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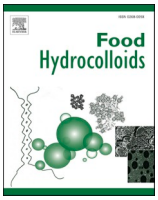

Characterising the concentration-dependent behaviour of heat-treated sunflower oleosomes at an air-water interface

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ABSTRACT

The behaviour of fresh and heat-treated sunflower oleosomes (oil bodies) at the air-water interface was studied using a force tensiometer with a conventional method and a new approach based on the fresh interface formation. Oleosomes were recovered using a wet milling process and washed to produce washed oil bodies (WOB), then dispersed as rinsed-washed oil bodies (RWOB) in ultra-pure water. Thermal treatment of oleosomes at 75 °C for 5 min did not alter the quality of oleosomes so it was used as the pasteurisation treatment. All oleosome preparations, including fresh RWOB, reduced surface tension (ST) at the air-water interface. Fresh RWOB exhibited faster, and more extensive ST reduction compared to heat-treated samples (HT-RWOB). Serum phase (a protein rich phase produced as part of the oleosome purification process) analysis indicated extraneous proteins contribute to ST reduction. ST measurements using HT-RWOB showed concentration-dependent ST reduction from 0.005% to 0.1% (w/v), with minimal changes at higher concentrations. Fitting a linear model on the concentration-dependent zone and concentration-independent zone showed that the cross over happens at the concentration of 0.1% (w/v) which is regarded as the critical packing concentration (CPC, the concentration by which the interface is saturated). Colloidal instability phenomena were observed at high concentrations. Particle size measurement and micrographs of emulsions before and after ST measurement indicate significant breakage and coalescence of oleosomes at high concentrations because of the formation of multiple layers at the interface after the CPC. This paper explores the impact of these changes on the ST and suggests possible explanations.

1. Introduction

Oleosomes (oil bodies or lipid bodies) are natural lipid droplets (emulsions) found in many plant sources, mainly plant seeds and nuts (Abdullah et al., 2020). In terms of plants, oleosomes can be found in every part of the organism; however, some parts such as seeds are the main storage source of such droplets (Song et al., 2017). Structural point of view, oleosomes consist of a triacylglycerol (TAGs) core surrounded with a monolayer of characteristic proteins and phospholipids (Tzen & Huang, 1992). This monolayer can provide oleosomes resistance to physicochemical stresses (Song et al., 2017). Oleosin, caleosin, and steroleosins are the most abundant proteins within the oleosome membrane. They play an integral role in the structural integrity and stabilisation of oleosomes (Nikiforidis, 2019). It has been reported that the specific proteins available within the oleosomes' membrane (mainly Oleosins) play a key role in oleosomes' stability (Abdullah et al., 2020). They are alkaline hydrophobic proteins with low molecular weight (around 20 kDa) that have a hydrophobic tail which penetrates the TAGs and an umbrella-shape hydrophilic part which covers the membrane (Jolivet et al., 2017). Oleosomes remain physically stable over a range of temperatures and pHs. Their membrane can protect the oil in the core from oxidation. Due to these attributes, and due to their credentials as a natural ingredient, they have been used in niche personal care applications, but there is a growing interest in their application in food and

Abbreviations: COB, Crude oil bodies; HT-RWOB, Heat-treated rinsed washed oil bodies; OB, Oil bodies; RWOB, Rinsed washed oil bodies; SDS-PAGE, Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis; ST, Surface tension; TAGs, Triacylglycerols; WOB, Washed oil bodies.

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pharma products.

Oleosomes have also been reported to be surface active at the air-water interface (the boundary between two different phases); this means that they can also migrate towards the air-water interface and stabilise it (Ntone et al., 2023). The surface activity of oleosomes can be attributed to the amphiphilic fragments in their membrane. Amphiphilic molecules have a specific structure which possess both hydrophilic and hydrophobic parts (Kresheck, 1975). They are able to stabilise emulsions by positioning their hydrophilic and hydrophobic moieties across an interface to reduce instability mechanisms (such as coalescence). Proteins, small molecular weight surfactants or particles (such as oleosomes) can all be surface active, although the mechanism of their action at the air-water interface can be different (Penfold & Thomas, 2010).

Pure water has been reported to have a surface tension of around 72.5 mN/m at 25 °C (Beattie et al., 2014). Surface active species can reduce the surface tension of water. The potential energy of the water molecules at the surface is usually higher than the energy of the molecules at the interior. Therefore, surface tension is defined as the amount of work required to bring sufficient molecules from the interior to the surface to expand it by unit area (Rosen & Kunjappu, 2012). The behaviour of oleosomes at interfaces has not been fully understood, although there have been some reports on the mechanisms of their positioning at the interface which provide good insights into this topic (Yang et al., 2021, 2022). Waschatko et al. (2012) reported that oleosomes tend to rupture, which leads to free oil and fragments of the membrane while coming to the air-water surface; while some research suggest that they can be intact while positioning at the interface (Karefyllakis et al., 2019). Yang et al. (2022) reported that when oleosomes and proteins are present in the system, oleosomes rupture at the interface which lead to a decrease in the foaming properties of the adsorbed proteins. These findings need to be verified to gain a better understanding of the oleosomes' behaviour at the interfaces. One hypothesis is that oleosomes' behaviour at interfaces is concentration-dependent and above a certain concentration (which we define as a critical packing concentration or critical packing parameter), their behaviour could be changed (due to the packing effects right at the interface). In addition, some phenomena such as packing, aggregation, and/or coalescence can affect the behaviour of oleosomes at the interface. The concentration-dependent behaviour at the interface is particularly interesting in foam stabilisation phenomena which can be used as a detrimental factor in several intended applications (such as ice cream, mousses, etc.).

The aim of this research is to study the effect of sunflower oleosomes on surface tension and gain a deeper understanding on the mechanism of their positioning and the factors affecting their behaviour at the air-water interface. In addition, a new method (based on the formation of fresh surface) has been developed to measure the changes of surface tension of oleosomes.

2. Material and methods

2.1. Raw ingredients and materials

Dehulled fresh sunflower (*Helianthus annuus*) seeds were supplied by Unilever (Colworth science park, UK). n-Hexane (purity of 99%) was purchased from VWR (VWR, USA). Sodium bicarbonate (purity of >99.7%) and sodium hydroxide (purity of 99%) were purchased from Honeywell (Honneywell Fluka, Germany). All the other reagents were at analytical grade and were from Sigma (Sigma-Aldrich, USA) or Merck (Merck Millipore, Darmstadt, Germany).

2.2. Preparation of oleosomes

The wet-milling recovery process of oleosomes or oil bodies is shown in Fig. 1. Sunflower oleosomes were prepared using a lab-scale extrusion juicer (Angel 8500s, Angel juicers, AM Naarden, the Netherlands) according to the method reported by De Chirico et al. (2020) & Romero-Guzmán et al. (2020) with some modifications. Firstly, seeds were soaked in sodium bicarbonate buffer (0.1 M, $NaHCO_3$, pH 9.5) in the ratio of 1:4 w/w at 50 °C for 1 h. After the mentioned time, the soaking buffer turned green because of the presence of chlorgenic acid in sunflower seeds which gets oxidised when exposed to the alkaline conditions. Then, the soaked seeds were drained and fed into the extruder. The initial extract from the extruder was called crude oil bodies (COB) and the resultant dry material (co-product) was named as cake. Then, COB was washed with $NaHCO_3$ buffer (0.1 M, pH 9.5; ratio of 1:7 w/w) and centrifuged (Beckman Avanti JXN-30 Series, Beckman Coulter, USA) for 35 min at 10.000×*g* at 4 °C. Following this, the top creamy layer was collected carefully and called as washed oil bodies (WOB). A further wash with deionised water (ratio of 1:7, 15% lipid-based) was repeated and the final cream was called as rinsed washed oil bodies (RWOB).

2.3. Thermal treatment of oleosomes

A thin layer technique was developed to pasteurise oleosomes. At first, oleosome suspensions (15% lipid-based) were made by adding the WOB cream into ultra-pure water. Then, samples were mixed by vortexing for 30 s. Four heating regimes were applied to the suspensions: (I) 63 °C/30 min; (II) 75 °C/0.5 min; (III) 75 °C/5 min; and (IV) 95 °C/6 min. A thin layer of the suspensions over a single-coated glass was exposed to the heating medium (water bath at 100 °C). Once the centre

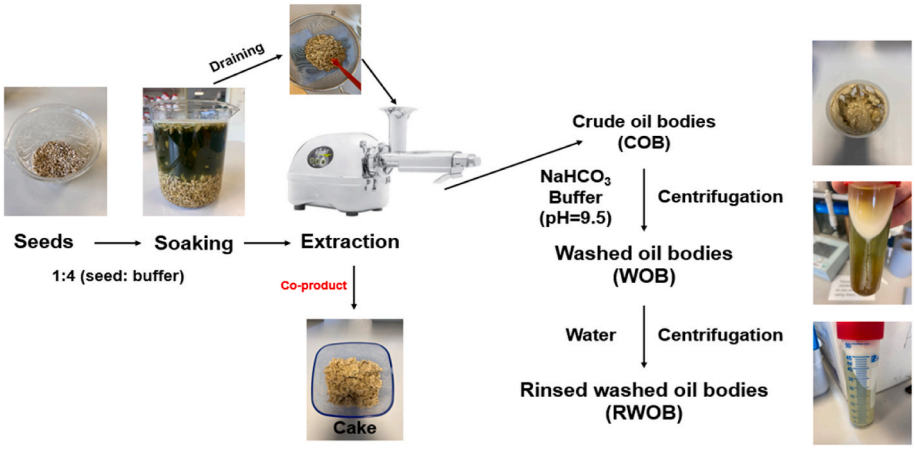

Fig. 1. A diagram of the steps involved in the recovery of oil bodied from sunflower seeds and their purification steps. Rinsed washed oil bodies (RWOB) it the final product of extraction.

of the sample reached the targeted temperature, they were transferred to another water bath (pre-set at the targeted temperature) and kept for the required amount of time, after which, samples were immediately cooled down in an ice-water bath. Finally, samples were centrifuged (10000×*g*, 35 min, 4 °C) (Beckman Avanti JXN-30 Series, Beckman Coulter, USA) and the top cream layer was collected and called HT-RWOB (heat treated rinsed washed oil bodies). The same process was repeated but without the heat treatment and the collected cream was named as fresh RWOB (non-heat treated rinsed washed oil bodies). All the resultant creams were tested for their protein and lipid content, protein profile, and total microbial content to determine the optimum heat treatment regime.

2.4. Purification of the serum

The serum phase after heat treatment and centrifugation was collected and purified as the following method: at first, hexane was added to the serum at the ratio of 1:2 to remove any remaining free oil. This was followed by the samples being vortexed for 30 s, followed by centrifuging (5000×*g*, 20 min, 4 °C) (Jouan CR3i multifunction Centrifuge, Thermo Fisher Scientific, Massachusetts, USA). Then, the upper layer and the middle layer (cream) were removed, and this process was repeated 2 more times on the lower phase (serum). After that, samples were filtered using a filter paper under vacuum. Finally, to ensure the serum was oleosome-free, a syringe filter (with the pore size of 0.45 μm) was used to filter the serum. The same procedure was used for the non-heat-treated serum (Fresh serum). All the purified samples were kept at 4 °C and analysed within 24 h of purification.

2.5. Proximate analysis of oleosomes

Total lipid and total protein contents were measured using the methods described in literature (De Chirico et al., 2018). Briefly, approximately 5 g of fresh oleosome samples (Fresh RWOB) were placed in a vacuum oven (Fistreem International Co. Ltd, Leicestershire, UK) (40 °C for at least 48 h or until constant mass value). For total lipid value, 0.2 g of the dried materials were weighed in microfuges and 1 mL of n-hexane was added to them. Then, all samples were grounded using a mini-bead beater (Mini-beadbeater-16, Biospec products, USA), following by centrifugation (Thermo Electron Cooperation, Fresco 21, Germany) and collecting the upper phase. The same procedure was repeated two more times and samples were dried under nitrogen until all the n-hexane was removed. Following this, the total lipid content was determined using Equation (1):

$$\textit{Total Lipid Content}\ (\%) = \frac{W_0 - W_1}{W_0} \times 100 \qquad \text{(Eq. 1)}$$

where W_0 and W_1 are the dry mass of the samples, before and after the delipidating process, respectively.

Protein contents were measured using Bicinchoninic acid (BCA) assay (Smith et al., 1985). First, 1 mL of 2% SDS solution was added to the dried delipidated samples. Then, all samples were vortexed for 30 s, followed by incubation at 60 °C for 30 min in a water bath. After cooling down the samples, they were centrifuged for 5 min at 4 °C at 17000×*g* (Thermo Electron Cooperation, Fresco 21, Germany). The supernatant was used for the protein quantification. Prior to sample analysis, a calibration curve was built, based on the standard solutions using bovine serum albumin (BSA).

2.6. Protein profile of the samples using sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The composition of protein extracts was evaluated *via* SDS-PAGE. First, samples were diluted in 1 mL of 2% SDS solution. Then, 20 μL of samples were mixed with 20 μL of reducing buffer (Laemmli buffer) and then heated for 5 min at 95 °C. Proteins were resolved by SDS-PAGE using 4–20% polyacrylamide gels (Criterion TGX Stain-Free precast gels, Bio-Rad, USA). SDS gels were positioned in a SE600 Bio-Rad separation unit and suspended in reducing buffer. The conditions for electrophoresis were as follows: voltage of 200 V for 45 min. After the electrophoresis process, the gels were imaged using Bio-Rad Gel DOC XR system (Bio-Rad, USA) with the gel activation time of 30 s.

2.7. Total aerobic microbial count

Total aerobic microbial count was measured according to the method previously described in literature (Aliyari & Rezaei, 2021). Briefly, six series of dilutions were prepared using the stock sample (original sample). Then, samples were applied to the counting medium (plate count agar, PCA which had been sterilised in an autoclave for 45 min at 121 °C) next to a Bunsen burner and were incubated for 48 h at 37 °C. Finally, the total number of colonies were counted, and total microbial content was calculated using Equation (2): Total aerobic colony cunt (ACC) was reported as colony forming units (CFU) per volume of sample (mL), where A is the number of colonies counted, D is the dilution factor, and V is the volume.

$$\textit{Total number of microorganisms}\left(\frac{CFU}{ml}\right) = A \times \frac{1}{D} \times \frac{1}{V} \qquad \text{(Eq. 2)}$$

All the measurements were repeated 5 times.

2.8. Lipase enzymatic activity

2.8.1. Enzyme solution from oleosome creams

Oleosome cream (1 g) was transferred in a 50 mL conical bottomtube and 20 mL of acetone was added. Then, samples were frozen (using liquid nitrogen), followed by an Ultra-Turrax (T18, IKA, Staufen, Germany) being used to break the oleosomes. After that, samples were centrifuged (6000×*g*, 20 min, 4 °C), and the supernatant was collected carefully. This washing protocol was repeated twice. The pellets were dried under nitrogen, and then suspended in sodium phosphate buffer (50 mM, pH 7.0) and 0.1% of Triton X-100 to avoid precipitations and agitated for 6h at 4 °C. Finally, samples were filtered using a syringe filter (pore size of 0.45 μm) and the clarified enzyme solution was stored at 4 °C and was used as the raw material for the lipase assay. All the experiments were carried on the same day.

2.8.2. Lipase assay

Lipase activity was measured using the method previously described by Ruiz et al. (2004) with a few modifications. Briefly, 20 mM of the p-NPL in 2-propanol solution was prepared and sonicated for 3 min. Then, a 1:10 (v/v) dilution in sodium phosphate buffer-Triton X-100 was made until the formation of a clear solution. Then, 1 mL of the enzyme solution (prepared previously) was mixed with the same amount of the substrate mixture (1 mM p-NPL, 5% 2-propanol, 0.6% Triton X-100, 50 mM sodium phosphate buffer). The final mixture was incubated at 40 °C for 90 min. After cooling down the samples in ice, the absorbance of samples was measured using the spectrophotometry procedure (at 405 nm) and was compared with the blank sample (the reaction mixture without the enzyme solution). Prior to the experiments, a standard curve was built using the commercial lipase. Results were reported as enzymatic activity unit (U) which is the activity of enzyme that can release 1 μmol of p-NP and normalised by the amount of protein in 1 g of the cream, considering the mass balance before and after centrifugation was maintained.

2.9. Surface tension measurements

Surface tension of the samples was measured using a force tensiometer (Attension Sigma 700, Biolin Scientific Inc., Sweden) using a Du

Nouy ring made of platinum and iridium with the radius of 9.58 mm and the thickness of 0.1850 mm. The Huh-Mason model was used as the correction factor. Two methods were used to evaluate the interfacial behaviour of oleosome O/W emulsions. In the first method, O/W emulsions (using HT and fresh creams) were prepared at different concentrations (0.005, 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1% w/v cream-based). The samples were next put into the measuring cuvettes, to begin the analysis, and the behaviour of the samples were recorded over time.

In the second method, ultra-pure water (49 mL) was added to the measuring cuvettes and analysis started. Then, immediately after beginning the measurements, 1 mL of the o/w emulsions were gently added to the edges of the cuvette by a pipette so that the final concentration of the samples was fixed at the previously mentioned concentrations. A control with only water added was used to confirm that there is negligible interface disruption with the addition of 1 mL. Precautions of time were taken to ensure that equilibrium was reached, and equilibrium was defined as 10 consecutive surface tension measurements with <0.5 mN/m deviation. Prior to measurements, density of all samples were measured using the density measurement apparatus provided by the same manufacturer (glass probe).

All the measurements were conducted at 20 °C and repeated at least 3 times.

2.10. pH value

pH value of the emulsions was determined using a pH meter (Mettler-Toledo AG FiveEasyTM FE20, 8603 Schwerzenbach, Switzerland). Prior to the measurements, the probe was calibrated at 20 °C and all the measurements were carried out at the same temperature.

2.11. Particle size distribution of oleosome emulsions

Particle size distribution of oleosome emulsions was assessed with the laser diffraction method according to the method reported by De Chirico et al. (2018). Briefly, O/W emulsion samples at certain concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0% w/v, cream-based) were applied to a laser diffraction particle size analyser (LS 13320, Beckman-Coulter, USA). The real refractive index was set to 1.47161 which is the refractive index of sunflower oil. Analysis was done once the obscuration value reached to the sufficient level.

2.12. Zeta potential measurements of oleosome emulsions

Zeta-potential of oleosome emulsions were measured using a litesizer DLS (Anton Paar light sizer 500, Anton Paar GmbH, Graz, Austria) at their native pH value (around 8.0). Firstly, the concentration of samples was fixed at 0.01% (based on lipid content) ((De Chirico et al., 2018), with slight modifications) and then injected into the measurement cells and placed in the equipment for analysis. All the measurements were done at 20 °C.

2.13. Bright field and fluorescence imaging of oleosome emulsions

An optical microscope (Nikon H600L, Japan) was used to capture the pictures from the oleosomes' microstructure according to De Chirico et al. (2018). Samples were positioned on a glass slide and covered with a coverslip. The magnification for imaging was 40 × .

For the fluorescence technique, a fluorescence microscope (EVOS FL Auto, Thermo Fisher Scientific, USA) was used according to Vardar et al. (2024). Samples were stained with Nile red (0.001%, w/v) and observed with a magnification of 40 × .

2.14. Statistical analysis

All the experiments were repeated in triplicate (except for those mentioned otherwise). A one-way analysis of variance (ANOVA) using SPSS software version 26 (SPSS software, V26, SPSS Inc., Chicago, USA) was used to study the data. The significant difference among samples were assessed based on a 95% confident limit using Duncan's test. All the graphs were plotted by OriginPro software version 2022b (OriginLab, MA, USA).

3. Results and discussion

To understand the behaviour of sunflower oleosomes at the air-water interface, two oleosome systems (fresh and heat treated) were considered. Thermal treatment can be important to see the real effects of oleosomes as it minimises the presence of external parameters (e.g., exogenous proteins, lipase, microorganisms, etc.) which can hugely impact the surface tension results (Whitnah, 1959) and cause a misunderstanding of the actual oleosome surface adsorption behaviour. In addition, the degree of thermal treatment can influence the oleosomes properties and even can lead to a loss in their quality (e.g., membrane protein denaturation, colloidal instability, etc.). Hence, thermal treatment conditions were optimised first to gain the best oleosome quality prior to surface tension measurements.

3.1. Thermal treatment optimisation

Heat treatment is a necessary step in food industries to make food-grade, safe materials. Heating oleosome preparations reduces the microbial load and endogenous enzyme activity (both of which can lead to significant quality deterioration), removes extraneous proteins, and can affect the tendency of oleosomes to remain separated or to aggregate.

Lipid values stayed high (93–95%, DWt) for all oleosome preparations (Supplementary file, Table S1). As RWOB is the last washing step (centrifugation + cream collection + drying), the purification of oleosomes leads to higher (dry-weight basis) lipid contents, compared with the first crude material (COB) (De Chirico et al., 2018). Protein content data of HT-RWOBs and fresh RWOB (Table 1) suggest that there was a significant decrease of protein content in the heat-treated samples, compared with the fresh RWOB and amongst the samples, it seems that in both extreme regimes (either long time and/or high temperature, i.e., 63 °C/30 min and 95 °C/6 min), the protein content was the lowest; while those samples treated at 75 °C seem to have had lower reduction rates. This significant reduction in the amount of protein shows that some exogenous proteins have been removed from the oleosome cream (Zhou et al., 2022). In addition, the protein content of crude oil bodies (COB) was measured as 19.52% (DWt). There was a significant decrease in the protein content from COB to Fresh RWOB which suggests exogenous proteins are removed during the washing/purification steps. Although during the recovery of oleosomes and subsequent washing

Table 1
Total protein content, aerobic colony count (ACC), and average particle size of non-heat-treated (Fresh) RWOB and Heat treated (HT) RWOBs.

Sample	Protein Content (%, DWt)	ACC (log CFU/mL)	D4,3 (μm)
COB	19.52 ± 0.58^{a}	N/A	N/A
Fresh RWOB	4.76 ± 0.10^{b}	9.98 ± 1.02^{d}	1.24 ± 0.20^{g}
HT-RWOB (63 °C/30 min)	1.60 ± 0.25^{c}	0.66 ± 0.13^{e}	3.85 ± 0.09^{h}
HT-RWOB (75 °C/0.5 min)	3.76 ± 0.11^{b}	1.11 ± 0.31^{f}	1.18 ± 0.07^{i}
HT-RWOB (75 °C/5 min)	3.54 ± 0.07^{b}	1.03 ± 0.17 ^{ef}	1.21 ± 0.12^{i}
HT-RWOB (95 °C/6 min)	1.78 ± 0.09^{c}	0.91 ± 0.10^{ef}	3.12 ± 0.09^{h}

*Error bars are expressed as mean ± STD.
**Values with different letters in a column show significant differences ($p < 0.05$).

steps, there is a reduction in the amount of proteins, heat treatment also seems to also cause a reduction. These findings agree with the results previously reported by Ding et al. (2020) who reported possible denaturation and unfolding of the surface proteins of oleosomes after thermal treatment. Data in Table 1 also suggest that treatment of oleosome suspensions at a mild temperature (75 °C) for a short amount of time (0.5 and 5 min) can preserve the proteins within the resultant cream after the washing step better compared to the other treatments (for example, protein content was around 4% after treating at 75 °C for 5 min and less than 2% after treating at 95 °C for 6 min).

These data suggest that protein denaturation caused by thermal treatment results in the proteins being released into the serum phase and separated from the cream during centrifugation. Therefore, when measuring protein content of the oleosome cream using BCA method, a reduction in the protein content of a thermally treated oleosome cream compared to a COB and RWOB was observed. The protein reduction in the cream is accompanied by an increase in the protein content of the aqueous phase after centrifugation (Data not shown). The serum from the fresh RWOB and HT-RWOB (75 °C/5 min) contain 8.0% and 10.2% of proteins, respectively.

The protein profile of RWOB and HT-RWOB creams is shown in Fig. 2. The band related to the fresh RWOB (line 1), shows bands around 20, 27, and 50 kDa which are representatives as oleosin, caleosin, and steroleosins, respectively. In addition, there are some bands in the higher molecular weight ranges which suggest the presence of exogenous proteins in the cream even in the last wash. Zhao et al. (2016) reported that although the protein composition is highly dependent on the extraction technique, some extrinsic proteins can inevitably remain in the cream even after several washing steps with $NaHCO_3$ buffer and/or pure water. However, as shown in Fig. 2, after doing the thermal treatment at 63 °C and 95 °C (lines 2 and 3, respectively), no obvious bands could be observed; and as protein content data suggests,-35 there is a significant loss in the amount of proteins. On the other hand, thermal treatment at 75 °C for 5 and 0.5 min in lines 4 and 5, respectively, could

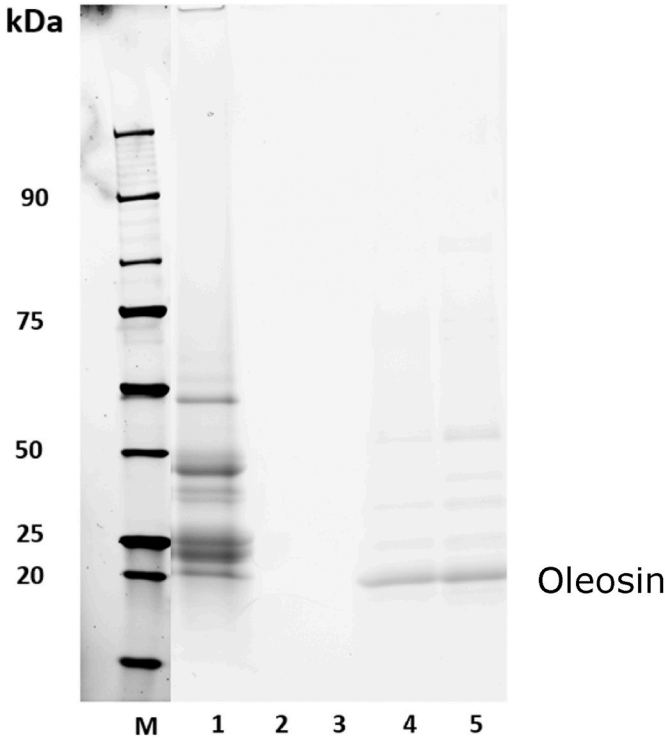

Fig. 2. SDS-PAGE analysis of the oleosome samples (M: standard; lane 1: fresh RWOB, 2: 63 °C 30 min, 3: 95 °C 6 min, 4: 75 °C 5 min, and 5: 75 °C 0.5 min). Lanes were loaded using equal volume of samples.

preserve the proteins more efficiently and more bands are observed compared to the other heating regimes. In addition, oleosins seem to still be available in the heat treatments conducted at 75 °C although in less quantity. Presence of oleosins in the final oleosomes after thermal treatment is important as they are reported to act as oleosomes' integral components to avoid coalescence. Oleosins seem to be temperature-sensitive like many proteins and denaturation of them is common when exposed to excessive heat (Huangfu et al., 2024).

Microbial load of the heat-treated samples compared to the control (fresh) is shown as Table 1. The control sample (fresh RWOB) had the highest number of total aerobic colony count because there was no thermal treatment applied to reduce its microorganisms; however, after applying heat to the samples, the aerobic colony count (ACC) reduced and the reduction was significantly higher in samples with either extreme time or extreme temperature (i.e., regimes of 63 °C for 30 min and 95 °C for 6 min). However, for all the heat treatment regimes, the total number of microorganisms were lower than the acceptable limit (100 CFU/mL). These results show that all the tested time/temperature regimes were effective enough to lower the total number of microorganisms to an acceptable value. Heating can slow down or stop the microbial activity by affecting the protein structure of the microorganisms' membrane and as a result, they can be deactivated (Tsai & Tikekar, 2023). Previous research has shown that at 4 °C, some microbial variants such as *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Aeromonas*, and *Achromobacter* are dominant (Ozer & Akdemir-Evrendilek, 2014) and applied heating regimes were able to reduce their number to an acceptable level.

Particle size distribution can be used to evaluate an emulsions stability (McClements, 2004). Particle size distribution of the HT-RWOBs versus fresh RWOB is shown in Table 1. The majority of the oleosomes were around 1 μm in diameter in both fresh and HT-RWOBs. However, there was a second peak observed in HT-RWOBs (distribution data not shown) when treated at 63 °C/30 min and 95 °C/6 min, which is probably due to oleosome flocculation (Ding et al., 2020) that led to higher D4,3 values. However, D4,3 did not change significantly with the other two thermal treatment regimes, which shows that the quality of oleosomes did not change by applying heat at 75 °C for 0.5 and 5 min. Considering the data shown in Table 1, the emergence of the second peaks in the extreme time and/or temperature could be related to the loss of oleosins (Fig. 2) in the cream after applying those heating regimes which negatively affected the stability of oleosomes.

Zeta potential can be used as an indication of the stability of the proteins that stabilise emulsions as the net charge density of the proteins at the oleosomes interface can be inferred from the electrophoretic mobility data (De Chirico et al., 2020). Zeta potential values were measured at pH value of around 8; across the samples the zeta potential did not vary significantly, staying in the region of between −29 and −36 mV (Supplementary file, Table S1). These values are still sufficiently high to prevent flocculation (i.e. maintain electrostatic repulsion), so there must be another reason for larger particle sizes emerging in heat treating oleosomes for a longer time or higher temperature. Although the protein profile showed a reduction in oleosins, other negatively charged small weight species present in oleosomes seem to contribute to the overall negative charge of oleosomes charge.

Results suggest that the thermal treatment regime at 75 °C for 5 min had the minimum change in the protein content while the ability to reduce the level of microorganisms to the acceptable content and the stability of HT-RWOB resulted from that treatment was high compared to the other heat treatments. As a result, that regime was selected for the rest of the experiments.

3.2. Enzymatic activity

Lipase enzymes hydrolyse TAG molecules, releasing free fatty acids. Thus, removing or inactivation of lipase can prolong shelf-life and postpone samples' degradation. De Chirico et al. (2020) reported that

the alkaline pH value of the soaking buffer ($NaHCO_3$) can prevent the carry-over of the enzymes and exogenous seed materials to the oleosomes; however, enzymatic activity cannot be stopped completely, and it is still found in the oleosomes (Katavic et al., 2006; Zhao et al., 2016). Bhatla et al. (2009) reported that in some seeds, lipase seems to have a physical association with the oleosomes, probably because of the bindings between the enzyme and oleosome membrane proteins.

The activity of lipase enzymes present in a RWOB oleosome preparation is reduced on heat treatment (75 °C for 5 min); the reduction of lipase activity increases with the time that oleosomes are held at 75 °C, and this reduction appears to be linear over heating time (5 min, Fig. 3). The initial enzymatic activity at time 0 was 0.093 U/mg, dry-weight basis (DWt), while after heat treatment at 75 °C for 5 min, the activity was reduced to 0.008 U/mg DWt. One possible scenario is that due to the structural change of the enzyme, it moved to the serum phase during the centrifugation and/or, the structural change (because of the heat treatment) could inactivate the activity of the enzyme. The data also indicates that a 5 min treatment of an RWOB suspension at 75 °C is high enough to inactivate almost all the lipase.

3.3. Effect of oleosomes on surface tension at the air-water interface

Surface active species can be adsorbed at the interfaces and reduce the surface tension of water (Prosser & Franses, 2001). The adsorption process starts with diffusion and followed by re-arrangement and orientation over time to reach the equilibrium state. It has been reported that oleosomes and their membrane fragments are surface active materials (Ishii et al., 2017). However, exogenous materials and enzymes (mainly lipase, due to releasing free fatty acids) could possibly affect the interfacial behaviour. To check this hypothesis, a comparison between thermally treated and non-thermally treated samples was conducted. Thermal treatment is an anticipated industrial process that would stabilise oleosomes by deactivating enzymes and reducing the microbial load. The effect of non-heat treated oleosomes on surface tension is shown in Fig. 4. Prior to the measurements, the surface tension of ultra-pure water was measured (20 °C) as 72.82 ± 0.05 mN/m. The change in the values of surface tension reflected the concentration of the oleosome emulsions. However, when comparing the trends with those which were thermally treated (HT-RWOB, Fig. 4), the time needed to reach the equilibrium was significantly lower than for fresh RWOB samples. For example, at concentration of 1% HT-RWOB, it took only 35 min to reach the equilibrium; while for the same concentration fresh RWOB, 298 min was needed to reach the equilibrium. The difference in

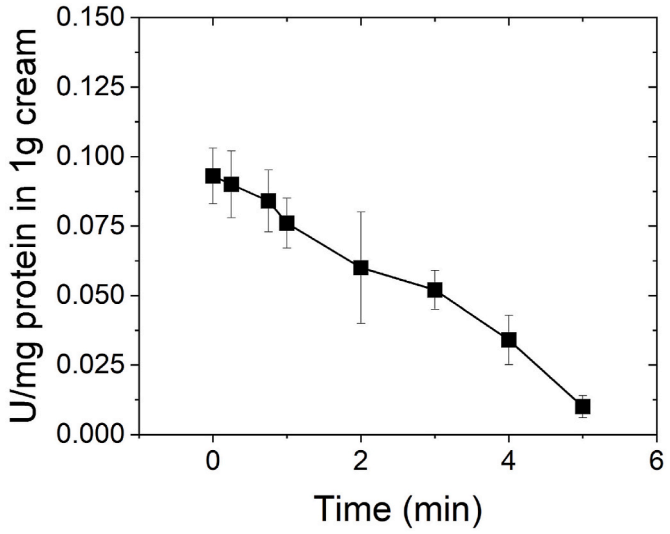

Fig. 3. Lipase activity of oleosome suspension (15% lipid-based) during the thermal treatment at 75 °C for up to 5 min.

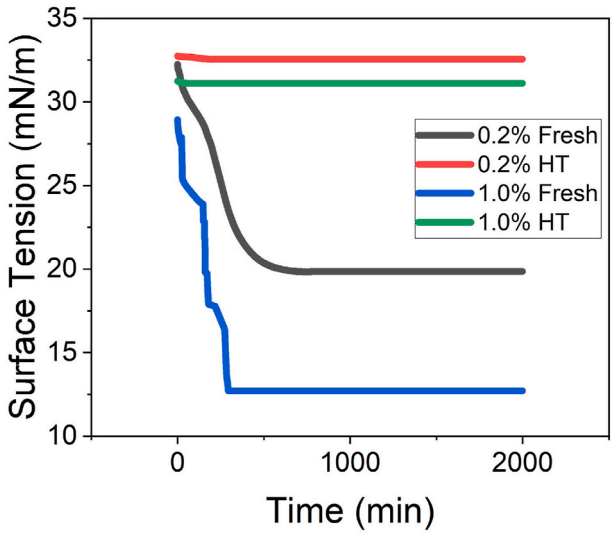

Fig. 4. Comparing the effect of fresh and HT-RWOB (two oleosome O/W concentrations) on surface tension (Temperature of the measurements: 20 °C).

the time for samples to saturate the whole surface area could be due to several reasons. Firstly, microorganisms present in the samples can have high surface activity because of their hydrophilic/hydrophobic structure (Shou et al., 2023; Villanueva et al., 2023). Secondly, many of the extraneous (such as globulins) and integral proteins (such as oleosins) are considered to be surface active (Yan et al., 2022); and thirdly, free fatty acids formed by various factors (mainly lipase) can move to the surface due to their strong surface activity (Waraho et al., 2011). Although a full understanding of the behaviour of these species is difficult due to the complexity of their interactions, It has been reported that exogenous proteins can make bridges between oleosome and as a result, stop or delay them from moving towards the interface (by limiting their movements) which can also explain the difference in the results from the fresh and HT samples (Karefyllakis et al., 2019).

As shown in Fig. 4, it is not possible to collect the data in the initial moments of the experiments for the different systems, which is due to the nature of the instrument and applied method (Kitabatake & Doi, 1982). As measuring interfacial tension with the force tensiometer involves making samples and inserting them into the cuvettes and then starting the run, there are always some data losses as surface active compounds rapidly diffuse to the interface. Waschatko et al. (2012) reported that because of the buoyancy effect and the natural structure of oleosome as amphiphilic materials, they can be adsorbed to the interface very fast. That is why a very steep declining profile of surface tension has been observed in most of the tested concentrations. As sunflower oleosomes are considered as micron or sub-micron droplets, their diffusion rate is quite fast. According to the Stokes-Einstein law (Equation (3)), there is an inverse relation between the diffusion rate and the radius of the particle.

$$D = \frac{kT}{6\pi r\mu} \quad \text{(Eq. 3)}$$

Where, D stands for the diffusion rate, k is the constant of Boltzmann (1.38×10^{-23} m^2 kg s^{-2} K^{-1}), T is the temperature, r is the particle's radius, and μ is the viscosity.

It should be noted that Stokes-Einstein law considers particles are in the form of spheres, which is valid for oleosomes that have been reported to be mainly spherical particles (Nikiforidis, 2019).

In order to have a better understanding of the initial adsorption, we proposed a hypothesis based on creating a fresh surface by adding the emulsions to water when the Du Nouy ring was already positioned at the air-water interface. The new proposed method was to apply 1 mL of

samples to the edges of the cuvette (which had been filled with 49 mL of ultra-pure water) after running the experiment, without disrupting the interface. The key difference among the conventional and our proposed method is that in the conventional method, the whole sample (in this case O/W emulsions) goes to the measuring cuvette and then, the movement of the ring starts to determine the force per unit area. However, the new method involves in applying the sample when the probe has already started its movement. Thus, a fresh surface can be made and as a result, the initial changes in ST can be monitored better. Data in Fig. 5 show the behaviour of the HT-RWOB samples running through the new developed method. There was a concentration-dependent behaviour of oleosomes with the new assay (Fig. 5) and it could be seen that with the new method (Fig. 5), data loss was lower than the conventional method (Fig. 4) and all the concentrations had higher initial surface tension starting values. This new method also did not disrupt the interface as ultra-pure water was also tested as a negative control. As a result, higher initial values of surface tension could be observed with the creation of fresh interface during running the experiment without disrupting the interface. This can be of great importance as monitoring the initial behaviour (reduction) of surface tension can give us an insight into the oleosomes' adsorption kinetics to the surface and/or interface.

Regarding the stabilisation of the air-water surface by oleosomes, there are two different scenarios proposed in the literature: the first scenario suggests that oleosomes rupture and coalesced free oils along with the membrane fragments stabilise the interface (Waschatko et al., 2012); the second scenario explains that oleosomes remain intact while moving towards interface and stabilise the interface by themselves as intact particles (Bettini et al., 2014). To understand the behaviour more, more tests have been done on the HT-RWOB O/W emulsions (as with those samples, a better understanding of the surface tension will be gained due to removing the other surface-active species described above).

Regarding the difference in the time needed for each sample to reach the equilibrium state (Fig. 6a), as oleosomes concentration increases, the time needed for the surface tension values to reach the equilibrium decreases. This observation can be explained because of the Brownian motion behaviour: when there are higher number of particles available in the system, the availability of them to move towards the interface is higher (Ho et al., 2022); compared to the low concentrations where the number of droplets is low and they need to travel a longer distance to get to the interface and as a result, there will be a longer time required for them to saturate the surface area completely.

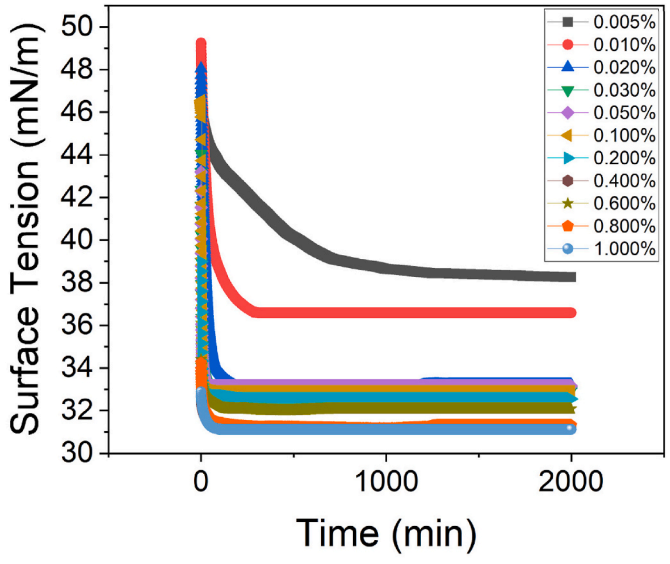

Fig. 5. Surface tension of HT-RWOB oleosome O/W emulsions at different concentrations using the newly proposed method.

In addition, at higher concentrations, if there is any breakage rupture of the droplets at the interface, membrane fragments can aggregate, and can also move towards the surface very fast and saturate the interface. Oleosins, for example, are able to form aggregates (because of their hydrophobic structure) and those formed micelles can position themselves at the interface (Vargo et al., 2014); however, their displacement from the membrane is not a common thing to happen; so, it can be assumed that in case of oleosome rupture at the interface, free oil and micelles of oleosin/phospholipids are responsible for saturating and stabilising the interface (Nikiforidis & Kiosseoglou, 2011). Another possible scenario is that due to the thermal treatment, oleosins were denatured and because of the structural change (possible unfolding in the hydrophobic region), they either make clusters with TAGs and/or phospholipids or with other oleosin molecules. These clusters could then adsorb to the interface (Waschatko et al., 2012).

According to data displayed in Fig. 6b, the values of surface tension reduced as the concentration of oleosomes was increased. Above 0.1%, there was not significant changes in the surface tension values at the equilibrium. The low values of surface tension of oleosomes compared to other surface active components can be because of the instability mechanisms (primarily partial coalescence) happening during the extraction of oleosomes which leads to the less stable droplets which can saturate the interface (Karefyllakis et al., 2019).

To calculate a critical packing concentration of oleosomes at the air-water interface, the equilibrium surface tension for the series of concentrations were plotted against the concentration on a semi-log plot. Double linear regressions were fitted, where, the first regression was below the critical packing concentration, a regime where the surface tension is dependent on oleosome concentration, while the second regression fitted the data above critical packing concentration, where the surface tension is independent of oleosome concentration. The critical packing concentration was then determined from the intersection between the concentration-dependent and -independent regions, i.e., concentration of 0.1% (w/v) (Fig. 7). The scaling factor for the packing in the concentration-dependent region was observed to be $\sigma_{equil.}^{\sim} \approx c^{0.36}$ (Fig. 7, inset), defined as non-logarithmic surface tension increase by least-square fitting and employing the Levenberg-Marquardt algorithm to a Power-law equation, $\sigma = C_p c^{n-1}$, where C_p is the packing coefficient ≈ ca. 6 (dimensionless), c is the oleosome concentration, and n is the oleosome packing index. This was significantly slower in the concentration-independent region $\sigma_{equil.}^{\sim} \approx c^{0.05}$. We postulate that the mass transport of oleosomes from an initially uniform bulk phase onto a freshly created air-water interface in the quiescent solution could be described in terms of adsorption consisting of at least two processes: (i) diffusion between the bulk and subsurface and (ii) adsorption-desorption between the subsurface and interface. Beyond, c > 0.1 % (w/v) the interfacial phenomenon would be predominantly governed by the latter process. At packing concentrations >0.8%, w/v, it appears that high oleosome packing and adsorption at the interface results in a contact-enhanced flocculation, enriching the subsurface and the interface with coalesced oil droplets. We captured this phenomenon of contact-enhanced coalescence at > 0.8–1.0% (w/v) oleosome packing concentrations using laser diffraction and complementary microscopy, and they are discussed in Sections 3.5 and 3.6.

3.4. The effect of serum phase composition on surface tension

Oleosome emulsion are dispersions containing droplets, i.e. oleosomes, and a continuous aqueous phase with natural seed proteins, with these playing a role on the surface behaviour of the emulsions. To elucidate the role of the continuous phase only (i.e., oleosome-free serum phase obtained after centrifugation and filtering), surface tension measurements were performed using the serum phase only. Data shown in Fig. 8 refer to the effect of the oleosome-free serum phase on surface tension. The main compounds available in the clarified serum

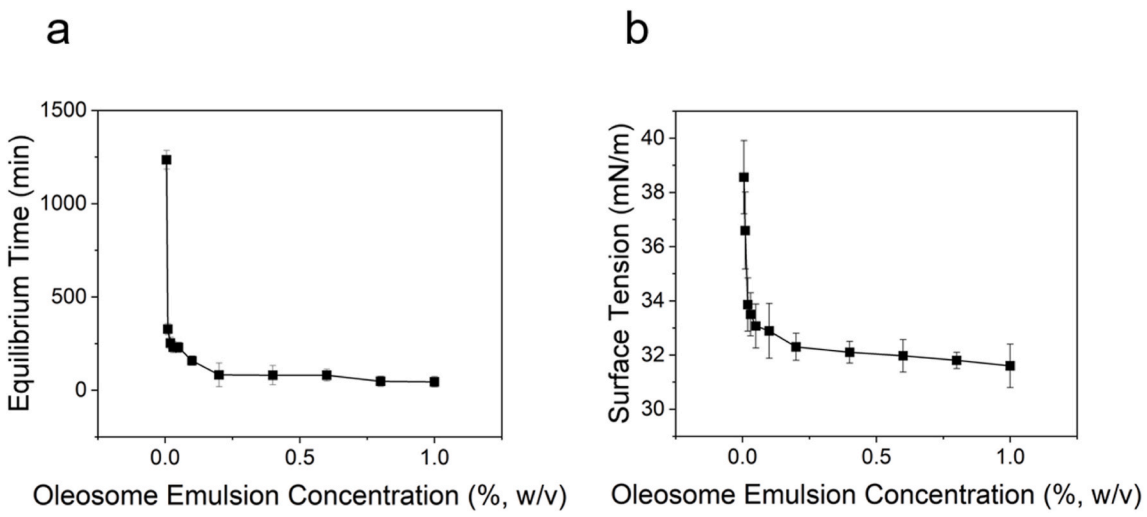

Fig. 6. (A): Time required for each emulsion concentration to reach the equilibrium state; (B): values of surface tension at the equilibrium for each emulsion concentration.

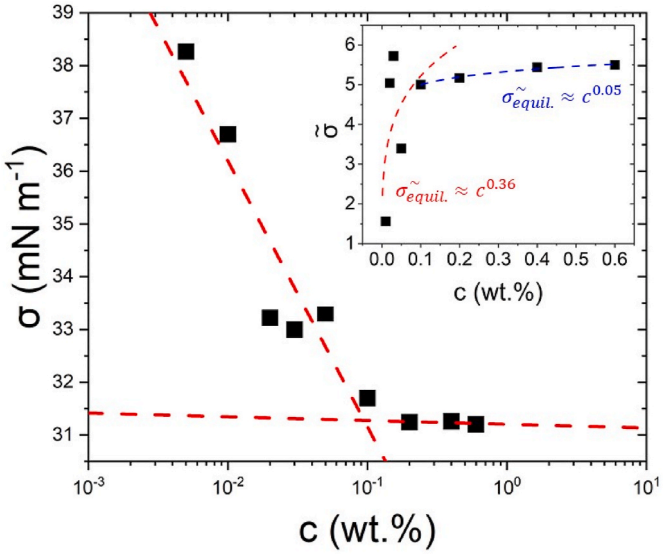

Fig. 7. A semi-log plot of surface tension vs. oleosome concentration series. The cross over point (i.e., concentration of 0.1% w/v, is the critical packing concentrations of oleosomes at the air-water interface).

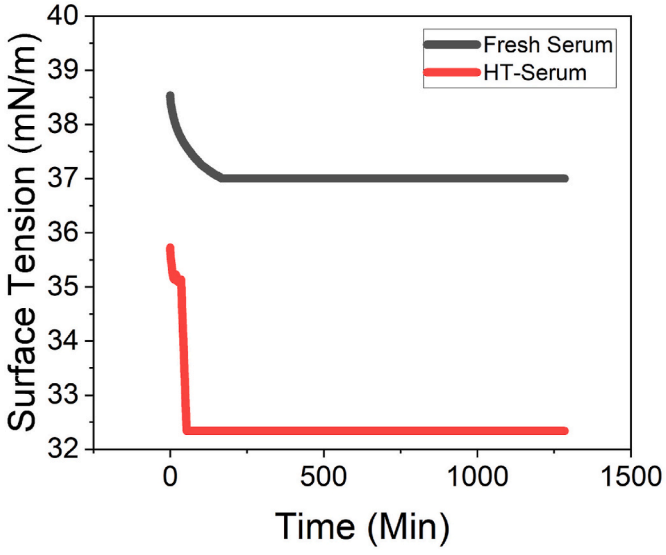

Fig. 8. The effect of fresh and HT serum on surface tension.

are water-soluble proteins such as globulins and albumins (Kaur & Ghoshal, 2022). Prior to measuring the surface tension of serums, their protein content was measured, and it was found out that HT-serum had higher protein content compared with the fresh serum. This is likely due to the denaturation of proteins and possible changes in their secondary and tertiary structure, meaning they have been more readily solubilised into the aqueous phase (Ding et al., 2020) and as a result, they moved from the cream phase into the serum phase. Furthermore, the mass balance was maintained during the whole process of the purification, considering the protein content of the first wash (WOB), RWOB, serum, and pellet phases. As shown in Fig. 8, both serums (HT and fresh) could reduce the surface tension and showed surface active behaviour. In addition, there was a difference in the time by which HT and fresh serums reached the equilibrium which shows that the rate of decay in the surface tension was different between these samples (Kitabatake & Doi, 1982). This observation can be explained by the protein content of HT and fresh serum. After thermal treatment, there was an increase in the protein content of the serum phase compared to the fresh serum (data not shown) due to protein structural change and a rise in the water solubility (mainly because of the exposure of amino acid residues to the environment). In a study, Poirier et al. (2021) described that sunflower seed storage proteins (mainly globulins and albumins) are able to saturate the interface and create a monolayer; then, they can create a thick film layer if they are left for a long time. This process can also be facilitated by the presence of other compounds such as polyphenols.

3.5. Particle size distribution

Particle size distribution of the samples is shown in Fig. 9. According to the results, for most of the concentrations, the major peak was around 1 μm which is comparable with the control and shows that they remained intact even after running the surface tension measurements (De Chirico et al., 2018). Karefyllakis et al. (2019) showed that sunflower oleosomes are usually spherical droplets ranging from sub-microns to 1 μm which is in line with the obtained results of this study. However, both high concentrations (0.8% and 1.0%) showed a second peak around 10 μm which is probably because of the partial or complete coalescence. These findings also suggest that at both high

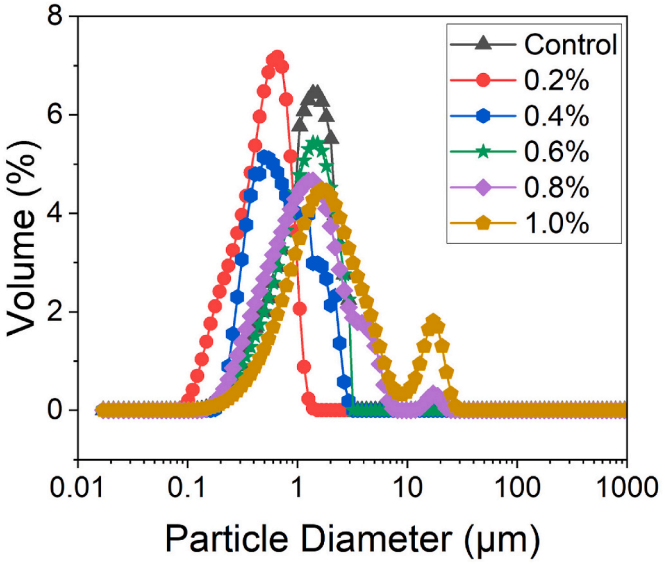

Fig. 9. Particle size distribution of oleosome emulsions after measuring surface tension (after the equilibrium point) compared to the control (freshly made emulsion).

concentrations (i.e., 0.8 and 1.0 %, w/v), oleosomes might have broken and their core oil released, resulting in the formation of larger droplets mainly due to strong hydrophobic forces (Waschatko et al., 2012). Karefyllakis et al. (2019) also reported that the presence of exogeneous proteins, even at a low concentration after washing steps, as (confirmed *via* SDS-PAGE analysis (Fig. 2)), can act as a bridge between two particles. Thus, although the amount of total protein decreased significantly after the thermal treatment and further centrifugation, the presence of even low concentrations of exogenous proteins could promote the aggregation of oleosomes, leading, possible to coalescence.

3.6. *Brightfield and fluorescence micrographs*

To validate the data observed with the particle analyser (Fig. 9), light micrographs were captured, and the results are shown in Fig. 10a. Oleosome emulsions were observed at different concentrations (from 0.2 to 1.0%) after doing the ST analysis. As light microscopy pictures in Fig. 10a show, there was no signs of colloidal instability (e.g., coalescence, aggregation, etc.) observed at low concentrations; however, pictures of the two last concentrations (0.8 and 1.0%) showed some larger droplets which could be a sign of oleosome rupture.

In addition, to confirm the hypothesis of oleosomes' rupture at high concentrations, the sample with the concentration of 1% was chosen for the fluorescence images and the images were compared with the freshly made emulsions at the same concentration. As Nile red is a hydrophobic stain, it can be used to observe any free hydrophobic part (for example TAGs) present in the sample (Fowler & Greenspan, 1985). As the

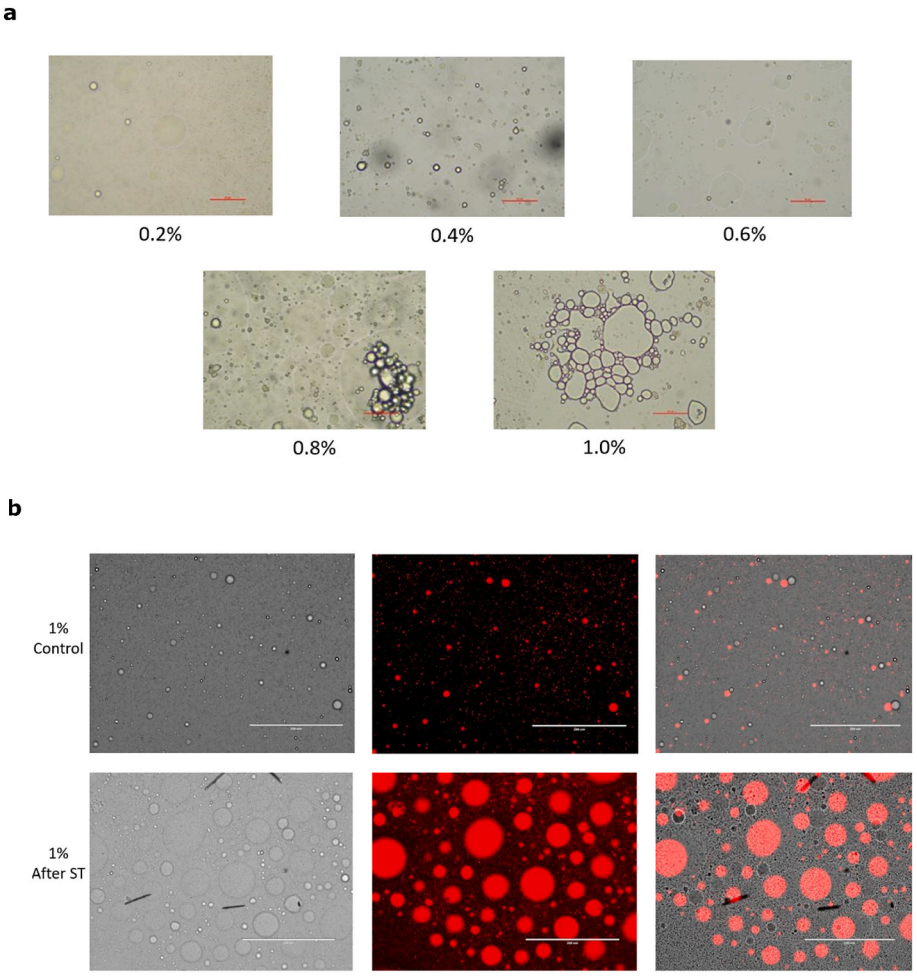

Fig. 10. Light (a) and fluorescence (b) micrographs of oleosomes at different concentrations (Scale: A) 50 μm; B) 200 μm). Light micrographs were taken after ST experiments.

fluorescence micrographs show (Fig. 10B), there are significantly higher amount of coalesced oil in the emulsion analysed after running surface tension measurements in comparison with the fresh emulsion. These pictures are in line with what was observed in the particle size experiment and they, together, confirm that at high concentrations (0.8 and 1.0%), oleosomes seemed to rupture while saturating the interface. Pickering particles can stabilise the interface through a mechanism called Pickering Stabilisation which means that solid particles can stabilise the interface without any deformation upon the adsorption to the interface. Small oleosome particles seem to act a Pickering stabiliser as they remained intact while positioning themselves at the interface (Abdullah et al., 2020).

4. Conclusion

This study has two phases: at first, the impact of heat treatment on the surface adsorption of oleosomes was investigates and then, the behaviour of heat-treated oleosomes at the interface was studied as a function of their concentration. Prior to the experiments, 15% (lipid-based) oleosome suspensions were made and the best heating treatment (HT) to attain the minimally damaged emulsions was selected. Fresh RWOB showed much longer equilibrium time compared to the HT-RWOB. This observation was confirmed by observing a significant decrease in the lipase activity (which involves in lipid hydrolysis) of oleosomes during the heat treatment. According to the surface adsorption behaviour of HT-oleosomes, it can be stated that their behaviour is highly concentration-dependant means that below the critical packing concentration (CPC), they form a monolayer at the interface and remain intact and above the CPC, (due to the formation of multiple layers) they rupture, and this causes colloidal destabilisation at the interface. The data suggests that the mechanism of the oleosome stabilisation at the interface is a combination of the ruptured and intact oleosomes. Concentration-dependant behaviour at the air-water interface could have important implications for the behaviour of surface-active materials when foams are formed within a system. This phenomenon, thus, is important for several intended applications (such as ice cream, mousses, etc.). The formation of a protein/phospholipid or intact oleosomes film-like materials at the interface can be of great interest in terms of applications in food industry as interface stabilisers which need more research in the future by measuring the critical force needed to break the oleosome droplets and to also have a better understanding of their interfacial behaviour through further experiments such as contact angle.

CRediT authorship contribution statement

Amin Aliyari: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Vincenzo di Bari:** Writing – review & editing, Supervision, Conceptualization. **Liam P.D. Ratcliffe:** Writing – review & editing, Supervision, Funding acquisition. **Pallab Kumar Borah:** Writing – review & editing, Software. **Yuanzhang Dong:** Writing – review & editing. **David Gray:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110896.

Data availability

Data will be made available on request.

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