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CaCl₂ supplementation of hydrophobised whey proteins: Assessment of protein particles and consequent emulsions

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ABSTRACT

Hydrophobised whey protein particles were prepared through successive acetylation and heat treatment practices, and the particle characteristics were modulated by CaCl₂ supplementation. Then, the usefulness of the hydrophobised protein particles for emulsification of a docosahexaenoic acid-rich oil was compared with that of heat-denatured whey protein. Addition of CaCl₂ into hydrophobised whey protein resulted in smaller protein particles and lower ζ-potential and interfacial tension values. It also decreased the creaming stability of the consequent emulsions. It was argued that besides Ca²⁺-protein charge interactions, Cl⁻ anions bind to the hydrophobised particles, and the Pickering stabilisation of oil does not rely on interfacial tension reduction. Compared with heat-denatured whey protein, hydrophobised whey protein afforded a lower protection to oil against oxidation; the peroxide value of the oil emulsified using hydrophobised protein was higher during storage.

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1. Introduction

Whey proteins contain substantial amounts of branched chain amino acids and are physiologically beneficial; for instance consumption of whey proteins combined with resistance training accelerates fat loss in humans (Lockwood et al., 2017) and causes increased muscle gain in untrained rats (Wróblewska et al., 2018). Moreover, whey proteins are widely used for technological purposes such as formation of cold-set and heat-induced gels (Egan, O'Riordan, O'Sullivan, & Jacquier, 2014; Oztop, McCarthy, McCarthy, & Rosenberg, 2014), modification of viscosity (Patocka, Cervenkova, Narine, & Jelen, 2006) and as fat replacer (Akalın, Karagözü, & Ünal, 2008). Heat treatment of whey proteins at non-gelling condition produces protein particles, which have been exploited as Pickering stabiliser of foams (Schmitt, Bovay, & Rouvet, 2014), oil-in-water (O/W) (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014) and water-in-water (Nguyen, Nicolai, & Benyahia, 2013) emulsions.

The functionality of whey protein particles is further tailored via complexation with oppositely charged polysaccharides and ions. Monovalent and multivalent salts including NaCl, MgCl₂ and AlCl₃

(Ince-Coskun & Ozdestan-Ocak, 2020), as well as ZnCl₂, MnCl₂, and CaCl₂ (Mohammadian & Madadlou, 2016) are used to tune the technological functionality of whey protein particles. CaCl₂ has also been used to produce soy protein particles through a non-thermal process, consisting of two crosslinking steps: ionic crosslinking by CaCl₂ and subsequent amine-aldehyde covalent crosslinking by glutaraldehyde. The produced protein particles were successfully used for Pickering emulsification of soy oil in water (Liu, Ou, & Tang, 2017). However, glutaraldehyde is irritant, and cytotoxic. Therefore, the utilisation of glutaraldehyde and other hazardous chemical crosslinkers is not favoured by consumers.

Although food-grade amine-aldehyde crosslinkers such as citric acid can be alternatively applied (Abaee, Madadlou, & Saboury, 2017; Reddy, Li, & Yang, 2009) in combination with ionic crosslinking to particulate plant and whey proteins, other opportunities to produce and tune whey proteins particles are available. For example, recently, hydrophobised whey protein particles were produced through a food-grade, simple and inexpensive method, which includes whey protein pre-acetylation at room temperature and subsequent acetylation-heat treatment (Madadlou, Flourey, & Dupont, 2018). Acetylation is known to append ester carbonyl groups to proteins (Zhao, Ma, Yuen, & Phillips, 2004), increase their

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hydrophobicity and cause conformational changes (Lakkis & Villota, 1992a; Shah & Singhal, 2019). The subsequent heat treatment resulted in formation of protein particles.

Surface tension measurements at the air–water interface indicated that the hydrophobised whey protein particles are adsorbed at the interfacial layer and can be used for stabilisation of water-in-water emulsions (Madadlou, Saint-Jalmes, Guyomarc'h, Flourey, & Dupont, 2019). However, the particles hold many more premises. A combinatory application of hydrophobised whey protein particles and ionic crosslinking by CaCl_2 may be advantageous to tune the protein particles functionality as O/W Pickering emulsifiers. In the present communication, we report the influence of CaCl_2 supplementation on the hydrodynamic size, electric charge, and oil-water interfacial tension of the protein particles. The role of Cl^- anions was particularly taken into consideration when discussing the observations. The importance of halide anions in development and modification of salt-supplemented protein networks (gels and particles) has been scarcely considered, whereas in addition to Ca^{2+} cations, Cl^- anions are capable to interacting with proteins, thereby influencing protein network characteristics (Farjami, Madadlou, & Labbafi, 2016). Subsequently, the applicability of the hydrophobised whey protein particles as Pickering emulsifiers for a docosahexaenoic acid (DHA)-rich oil was assessed. To the best of our knowledge, there is no report in the literature about the interfacial and emulsification properties of hydrophobised (acetylated-heat denatured) whey proteins.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was a gift from Lactalis Ingredients (Lactalis Group, Bourgbarré, France). It had 90% protein, 5.1% moisture, 3.0% lactose, and 1.97% ash contents (Madadlou et al., 2018). DHA-rich oil was purchased from Polaris (Polaris, Quimper, France); it comprised 678 mg g^{-1} DHA and 23 mg g^{-1} eicosapentaenoic acid (EPA) as triacylglycerols. Deionised water was used throughout the research, and all the chemicals used were of analytical grade.

2.2. Fabrication of hydrophobised and heat-denatured WPI particles

Hydrophobised WPI particles were prepared according to the process described by Madadlou et al. (2018), with minor modifications. At first, a WPI solution (65 mg mL^{-1}) was prepared by adding deionised water to WPI powder and stirring for 4 h at 20°C ; sodium azide was added (1000 ppm for peroxide value and creaming index determination, 100 ppm for other experiments) to inhibit microbial growth. Then the solution was left overnight at 4°C to ensure full hydration of whey proteins. WPI hydrophobisation was accomplished by the following steps: 9.5 mL of native WPI solution (65 mg mL^{-1}) was charged with acetic anhydride (100 μL) and then gradually with 7 M NaOH (a total of 280 μL) with continue stirring. Subsequently, the solution was stirred for an additional period of 30 min; during this time, the WPI solution was occasionally supplemented with 1 M NaOH to maintain pH value in the range of 8.1–8.5, facilitating acetylation. Later, the solution (pH = 8.5) was heated at 80°C while being stirred at 800 rpm for 20 min. After heat treatment, the solution was cooled using an ice bath and pH was adjusted to 8.0 by adding 10–20 μL of 1 M NaOH.

A heat-denatured WPI sample was also made by simply heating the native WPI solution (adjusted to pH 8.2 with 1 M NaOH) at 80°C for 20 min (Law & Leaver, 2000). Then, the solution was cooled using an ice bath and pH was adjusted to 8.0 by adding 0–10 μL 1 M

NaOH. The hydrophobised (i.e. acetylated and heat-denatured) and heat-denatured WPI dispersions were diluted by adding deionised water to a final concentration of 10 mg mL^{-1} . Finally, CaCl_2 (500 mM) was gradually added into the various samples to be investigated, to reach a final Ca^{2+} concentration of 0–7 mM.

2.3. Protein particle characterisation

2.3.1. Particle size and ζ -potential measurement

The particle size and ζ -potential of hydrophobised WPI and heat-denatured WPI were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). A laser wavelength of 633 nm at a backscattering angle of 173° was applied for size measurements at 20°C .

2.3.2. Surface hydrophobicity measurement

The surface hydrophobicity of heat-denatured and hydrophobised WPIs were measured according to the method published by Alizadeh-Pasdar and Li-Chan (2000) with some modifications. A stock solution of $1.41 \times 10^{-3} \text{ M}$ 6-propionyl-2-(N–N-dimethylamino)-naphthalene (PRODAN) was prepared in methanol, transferred to screw-capped vials, and wrapped in aluminium foil to avoid exposure to light. The solution was stored in a freezer ($\leq -10^\circ\text{C}$) until the day of experiment, when it was held in ice throughout the experiment. The excitation/emission slits and wavelengths of the spectrofluorometer (FLX-Xenius, SAFAS, Monaco) were set at 5 nm/5 nm and 500 nm/600 nm, respectively. To successive samples containing 4 mL of diluted WPI was added 10 μL of PRODAN stock solution, which was mixed well by vortexing. After 30 min in the dark, the relative fluorescence intensity (RFI) of each solution was measured, starting from blank (deionised water plus probe) and then the lowest to the highest protein concentration (0–2 mg mL^{-1}); the fluorometer quartz cell was rinsed between samples with a small volume of the solution to be measured. RFI values of buffer and protein dilution blanks (no PRODAN) were also measured. The RFI of each protein dilution blank was subtracted from that of corresponding protein solution with PRODAN to obtain net RFI. The initial slope (S_0) of the net RFI versus protein concentration (percent) plot was calculated by linear regression analysis with Microsoft Excel and used as an index of the protein surface hydrophobicity.

2.3.3. Interfacial tension (γ) measurement

The tension at the interface between DHA-rich oil and WPI dispersion was measured by the pendent drop method using a pendent drop tensiometer (Tracker; Teclis-Scientific, Civrieux d'Azergues, France). After formation of a fresh WPI drop (15 μL) at the tip of a syringe, the time evolution of γ was studied for 720 s. The drop profile was determined by image analysis, from which γ was derived. Under mechanical equilibrium of capillary and gravity forces, the Laplace equation relates the pressure difference across the interface, the γ and the interface curvature. These experiments were carried out at 20°C . After experiments, the following kinetic model was used to describe the variation of γ at the oil/water interface:

$$\ln[(\gamma_t - \gamma_c)/(\gamma_w - \gamma_c)] = -kt \quad (\text{Eq. 1})$$

where γ_w and γ_c are values of γ at times 0 and 120 s after droplet formation, γ_t is the value of γ at time t and k is the rate constant (Panizzolo, Mussio, & Añón, 2014).

2.4. Emulsion preparation

Emulsion samples were prepared by a two-step homogenisation process. DHA-rich oil was gradually added into the WPI dispersion (10 mg mL^{-1}) while being stirred (IKA T 10 basic Ultra154 turrax, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 2000 rpm. Then, mixing was continued at 20,000 rpm for 6 min. The volume ratio of oil-to-WPI dispersion was 0.6:0.4. The resulting coarse emulsion (30 mL) was homogenised within an ice bath using an ultrasonic homogeniser (Q700 Sonicator, Qsonica sonicators, USA) with a standard tip (3.2 mm diameter) immersed 1/2 in a beaker of 40 mm diameter (50 mL volume). The emulsion was simultaneously stirred with a magnetic stirrer to ensure homogeneity. Each sample was sonicated at 20 kHz and an amplitude of 40% for 5 min. The O/W emulsions obtained were subjected to analysis.

2.5. Characterisation of emulsions

2.5.1. Droplet size determination

Droplet size ($d_{4,3}$) of emulsions was determined using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The refractive index of oil phase and water phase used in the mathematical analysis were 1.45 and 1.33, respectively. The equipment works with two different wavelengths (He/Ne laser: 633 nm; electroluminescent diode: 466 nm). This wavelength combination takes into account the effect of post-diffusion and enhances sensitivity to small particles.

2.5.2. Optical microscopy

Freshly formed emulsions were observed under an optical microscope (Olympus BX51TF, Hamburg, Germany) with $40\times$ objective. The imaging was carried out at non-fluorescent mode (bright field microscopy) and using the Archimed software (version 7.1.1, Microvision Instruments, Evry cedex, France) at 20°C .

2.5.3. Creaming stability measurements

The creaming stability of prepared emulsions was studied following the method of Onsaard, Vittayanont, Srigam, and McClements (2006). Briefly, 27 g of emulsion samples were transferred into a test glass (with 24 mm internal diameter and 72 mm height), tightly sealed with a plastic cap to prevent evaporation, and then left to stand at 20°C for 8 days. The emulsions separated into a top "cream layer" and a bottom "serum layer" (H_s). The extent of creaming was characterised by the creaming index (CI) = $100 \times (H_s/H_e)$, where H_s and H_e are the height of the serum layer and total height of the emulsions in the tubes, respectively.

2.5.4. Rheological behaviour

Rheological assessment of the emulsions was performed immediately after emulsion preparation using a MCR 301 rheometer (Anton Paar, Graz, Austria) equipped with a cone-plate geometry (50 mm diameter, cone angle = 1.996°) at 20°C . After equilibration, flow curves were determined at an increasing shear rate from 0.1 to 100 s^{-1} . Herschel-Bulkley model was used to describe the flow behaviour of emulsions:

$$\tau = \tau_0 + K(\dot{\gamma})^n \quad (\text{Eq. 2})$$

where τ and τ_0 were shear stress and yield stress, respectively, and $\dot{\gamma}$ was the shear rate; n and K were the flow behaviour index and the consistency index, respectively.

2.5.5. Oxidative stability

After formation, all emulsions were stored at 20°C for 8 days. A control sample containing only DHA-rich oil was also analysed. At

first, the oil was extracted from emulsions by adding 0.3 mL emulsion to 6 mL chloroform/methanol (2:1, v/v). The mixture was mixed 15 min at 30 rpm and then was supplemented with 1.26 mL 0.9% NaCl solution. The solution was mixed again for 15 min and rested for 30 min. Then the supernatant was removed and the subnatant was collected, filtered and treated with nitrogen to volatilise the solvent. Subsequently, 0.2 mL absolute ethanol was added to the sample and gassed under nitrogen ensure that all solvent and water were removed from the sample.

The peroxide value (PV) of emulsions was measured by an ISO standard method with some modifications (Shantha & Decker, 1994). Briefly, a volume of 100 mL of a chloroform/methanol mixture (7:3, v/v) was added to both the extracted oil and non-emulsified oil (0.100–0.110 g). After resting for a short time, 9.9 mL of the oil-solvent mixture was supplemented with $50 \mu\text{L}$ 3.94 M ammonium thiocyanate solution, the mixture vortexed for 4 s, then $50 \mu\text{L}$ 0.072 M iron (II) solution was added and the mixture was vortexed again for 4 s. After incubation for 10 min at 20°C , the absorbance of the samples was read at 500 nm using a UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA, USA).

2.6. Statistical analysis

Samples were produced at least three times, and the experiments were performed in triplicate. The results were analysed by one-way ANOVA with SPSS software version 17 (International Business Machines, Armonk, NY, USA) using Duncan's test at a significance level of $P < 0.05$.

3. Results and discussion

3.1. Characterisation of protein particles

3.1.1. Particle size

The hydrophobisation process resulted in larger protein particles compared with obtained with only heat denaturation (Fig. 1). Grafting acetyl moieties onto the surface of native whey proteins before heat treatment could enhance hydrophobic interactions between proteins, resulting in larger particles during the subsequent heat treatment. It is already indicated that roughly 90% of available amino groups on whey proteins undergo acetylation by the method used in the current study (Madadlou et al., 2018).

The particle size of the heat-denatured WPI did not significantly change by CaCl_2 addition up to 5 mM. However, the particle size increased nearly 20% when CaCl_2 concentration increased from 5 to 7 mM (Fig. 1). The small change of particle size suggests that protein particles did not aggregate; rather the hydration extent of the particles increased by CaCl_2 addition at 7 mM. Kosmotropic cations such as Ca^{2+} have a stronger affinity to anions than water; this is valid not only for anions in bulk solvent but also for aspartate and glutamate residues on the surface of a protein. Protein- Ca^{2+} interactions neutralise the favourable structure-making effect of protein anions on water (Eggers & Valentine, 2001) and cause disruption of the hydrogen bonds between protein and water, i.e., protein partially dehydrates. Concomitantly, the attached Ca^{2+} to protein binds to the water in immediate vicinity, which opposed (at low Ca^{2+} concentrations) and eventually dominated the dehydration effect of Ca^{2+} binding to protein, leading to a higher hydrodynamic diameter at the highest CaCl_2 concentration (7 mM). It is noteworthy that at CaCl_2 concentrations greater than 7 mM the heat-denatured protein particles extensively aggregated (results not shown).

In contrast to the results obtained for the heat-denatured WPI, the particle size of the hydrophobised WPI gradually (though not consecutively) decreased with increasing CaCl_2 concentration throughout the whole concentration range between 0 and 7 mM

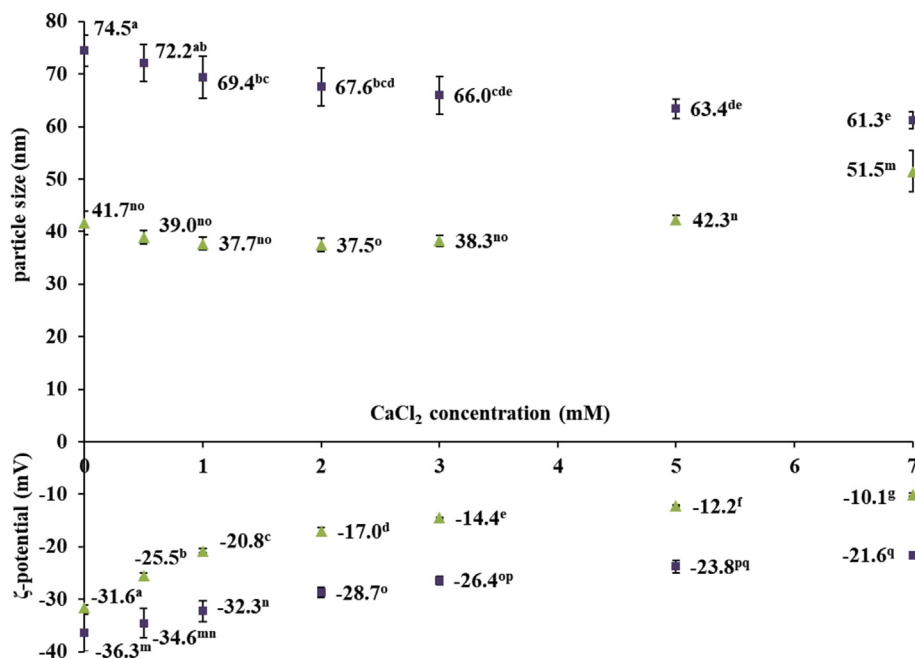


Fig. 1. Particle size and ζ -potential value of hydrophobised (■) and heat-denatured WPI (▲) samples as influenced by CaCl₂ concentration. Values with different lowercase letters for each sample were significantly different ($P < 0.05$). Error bars indicate standard deviation.

(Fig. 1). A total size reduction of approximately 20% is observed for hydrophobised WPI particles when the added CaCl₂ concentration increased from 0 mM to 7 mM. The small size change indicates that the protein particles did not disaggregate; rather they progressively dehydrated (i.e., became less hydrated) with increasing the CaCl₂ concentration.

The dehydration of the hydrophobised WPI particles as a consequence of CaCl₂ supplementation and comparison with changes in the hydrodynamic size of heat-denatured WPI particles indicates that acetylation of whey proteins influenced the protein-salt interaction. It is possible to argue that the counter ions of Ca²⁺, i.e., Cl⁻ anions, must be taken into account. It has been reported that at a given ion normality of 50 mM, Cl⁻ is among the weakest anions that can associate with the protein surface (Zhao, 2016). Nonetheless, grafting acetyl moieties onto whey proteins could strongly increase Cl⁻ binding to proteins via a phenomenon called hydrophobic concavity, i.e., hydrophobic interactions within a nonpolar microcavity. Being a low-charge-density ion, Cl⁻ can stick to hydrophobic protein ensembles (Farjami et al., 2016). Hydrophobic concavity accounts for the selective binding of Cl⁻ anions over phosphates and carboxylates (that rank far above Cl⁻ in the Hofmeister series) to macrocyclic hosts. Likewise, interactions between Cl⁻ and hydrophobic amino acids play a crucial role in functionality of chloride-selective protein channels of cell membranes. In such channels, a Cl⁻ ion may be bound by just a few H-bond donors but it is surrounded by hydrophobic amino acid residues and contacts with hydrophobic CH₂ groups (Dabrowa, Ulatowski, Lichosyt, & Jurczak, 2017). The binding of Cl⁻ to acetylated protein particles most likely occurred through complexation of ions within the hydrophobic pockets made of neighbouring acetyl groups. The anions that bind to hydrophobic concavities completely or at least partially lose their solvation shell water molecules (Sokkalingam, Shraberg, Rick, & Gibb, 2016), which explains the decrease of WPI particles hydration due to Cl⁻ binding at CaCl₂ concentrations between 0 and 7 mM.

3.1.2. ζ -potential

Acetylation of proteins with acetic anhydride involves covalent attachment of acetyl moieties to free amino groups of proteins

(Lakkis & Villota, 1992b). As expected, the hydrophobised WPI had a more negative ζ -potential value compared with the heat-denatured WPI (Fig. 1), which is attributed to consumption of positively charged free amino groups during the acetylation process.

Addition of CaCl₂ reduced the absolute value of ζ -potential of both heat-denatured and hydrophobised protein particles (Fig. 1). Negatively charged amino acid residues, namely aspartate and glutamate bind to Ca²⁺ ions, which decreases the ζ -potential. It is noteworthy that the maximum alteration of ζ -potential due to CaCl₂ supplementation was much more significant for the heat-denatured WPI (nearly 3 fold) than the hydrophobised WPI (nearly 1.7 fold) when the concentration of CaCl₂ was increased from 0 to 7 mM. This is in accordance with our hypothesis on the concavity pocketing of Cl⁻ anions into the acetylated (hydrophobised) protein particles.

3.1.3. Surface hydrophobicity

As expected, grafting acetyl moieties onto whey proteins increased the surface hydrophobicity from ~119 for the heat-denatured WPI to ~128 for the hydrophobised WPI at 0 mM CaCl₂ concentration. The addition of CaCl₂ did not significantly influence the surface hydrophobicity of the samples (results not shown). PRODAN is an uncharged aromatic probe and is advantageous for measuring protein hydrophobicity over ionic probes such as *cis*-parinaric acid (CPA) and 1-anilinonaphthalene-8-sulfonic acid (ANS) (Alizadeh-Pasdar & Li-Chan, 2000). Similar to our observation, increasing ionic strength did not influence the surface hydrophobicity of ovalbumin measured by PRODAN but increased that of ovalbumin measured by ANS (Haskard & Li-Chan, 1998), which is negatively charged.

3.1.4. Interfacial tension

The time-dependent variation of γ at the interface between DHA-rich oil and WPI dispersions is presented in Fig. 2A. For the freshly formed pendant drop (at time ≈ 0) and in the absence of added CaCl₂, there was not a statistically significant difference between the γ values of the heat-denatured and hydrophobised WPI

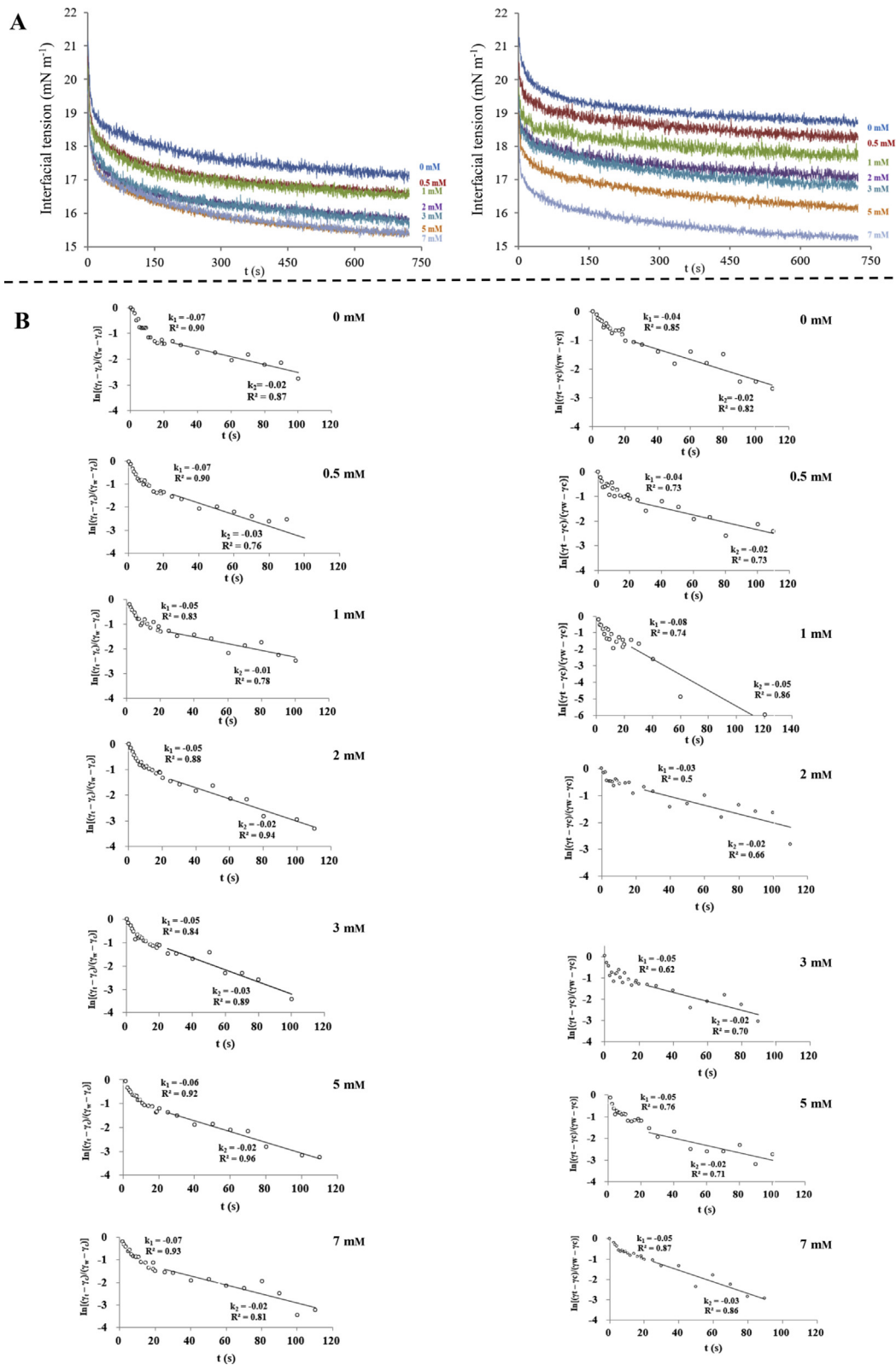


Fig. 2. Oil-water interfacial tension (A) and interfacial tension decay rate (B; k , the slope of the line) of WPI samples: lefthand panels, heat-denatured WPI; righthand panels, hydrophobised WPI. Concentrations (mM) refer to the added CaCl₂ into WPI dispersions.

samples; both WPIs had a γ value of approximately 21.1 mN m⁻¹, which subsequently decreased with droplet ageing. Supplementation with CaCl₂ decreased γ at any given time for both WPIs. We presume that ζ -potential reduction of protein particles due to CaCl₂ addition (Fig. 1) caused a more efficient packing of protein particles at the interface, reducing the γ . Besides, it is known that cations such as Ca²⁺ increase the contact angle of adsorbing particles at the oil-water interface, thus the interfacial tension decreases more substantially. The influence of cations on contact angle is associated with decrease of particles hydration level (Wen, Sun, & Bai, 2018), which may explain the smaller differences between the γ values of heat-denatured WPI (for which the hydrodynamic size did not decrease between 0 and 5 mM CaCl₂ and slightly increased at 7 mM CaCl₂) compared with the greater differences between the γ values of hydrophobised WPI (for which hydrodynamic size decreased between 0 and 7 mM CaCl₂) as a function of CaCl₂ concentration (Fig. 2A). This is in accordance with the results of Fan et al. (2017) who reported that the tension at the oil-WPI interface decreased with increasing CaCl₂ concentration.

Analysis of γ values using Eq. (1) resulted in two γ reduction constants: k_1 which refers to the rapidly falling period of γ in the initial 20 s and k_2 which refers to the slowly falling period of γ over the rest of interface ageing (Fig. 2B). Following Panizzolo et al. (2014) Eq. (1) was applied to time periods between 0 and 120 s of droplet ageing. Although the model was suitable to characterise γ reduction rates of the heat-denatured WPI, i.e., it had high R² values especially for k_1 , the hydrophobised WPI did not properly fit to the model (Fig. 2B). Nonetheless, the following main results can be concluded from the modelling practice: as expected, k_1 was always higher than k_2 for both WPI samples; in general, k_1 values of the heat-denatured WPI were higher than those of the hydrophobised WPI; k_1 values of neither of WPIs were influenced by CaCl₂ concentration (Fig. 2B).

The higher k_1 compared with k_2 , and invariable k_1 values at different CaCl₂ concentrations, indicate fast absorption of protein particles to the interface of freshly formed pendant drops irrespective of the added CaCl₂ concentration. Therefore, though γ decreased with increasing CaCl₂ concentration at any given time (Fig. 2), the reduction rate of γ in the rapidly falling period (i.e., k_1) was not influenced by CaCl₂ concentration.

The higher k_1 (Fig. 2B) values of the heat-denatured WPI than the hydrophobised WPI was caused by the lower absolute ζ -potential value (Fig. 1) of the former, which presumably lead to more rapid packing of the heat-denatured protein particles at the interface, in comparison with the hydrophobised WPI.

Whereas small molecule surfactants can cause roughly a 10-fold reduction of γ at the oil-water interface (Posocco et al., 2016), the highest extent of γ reduction in the current study was merely ≈ 0.25 fold compared with the corresponding initial value (i.e., at time ≈ 0). Actually, Pickering-type stabilisation of emulsions does not rely on γ reduction. In fact, particle trapping at the oil-water interface does not cause appreciable changes in the interfacial tension. Rather particles position at the continuous side of the droplet interface and stabilise emulsions (Vignati, Piazza, & Lockhart, 2003). However, the stability of Pickering emulsions is proportional to γ . According to the following equation (3), the higher the γ , the higher the energy required to desorb Pickering particles from the interface:

$$E = \pi R^2 \gamma_{ow} (1 + \cos\theta)^2 \quad (\text{Eq. 3})$$

where E is the energy required to remove particle, R is particle radius, γ is tension at the oil-water interface, and θ is the three-phase contact angle (Binks & Lumsdon, 2000). The reduction of γ with increasing CaCl₂ concentration in the present study is

therefore expected to cause a lower emulsion stability, and result in destabilisation phenomena such as creaming.

3.2. Characterisation of pickering emulsions

3.2.1. Bulk and microscopic appearance, and droplet size

Fig. 3A shows the bulk appearance of oil-WPI dispersion mixes after emulsification practices, i.e., mechanical stirring and ultrasonic homogenisation. WPI concentration in the aqueous phase of mixes was 10 mg mL⁻¹ and the proportion of the DHA oil in mixes was 60% (v/v). The mixture of oil and heat-denatured WPI supplemented with CaCl₂ at 3 mM transformed to a soft gel during ultrasonic homogenisation, failing to make an emulsion. As well, the mixture of oil and hydrophobised WPI supplemented with CaCl₂ at 6.5 mM was highly viscous and oil was not readily dispersed in the aqueous phase, therefore, after preparation a portion of oil did not emulsify and remained at the surface (Fig. 3A). These samples were not subjected to additional analysis.

The emulsion samples made using heat-denatured WPI without added CaCl₂ and hydrophobised WPI supplemented with 0, 3 and 5.5 mM CaCl₂ were stored for a period of 8 days. The sample made using the hydrophobised WPI with 0 mM added CaCl₂ did not show any extent of creaming; whereas, the rest of the samples slightly creamed, i.e., those prepared using the hydrophobised WPI with 3 or 5.5 mM CaCl₂ and the heat-denatured WPI with 0 mM CaCl₂. The creaming index of the phase separated emulsions reached 1.7% (Fig. 3B) after 2 days and remained unchanged over the rest of the storage period. In accordance with these observations, Fan et al. (2017) reported that CaCl₂ supplementation of heat-denatured WPI at concentrations above 0.2 mM caused creaming of the subsequent O/W emulsion.

Creaming of an emulsion indicates droplets flocculation (Dickinson, 2009). Therefore, the oil droplets covered by the hydrophobised protein particles without added CaCl₂ were fully stable to flocculation/aggregation, and CaCl₂ supplementation of the hydrophobised WPI reduced the flocculation stability of the emulsion droplets, which is in line with reduction of γ with increasing CaCl₂ concentrations. Electrostatic repulsion between the protein particle-coated oil droplets could be the main cause of the considerable flocculation stability of the emulsion made using the hydrophobised WPI without added CaCl₂. The absolute ζ -potential value of the hydrophobised WPI particles was ≈ 36 mV, which is quite high. It decreased to ≈ 26 mV upon CaCl₂ addition at 3 mM (Fig. 1).

A weaker electrostatic repulsion between the oil droplets can rationally lead to droplet flocculation and emulsion creaming, as observed in the current study. However, electrostatic repulsion between oil droplets was not most probably the sole factor affecting the flocculation/creaming. Heat-denatured WPI without added CaCl₂ had an absolute ζ -potential value of ≈ 32 mV, which is higher than those of the hydrophobised WPI with added CaCl₂ at 3–7 mM (Fig. 1). Yet the emulsion samples made using the heat-denatured WPI without added CaCl₂ and hydrophobised WPIs supplemented with 3 and 5.5 mM CaCl₂ had comparable extents of creaming (Fig. 3B). It can be argued that protein particle size also influenced the flocculation stability of the Pickering emulsions. The hydrophobised WPI had larger particles than the heat-denatured WPI (Fig. 1). Larger particles could cause a more effective steric hindrance between the particle-coated oil droplets. Hence, a longer-range steric hindrance counterbalanced the weaker electrostatic repulsion in emulsions made using the CaCl₂-supplemented hydrophobised WPI when compared with the stronger electrostatic repulsion but shorter-range hindrance in CaCl₂-free heat-denatured WPI. Such a counterbalancing of the stabilisation mechanisms led to comparable creaming extents between the

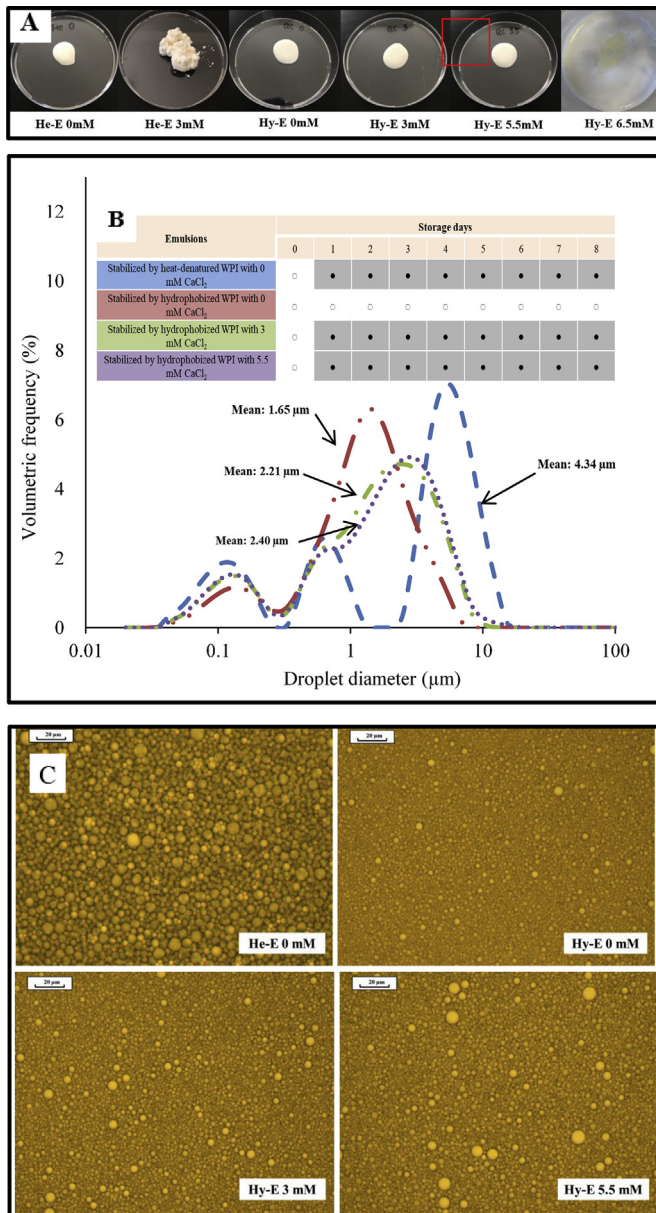


Fig. 3. Photographs (A) of oil-WPI dispersion mixes after emulsification using the heat-denatured WPI (He-E) and the hydrophobised WPI (Hy-E) supplemented with different CaCl₂ concentrations (0, 3, 5.5 and 6.5 mM); creaming index (B); empty symbols mean no creaming, full symbols mean creaming and the creaming index was 1.7% and droplet size distribution of the emulsions stabilised by either heat-denatured (—, 0 mM CaCl₂) or hydrophobised WPI (—, 0 mM CaCl₂; —, 3 mM CaCl₂; —, 5.5 mM CaCl₂) microscopy images (C) of He-E and Hy-E samples with different CaCl₂ concentrations. Scale bars show 20 µm.

emulsions. The importance of steric hindrance to prevent extensive flocculation of oil droplets in Pickering emulsions has also been shown for low-charged (ζ -potential ≈ -16 mV) starch nanoparticles (Ge et al., 2017).

The droplet size of freshly prepared emulsions analysed by the light scattering technique lies in the order of heat-denatured WPI > hydrophobised WPI supplemented with CaCl₂ at either 3 or 5.5 mM > hydrophobised WPI supplemented with CaCl₂ at 0 mM (Fig. 3B). The same trend is perceived in optical microscopy images of emulsion samples immediately after preparation (Fig. 3C). Obviously, the droplet size order does not follow the order of γ (Fig. 2A). As mentioned earlier, particle trapping at the oil-water

interface does not appreciably influence γ at the oil-water interface; vice versa, lower γ values caused by CaCl₂ addition resulted in lower desorption energy for particles (Binks & Lumsdon, 2000).

3.2.2. Flow behaviour

Fig. 4 shows the flow behaviour of the emulsion samples stabilised by either heat-denatured or hydrophobised WPIs. The viscosity of emulsions decreased with increasing shear rate, indicating that all samples were shear thinning, which is attributed to alignment of droplets in direction of the applied shear field (Soltani & Madadlou, 2015). The emulsion samples stabilised by the heat-denatured and hydrophobised WPIs without added CaCl₂ (0 mM) had statistically indifferent yield stresses (τ_0) and consistency coefficients (K); however, the flow behaviour index (n) of the sample stabilised by the hydrophobised WPI was lower than that of the emulsion stabilised by the heat-denatured WPI (Table 1). The latter indicates that the hydrophobised WPI-stabilised oil droplets aligned more easily than the heat-denatured WPI-stabilised droplets in direction of shear field. The difference in shear thinning property could be caused by the smaller droplet size of the emulsion stabilised by the hydrophobised WPI (Fig. 3), as well as dissimilar inter-droplet electrostatic repulsion. A higher electrostatic repulsion between droplets might render the emulsion stabilised by the hydrophobised particles more easily thinning with increasing shear rate.

Addition of CaCl₂ into WPI at 3 and 5.5 mM did not influence the K value of the emulsion made using the hydrophobised WPI (Fig. 4B). Likewise, Keowmaneechai and McClements (2002) reported that supplementation with CaCl₂ at 2 mM did not affect the viscosity of model beverages based on whey protein-stabilised O/W emulsions. Nonetheless, the flow behaviour index (n) and τ_0 of the hydrophobised WPI-stabilised emulsion was higher when WPI was supplemented with 5.5 mM CaCl₂ compared with 0 mM (Fig. 4B). We attribute the lower shear-thinning property to reduced electrostatic repulsion between oil droplets (due to lower ζ -potential absolute values, Fig. 1) and the increased τ_0 to the presence of larger droplets (Fig. 2B) when the added CaCl₂ into WPI increased from 0 mM to 5.5 mM.

3.2.3. Oxidative stability

The oxidative stability of emulsions was investigated for 8 days. All emulsions were kept at 20 °C immediately after preparation and the emulsified oil was extracted at fixed time intervals for PV measurements. Non-emulsified DHA-rich oil was also analysed for comparison purposes. The DHA-rich oil emulsified using the heat-denatured WPI had the lowest PV among all samples throughout the whole storage period (Fig. 5), which is attributed to the anti-oxidative effect of exposed thiol groups in the heat-denatured protein. Heat denaturation exposes the previously hidden sulfhydryl groups, which contribute to protein aggregation by $-SH/S-S$ exchange reactions and have a central role in protein-based redox systems (Ulrich & Jakob, 2019).

The highest PV belonged to the non-emulsified oil at the end of the storage time. However, the oil emulsified using the hydrophobised WPI without added CaCl₂ (i.e., 0 mM CaCl₂) had a higher PV than the non-emulsified oil in the first storage days (Fig. 5). The underlying mechanism for this observation is not clear and further systematic studies are required to reveal the effect of hydrophobised WPI on oil oxidation. Nevertheless, it is worth mentioning that the two-step mechanical-ultrasonic emulsification process which was applied in the current study could enhance oil oxidation, leading to higher PV at the early days of storage in comparison to the non-emulsified oil (i.e., no mechanical-ultrasonic treatments). Mechanical agitation of oil-water mixtures causes air incorporation into the emulsion, and

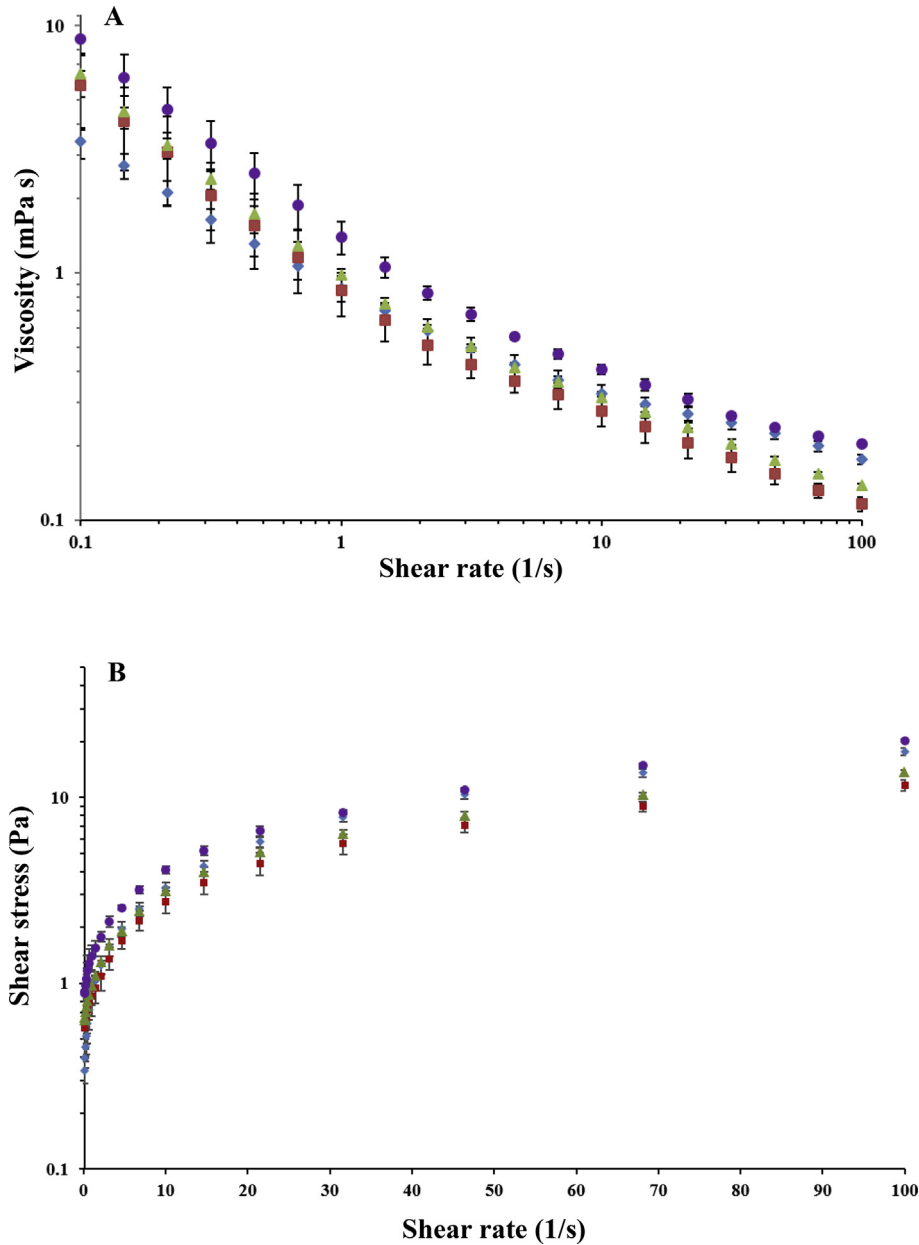


Fig. 4. Viscosity (A) and shear stress (B) of emulsions stabilised by either heat-denatured (\blacklozenge , 0 mM CaCl_2) or hydrophobised WPI (\blacksquare , 0 mM CaCl_2 ; \blacktriangle , 3 mM CaCl_2 ; \bullet , 5.5 mM CaCl_2) as a function of shear rate. Error bars indicate standard deviation.

Table 1
Herschel-Bulkley model of flow behaviour of emulsions.^a

Emulsion	τ_0 (Pa)	K (Pa s ⁿ)	n	R ²
He-E 0 mm	0.24 ^b	0.57 ^a	0.74 ^b	0.99
Hy-E 0 mm	0.40 ^b	0.49 ^a	0.68 ^c	0.99
Hy-E 3 mm	0.49 ^b	0.52 ^a	0.70 ^{bc}	0.99
Hy-E 5.5 mm	0.90 ^a	0.48 ^a	0.81 ^a	0.99

^a Abbreviations are: He-E, emulsions stabilised by heat-denatured WPI; Hy-E, emulsions stabilised by hydrophobised WPI.

ultrasonic irradiation proceeds by formation of acoustic cavities, as well formation of highly reactive radical species (Madadlou, Mousavi, Emam-djomeh, Ehsani, & Sheehan, 2009), which could enhance oil oxidation. The prooxidant effect of the emulsification process was most probably masked by the thiol groups present in

the heat-denatured WPI, but the hydrophobised WPI which likely had lower exposed thiol groups (due to extensive hydrophobic interactions before heat treatment) could not prevent early oil oxidation. A difference between the thiol groups contents of hydrophobised and heat-denatured WPIs has previously been noticed (Madadlou et al., 2018).

When CaCl_2 concentration increased from 0 to 5.5 mM, the hydrophobised WPI conferred a better protective effect on the emulsified oil, associated with the more compact packing of protein particles at the oil-water interface (Fig. 2). Besides, CaCl_2 supplementation increased the droplet size of the emulsion stabilised by the hydrophobised WPI. The surface area-to-volume ratio of larger droplets is lower than smaller droplets. Lethuaut, Métro, and Genot (2002) observed that higher interfacial areas of smaller droplets resulted in higher oxidation rates.

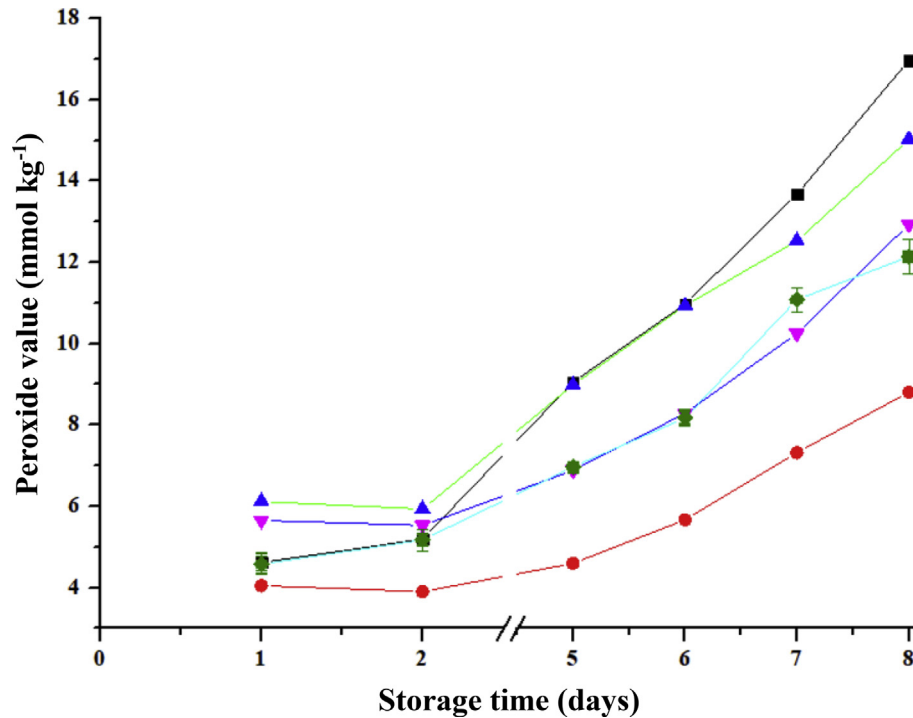


Fig. 5. Peroxide value of DHA-rich oil (■) and its emulsions stabilised by either heat-denatured (●, 0 mM CaCl₂) or hydrophobised WPI (▲, 0 mM CaCl₂; ▼, 3 mM CaCl₂; ◆, 5.5 mM CaCl₂) during storage at 20 °C.

4. Conclusion

Supplementation of WPI with CaCl₂ after heat-denaturation or hydrophobisation had significant influences on protein ζ-potential and the characteristics of resulting emulsions. We hypothesised that in addition to the expected charge interactions between Ca²⁺ ions and negatively charged proteins, Cl⁻ interacted with hydrophobised protein particles through grafted acetyl moieties. This hydrophobic concavity of Cl⁻ might be exploited at selective binding of Cl⁻ in the presence of other anions when designating artificial cells membranes; Cl⁻ is the sole inorganic anion used by cells at transmembrane gradients.

CaCl₂ supplementation of both heat-denatured and hydrophobised WPIs resulted in slightly lower γ values at the oil-water interface at any given time and did not affect γ reduction rate. Therefore, we conclude that the effects of CaCl₂ on emulsion properties (creaming, droplet size, rheology, and oxidative stability) are rather associated with changes at particles size and ζ-potential than γ.

CaCl₂ supplementation of hydrophobised WPI decreased the susceptibility of DHA-rich oil to oxidation during emulsification process. Further studies are required to address why hydrophobised WPI (without added CaCl₂) had higher peroxide values in early storage than non-emulsified oil, whereas, the oil stabilised using heat-denatured WPI had the lowest peroxide value throughout storage. At present, we attribute this observation to the less count of (surface) thiol groups in hydrophobised WPI particles and the prooxidant effect of mechanical-ultrasonic emulsification on oil.

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