrophilic amino acids, with a side chain faced toward polar water molecules, contribute the most to the electrical charge around the protein. The effect of polyphenol content and polyphenol type on the ζ-potential of the conjugates is shown in Table 1. All conjugates had a higher absolute value of ζ-potential than LMPC ($p < 0.05$). The zeta potential of LMPC-CA conjugates was slightly reduced to around −31 mV compared to −28 mV for LMPC-control. The ζ-potential of LMPC-TA conjugates further decreased with increasing concentration, reaching −34 mV for LMPC-TA150. The slight increase of negatively charged groups on the protein surface can be attributed to the interactions between negatively charged quinone form of the polyphenols and positively charged amino acids (such as lysine and histidine) happening under alkaline conditions. As it can be seen in the following section, polyphenol-protein conjugation might cause some changes in the protein secondary and tertiary structure that may result in a different spatial distribution of the charged residues (Liu et al., 2023).

3.1.3. *FT-MIR spectroscopy*

FT-MIR spectroscopy has been widely used to identify conformational changes in protein. The FT-MIR spectra obtained for LMPC and the conjugates are presented in Fig. 1A. Three major bands were observed in the LMPC-control and conjugates spectra, a band at around 3272–3270 cm^{-1} (N—H and O—H stretching), the amide I band (1700–1600 cm^{-1}, C═O stretching) and amide II band (1600–1500 cm^{-1}, N—H bending and C—N bending). The reduction of intensity of the amide bands of LMPC-TA100 and LMPC-TA150 and the shift to higher wavelength of the amide I band of LMPC-TA150 might indicate

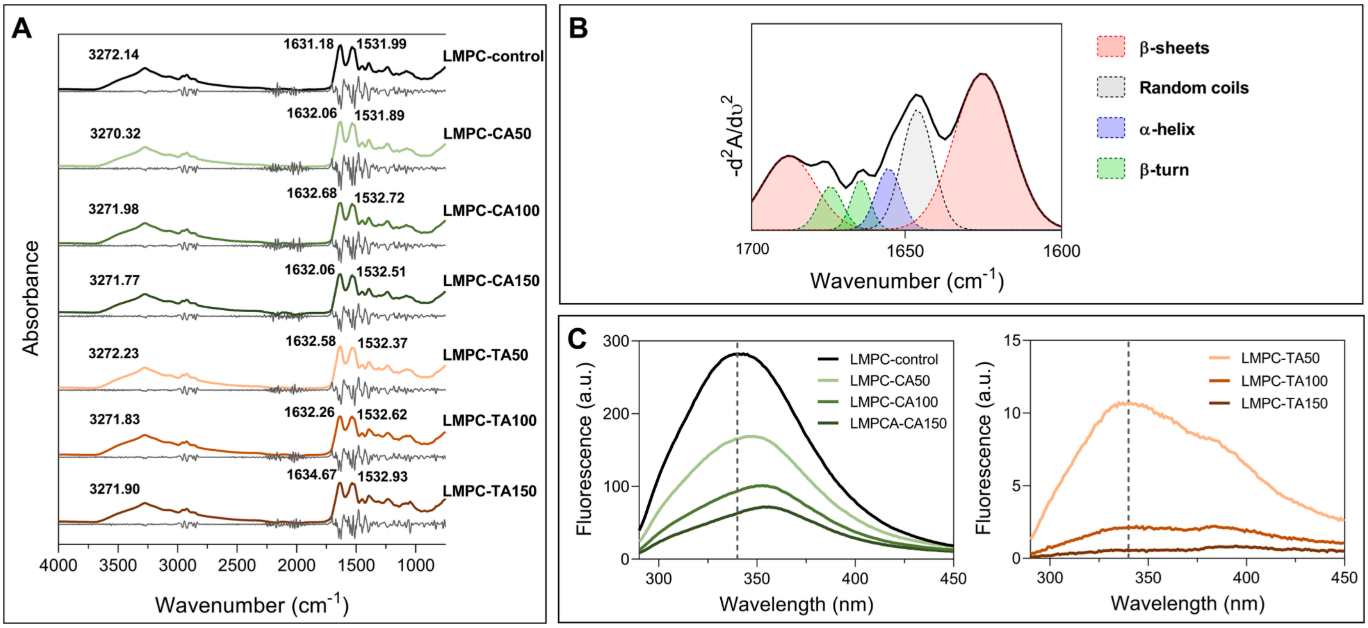

Fig. 1. Second-derivative (grey lines) and FT-MIR spectra (A), example of curve-fitted inverted second-derivative of the amide I region of LMPC-control (B), and intrinsic fluorescence spectra of LMPC and its conjugates (C). The dashed lines indicate the wavelength at which the maximum of fluorescence is obtained for LMPC-control.

changes in the protein structure of the conjugates.

The protein secondary structure can be determined by analyzing the amide I region of the spectra (Yang et al., 2015). The relative percentages of different secondary structures were obtained by correlating wavelength of the bands with secondary structures as follow: β-sheet (1618–1634 cm^{-1} and 1685–1696 cm^{-1}), random coil (1645–1646 cm^{-1}), α-helix (1653–1655 cm^{-1}) and β-turn (1663–1674 cm^{-1}) (Sui et al., 2018). An example of curve-fitted inverted second derivative is presented in Fig. 1B. The content of several types of secondary structures are reported in Table 2. The conjugation of LMPC with CA did not significantly change the secondary structure of the LMPC. However, the conjugation of LMPC to TA induced conformational changes, only for LMPC-TA150, with a decrease in β-sheet content and an increase of random coil content but no significant changes in α-helix and β-turn content. These results are in line with Parolia et al. (2022) who reported that the conjugation of lentil proteins with several polyphenols under alkaline conditions induced the same pattern of conformational changes. Even though lentil proteins are globular, they also found an increase of random coil content and a decrease of β-sheet content while the α-helix and β-turn contents remained unaffected. Some of those changes have been linked to the behavior of the proteins at the interface, for instance, higher content of random coil implies higher flexibility which might facilitate adsorption at the oil–water interface (Chen et al., 2022).

3.1.4. *Intrinsic fluorescence*

Proteins possess three fluorescent amino acids, amongst them tryptophan is the major intrinsic fluorophore and has a high sensitivity to the local environment. Therefore, information about changes in tertiary structure and protein–polyphenol interactions can be obtained by analyzing changes in the emission spectra of tryptophan (Lakowicz, 1999). The effect of the type and concentration of polyphenol used in the LMPC-polyphenol conjugate on the intrinsic emission spectra of LMPC is shown in Fig. 1C. Regardless of the polyphenol, the conjugation induced a progressive reduction of the fluorescence intensity. It may be attributed to the covalent interactions between tryptophan and the polyphenols (Kroll et al., 2003). Besides, the conjugates prepared with TA showed a much lower fluorescence intensity than those containing CA. This might reflect stronger interactions between tryptophan and TA than CA. Alternatively, tryptophan residues could be more exposed to other quenchers after conjugation, such as lysine and histidine, indicating that the conjugation induced conformational changes. Moreover, when compared to LMPC-control, the wavelengths of maximum fluorescence shifted from 341 nm (LMPC-control) to 346, 352, and 356 nm for LMPC-CA50, LMPC-CA100, and LMPC-CA150, respectively. The fluorescence intensity obtained for the LMPC-TA conjugates was too low to be able to determine changes in maximum fluorescence. Red-shifts in emission maxima are usually attributed to the transfer of tryptophan residues to a more hydrophilic environment (Lakowicz, 1999). Therefore, the conjugation of LMPC with polyphenols may have induced structure changes. Similar findings, a significant decrease in fluorescence associated with a red-shift, have been observed previously with different type of proteins and polyphenols (Sun et al., 2020b; Chen et al., 2022).

Table 2
Content of secondary structures in LMPC-control, LMPC-CA and LMPC-TA conjugates. Different letters within a column indicate significant differences ($p < 0.05$).

Sample	Content of secondary structures (%)			
	β-sheet	Random coil	α-helix	β-turn
LMPC-control	62.83 ± 0.94^{a}	17.10 ± 2.06^{a}	9.36 ± 1.81^{a}	10.71 ± 0.90^{a}
LMPC-CA50	63.37 ± 1.33^{a}	$17.84 \pm 1.85^{a,b}$	8.81 ± 2.03^{a}	9.98 ± 0.78^{a}
LMPC-CA100	$62.11 \pm 1.18^{a,b}$	$17.64 \pm 1.90^{a,b}$	9.25 ± 1.42^{a}	11.00 ± 0.52^{a}
LMPC-CA150	62.35 ± 0.78^{a}	$18.72 \pm 1.20^{a,b}$	8.34 ± 1.16^{a}	10.57 ± 0.46^{a}
LMPC-TA50	$61.47 \pm 1.27^{a,b}$	19.67 ± 1.10^{b}	7.91 ± 0.82^{a}	10.95 ± 0.62^{a}
LMPC-TA100	$60.93 \pm 2.60^{a,b}$	$19.36 \pm 1.33^{a,b}$	8.86 ± 0.54^{a}	10.95 ± 2.20^{a}
LMPC-TA150	60.29 ± 0.55^{b}	20.03 ± 0.71^{b}	9.67 ± 1.38^{a}	10.53 ± 0.71^{a}

3.1.5. *Surface hydrophobicity*

The surface hydrophobicity gives us an indication of the hydrophobic groups present on the surface of the protein or protein–polyphenol conjugate in contact with the surrounding environment. It can be used to assess the changes in protein structure and, in turn, its ability to adsorb at the oil–water interface. The surface hydrophobicity was measured by determining the binding of the ANS probe to hydrophobic patches at the surface of single or conjugated LMPC. The conjugation of LMPC with CA did cause a significant but small reduction in protein surface hydrophobicity for LMPC-CA100 and LMPC-CA150 (Fig. 2A). However, the surface hydrophobicity drastically decreased between 53 % and 64 % when LMPC was conjugated with TA. Different reasons can explain the sharp decrease of surface hydrophobicity observed in LMPC-TA conjugates. First, TA is highly polar due to the presence of multiple hydroxyl groups within its structure whereas CA presents a much lower polarity. This characteristic might be responsible for the higher reduction in the surface hydrophobicity observed compared to LMPC-CA conjugates Finally, the conjugation of LMPC with polyphenols induces conformational changes, as FT-MIR spectroscopy (see Section 3.1.3) and fluorescence analysis (Section 3.1.4) confirmed, what can affect the exposure of the hydrophobic and hydrophilic groups present in the protein surface. Aewsiri et al. (2009) also reported that the conjugation of gelatin with three polyphenols induced significant reduction in surface hydrophobicity that varied according to the polyphenol type and concentration.

3.1.6. *Interfacial tension*

The equilibrium interfacial tension between sunflower oil and LMPC-control and LMPC-polyphenol aqueous solutions is shown in Fig. 2B. As expected, the presence of emulsifiers (WPI, single or conjugated LMPC) significantly reduced the interfacial tension between water and sunflower oil. Besides, it can be seen how the interfacial tension obtained for LMPC-control was significantly lower than the one for WPI, the protein considered as a reference. In line with this, protein concentrates of other insects, such as black soldier or mealworm have shown values of water-sunflower oil interfacial tension of 3.4 mN/m (Wang et al., 2021b) and 11.1 mN/m (Gould & Wolf, 2018), respectively.

The conjugation of LMPC with CA did not show a significant effect on the interfacial tension ($p > 0.05$). However, when LMPC was conjugated to TA, the interfacial tension increased significantly with TA concentration, reaching 14.18 ± 0.79 mN/m for LMPC-TA150. This behavior might be related to the decrease in surface hydrophobicity described in Section 3.1.5. A reduction in the hydrophobic patches on the surface of the LMPC-polyphenol conjugates might prevent the protein to properly adsorb at the oil–water interface. Likewise, Parolia et al. (2022) also reported a loss of surface hydrophobicity associated with an increased interfacial tension when lentil protein was conjugated with different plant polyphenols.

3.1.7. *Emulsifying activity*

The emulsifying activity (EA) enabled to firstly assess the ability of LMPC-polyphenol conjugates to stabilize O/W emulsions (Table 1). Unlike other examples of protein–polyphenol conjugation which lead to an increase in emulsifying capacity (Benjakul et al., 2021), no differences were obtained between the EA values of the LMPC-polyphenol conjugates and LMPC-control (53–55 %, $p > 0.05$). It is interesting to note that even though the interfacial tension values increased, and the surface hydrophobicity decreased when LMPC was conjugated with TA,

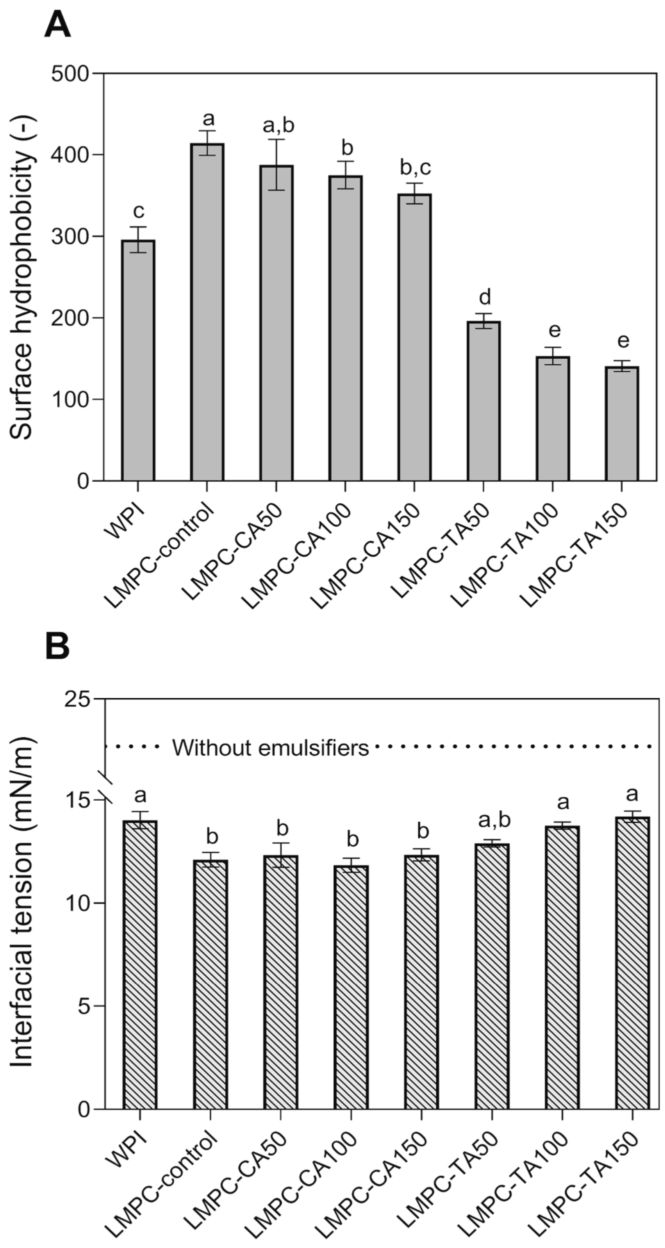

Fig. 2. Surface hydrophobicity of WPI, LMPC-control and conjugates (A) and interfacial tension at the oil-aqueous phase interface (B). Different letters mean significant differences in interfacial tension and surface hydrophobicity values ($p < 0.05$). The dotted line indicates the value of interfacial tension between phosphate buffer and sunflower oil.

it did not negatively impact the EA of the conjugates. Aewsiri et al. (2009) reported that the changes in EA found, for gelatin-tannic acid conjugates, depended on the concentration of tannic acid. At the lowest concentration studied, no changes in EA was observed even if the surface hydrophobicity of the conjugates was significantly reduced. Similar results were obtained by Chen et al. (2019) for porcine plasma protein hydrolysates-polyphenol conjugates. The loss of surface hydrophobicity may not have been sufficient to induce changes in EA, which could have been the case with higher concentration of polyphenols. The values of EA obtained are in range with emulsifying activities obtained for other insects. For example, Wang et al. (2021b) reported EA values of 60 % for Black Soldier Fly protein concentrate, nearly equal to WPI (59.2 %).

3.1.8. Antioxidant activity of the conjugates

The antioxidant activity of LMPC-control and LMPC-polyphenol conjugates was measured using DPPH and ORAC assays, both rely on the deactivation of radicals; the first is based on the transfer of a single electron while the second of a hydrogen atom (Prior et al., 2005). As Table 1 shows, the conjugation significantly improved the antioxidant activity of LMPC. The DPPH values obtained for the LMPC-CA and LMPC-TA conjugates ranged from 45 to 91 μmol TE/g and 319 to 874 μmol TE/g, respectively, while the DPPH value of LMPC was 19 μmol TE/g. Although for both conjugates, the DPPH scavenging activity increased with the polyphenol concentration, LMPC-TA conjugates presented much higher DPPH values than those for LMPC-CA conjugates. LMPC-TA50 had a DPPH value around 3.5 times superior to the one of LMPC-CA150. Similar results were obtained when porcine plasma protein hydrolysates were conjugated with TA and CA (Chen et al., 2019) what has been accounted for the higher number of hydroxyl groups in TA compared to CA (Sun et al., 2020a).

ORAC results followed a similar trend, with values for the LMPC-CA and LMPC-TA conjugates that ranged from 562.6 to 651.0 μmol TE/g and from 700.6 to 1380.1 μmol TE/g, respectively. Regardless of the polyphenol, conjugates showed significantly higher ORAC values that the LMPC-control, 473.3 μmol TE/g. As for the effect of the type of polyphenol, ORAC results for LMPC-TA conjugates were significantly higher than those for LMPC-CA conjugates almost at any polyphenol concentration. All in all, the antioxidant properties of LMPC were significantly improved after the conjugation with CA and TA, with LMPC-TA conjugates exhibiting the highest antioxidant activity.

3.2. Physical and oxidative stability of emulsions

Flaxseed O/W emulsions were prepared with LMPC-TA150 and LMPC-CA150 as they showed the highest in vitro antioxidant activity and comparable emulsifying activity as the other conjugates. The physical and oxidative stability of these emulsions was compared to that of flaxseed O/W emulsions prepared with WPI, a commonly used dairy protein, and LMPC-control as emulsifiers. To do so, the progress of droplet size distribution and the formation of diene hydroperoxides and TBARS, as indicators of the oxidation kinetics, were monitored over a 12-day period under accelerated oxidation conditions at 50 °C.

3.2.1. Physical stability of emulsions

Initially, all emulsions had comparable droplet sizes ($d_{4,3}$ from 11.5 to 11.9 μm) and a monomodal distribution (Fig. 3), implying that LMPC and their conjugates have emulsifying properties similar to WPI. All the emulsions exhibited creaming after one day, but remained stable throughout storage, with no visible oiling-off (Fig. 3 and Fig. S1, supplementary material) and an unchanged droplet size distribution of the emulsions stabilized with WPI, LMPC-control, and LMPC-CA150 after 12 days of storage. However, at day 12, the emulsions stabilized by LMPC-TA150 exhibited a small volume fraction of droplets of around 100 μm. The surface charge of the emulsion droplets stabilized with WPI, LMPC, and the conjugates LMPC-CA and LMPC-TA was followed during storage at 50 °C and presented in Fig. 3E. The initial surface charge was negative for all emulsions, with LMPC-control-stabilized emulsions exhibiting the lowest absolute value (33 mV versus 43, 43, and 50 for WPI, LMPC-CA150 and LMPC-TA150, respectively). An absolute value of ζ-potential >30 mV is generally linked to sufficient repulsive forces for the emulsion to remain physically stable. Throughout 12 days of storage, the absolute ζ-potential increased for LMPC-control, LMPC-CA150 and LMPC-TA150, with significantly higher absolute values at day 12 compared with the initial ones. Upon adsorption at the oil/water interface proteins undergo conformational changes which may result in changes in ζ-potential over time (Wicek & Chibowski, 2002).

3.2.2. Oxidative stability

Lipid oxidation is a major concern, especially in food products rich in unsaturated fatty acids, as it causes the development of unpleasant off

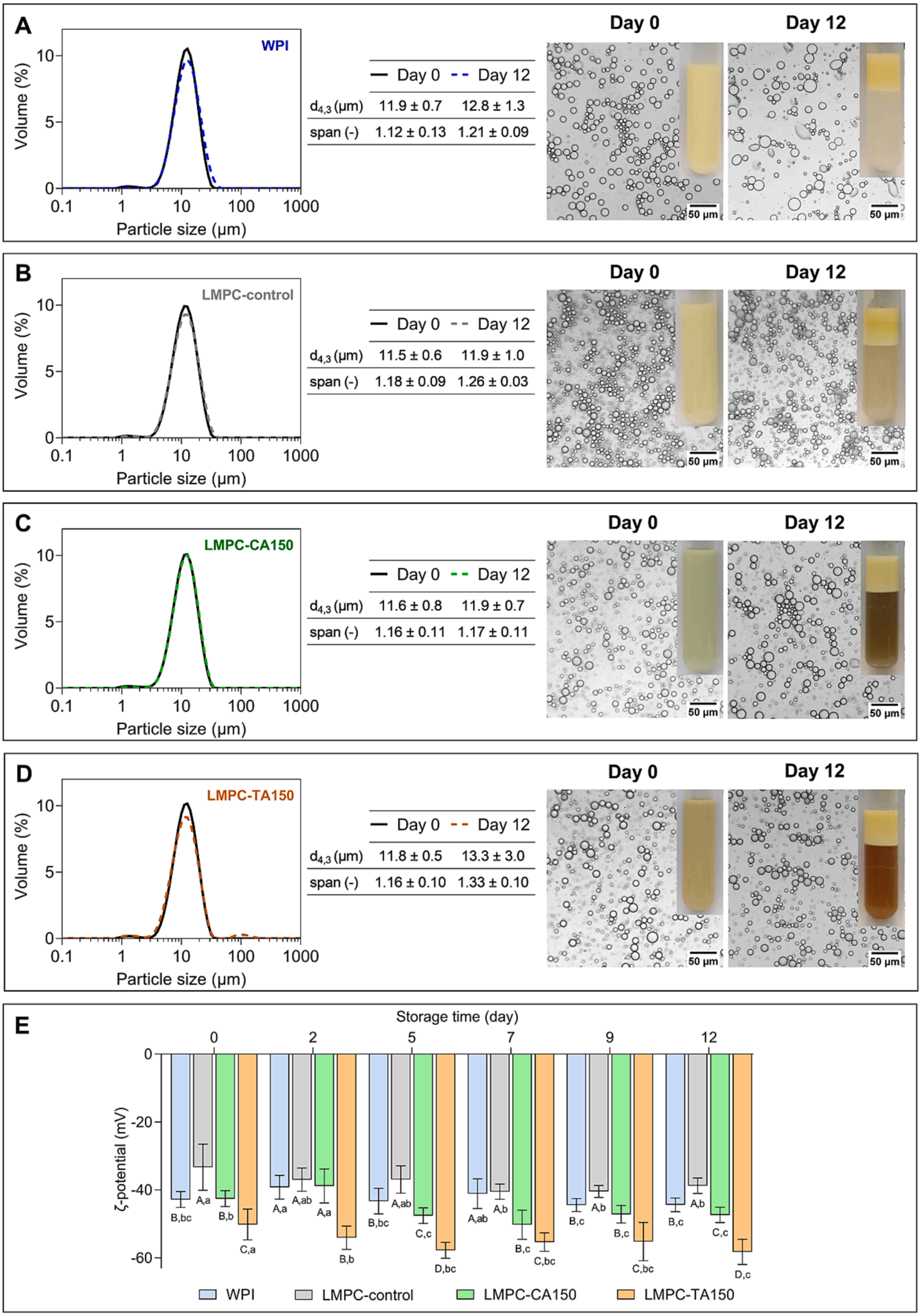

Fig. 3. Droplet size distribution, micrographs and visual appearance of initial emulsions and emulsions after 12 days of storage stabilized with WPI (A), LMPC-control (B), LMPC-CA150 (C) and LMPC-TA150 (D), and ζ-potential during storage of emulsions (E). Different uppercase letters indicate significant difference for the same storage time and different lowercase letters indicate significant differences for the same emulsifier used ($p < 0.05$).

flavors and alters their nutritional benefits. The influence of the conjugates LMPC-CA150 and LMPC-TA150 used as emulsifiers on the oxidative stability of flaxseed O/W emulsions was compared to that of emulsions stabilized with LMPC-control and WPI. To do so, the formation of lipid hydroperoxides and TBARS was monitored for 12 days, with measurements every 2 or 3 days (Fig. 4). While all emulsions showed similar hydroperoxide ($p > 0.05$) and TBARS ($p > 0.05$) levels at day 0, the hydroperoxide content increased steadily during the first 5 days, remaining constant at around 16–19 mmol hydroperoxide/kg oil until day 12 for all emulsions except those stabilized with LMPC-control. LMPC-control stabilized emulsions showed a slight decrease in the hydroperoxide content down to 13 mmol hydroperoxide/kg oil observed at day 12. As hydroperoxides are primary compounds of lipid oxidation, this sudden reduction can be the result of process kinetics when their formation rate becomes slower than their decomposition rate (Frankel, 2005).

TBARS levels increased during storage for all the emulsions, but significant differences were observed depending on the emulsifier used (Fig. 4B). From day 2 to day 12, TBARS level was significantly lower for the emulsions stabilized with LMPC-TA150 compared to those with LMPC-control, reaching values around 48 and 103 μmol/kg oil at day 12, respectively. No significant differences on the progress of TBARS levels were observed between LMPC-CA150- and WPI-stabilized emulsions throughout storage, reaching around 64–69 μmol/kg oil at day 12. As observed for the hydroperoxide content, LMPC-stabilized emulsions exhibited a decrease on the TBARS levels on day 12. This trend can be explained if we consider that the secondary oxidation products detected by the TBARS assay are reactive substances that can be further transformed into tertiary oxidation products, such as alkyl furans, ketones, or aldol condensation products (Grebenteuch et al., 2021). Accordingly, conjugates were found to be more effective in reducing the formation of secondary products of oxidation, as proved by lower TBARS values, as they were regarding the formation of hydroperoxides.

Once hydroperoxides are formed they can move to the oil–water interface as they are surface active and interact with metals present in the aqueous phases and decompose into alkoxyl or hydroxyl radicals. These radicals will participate to the oxidation of fatty acids and generate secondary products of lipid oxidation such as malondialdehyde, detected by the TBARS assay, which are responsible for rancid odors (Gutiérrez-del-Río et al., 2021). Based on these results, it appears that the conjugates LMPC-CA150 and LMPC-TA150 were able to inhibit the formation of alkoxyl or hydroxyl radicals, preventing them from undergoing further reactions.

Overall, the emulsifier' ability to reduce lipid oxidation followed the order of LMPC-TA150 > LMPC-CA150 = WPI > LMPC-control. It is noteworthy to point out that no significant differences in the oxidation rate were found between WPI and LMPC-CA150 stabilized emulsions. To understand the impact of the type of protein (WPI versus LMPC-control) and conjugate (LMPC-TA150 versus LMPC-CA150) used as emulsifier we should consider that lipid oxidation in O/W emulsions depends on several factors, including droplet size, which affects the overall surface area available for oxidation, the surface charge of emulsion droplets, the thickness of the interfacial layer, the presence of protein in the continuous phase, and the chemical properties of the protein (Gumus et al., 2017; Hu et al., 2003). According to Fig. 3, all the emulsions had similar droplet sizes throughout the storage period, suggesting that the difference in surface area was not the primary cause for the lipid oxidation differences. As for the surface charge of the droplets, it could affect the lipid oxidation because negatively charged droplets may attract prooxidant metals such as iron and copper. However, the results cannot only be explained by this factor as all emulsions remained negatively charged throughout storage, while those emulsions with the highest absolute value of ζ-potential (ranging from 50.2 to 58.3 mV), that is those stabilized with LMPC-TA150, showed the highest oxidative stability. On the contrary, the LMPC-control-stabilized emulsions, with the lowest absolute surface charge (ranging from 33.3 to 40.5 mV), oxidized the fastest. Other aspect to be considered is the non-adsorbed protein/conjugates which, being negatively charged, can attract cationic prooxidant metals preventing them to act at the droplet surface (Gumus et al., 2017). Moreover, the properties of the interface as a physical barrier, with protein–polyphenol conjugates able to create denser and thicker interfaces than the single proteins, should also be examined since they could help preventing the oxidation of oil (Yan et al., 2023).

4. Conclusion

The alkaline method enabled to successfully produce LMPC-polyphenol (CA and TA) conjugates with enhanced properties compared to LMPC for food applications as antioxidant emulsifiers. TA was found to have greater binding capacity to LMPC than CA. The conjugation of LMPC with both CA and TA induced changes, depending on polyphenol type and concentration, in the secondary and tertiary structures of the protein as confirmed by the reduction in fluorescence intensity and changes in random coil and β-sheet contents. The conjugation was associated with a significant reduction of the surface hydrophobicity of the conjugates leading to an increase of interfacial values for the LMPC-TA conjugates, without impairment of the emulsifying activity. Compared to LMPC-control, the conjugates presented enhanced antioxidant properties proven by the values of DPPH and ORAC assays. The use of the LMPC-polyphenol conjugates as emulsifier improved the oxidative stability of flaxseed O/W emulsion, while maintaining the physical emulsion stability. Hence, this study suggest

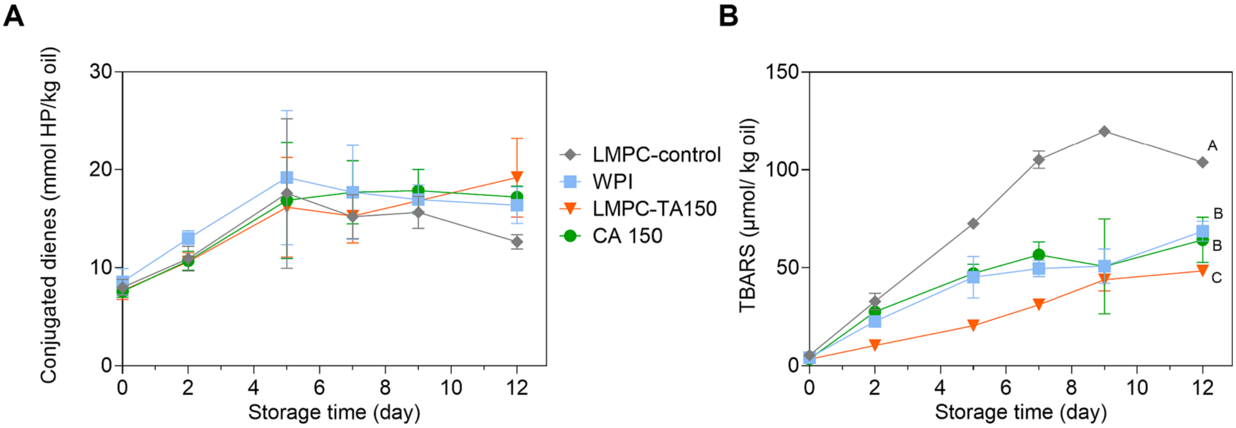

Fig. 4. Formation of conjugated diene hydroperoxides (A) and TBARS (B) during storage at 50 °C of O/W emulsions stabilized with WPI, LMPC-control, LMPC-CA150, or LMPC-TA150. Different letters indicate significant difference ($p < 0.05$).

that LMPC-polyphenol conjugates could be used as effective antioxidant emulsifiers in O/W emulsions rich in omega-3 fatty acids.

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CRediT authorship contribution statement

Aurélie Ballon: Conceptualization, Methodology, Investigation, Writing – original draft. **Maria-Paz Romero:** Resources, Investigation. **Luis E. Rodriguez-Saona:** Resources. **Sílvia de Lamo-Castellví:** Conceptualization, Funding acquisition. **Carme Güell:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Montserrat Ferrando:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137494.

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