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# Freeze-thaw stability of oilseed rape oleosome emulsions

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## ABSTRACT

This work investigated the stability of natural oleosome emulsions on freeze-thawing. Oleosomes were recovered from oilseed rape seeds following an aqueous extraction process using sodium bicarbonate (0.1 M). The final emulsions pH was adjusted to 9, 6 and 3 to achieve surface charge values of  $-50.3 \pm 1.6$ ,  $-20.0 \pm 2.4$ , and  $+37.5 \pm 0.5$  mV, respectively. The emulsions with lipid mass fraction of  $0.28 \pm 0.02$  were cooled to  $-20$  °C for up to 24 h and thawed at  $20$  °C for 18 h, and their freeze-thaw stability assessed quantifying amount of released oil (oil yield) due to emulsion destabilisation. The destabilisation of the oleosome emulsions at pH 9 and 6 increased with isothermal holding time at  $-20$  °C, whereas the emulsion at pH 3 destabilised more rapidly. Differential scanning calorimetry analysis of emulsions cooled from  $20$  °C to  $-20$  °C at  $-10$  °C/min, and held at  $-20$  °C for 8h, revealed how the continuous phase rapidly crystallised on cooling, whereas lipid crystallisation started after 2 h at  $-20$  °C and continued for the following 3 h. Oil yield data combined with differential scanning calorimetry curves suggest that the oleosome emulsions at pH 9 and 6 destabilised along with crystallisation of the dispersed lipid phase, whereas emulsions at pH 3 destabilised with the continuous phase crystallisation. It was hypothesised that oleosome emulsions at pH 9 and 6 ruptured by a mechanism of partial coalescence. At pH 3 the electrostatic interaction between phospholipids and oleosin molecules, the main components of oleosome interface, may be reduced resulting in a weaker interface which on cooling can be easily disrupted by ice crystals. Oil yield data for emulsions with increasing continuous phase mass fraction (0.50 and 0.70) suggest a lower extent of destabilisation than control (0.28 continuous phase). Increasing the number of freeze-thaw cycles from one to three did not cause significant increase in the oil yield.

## 1. Introduction

Oleosomes, or oil bodies, are sub-cellular lipid storage organelles present in all plant reproductive organs. They are oil droplets, made of triacylglycerols, surrounded by a phospholipid monolayer, in which specific structural proteins, mainly oleosins, are embedded (Frandsen et al., 2001; Huang, 1996; Tzen et al., 1992, 1993). Oleosins contribute to the oleosome stability by providing steric hindrance and electrostatic repulsion (Tzen and Huang, 1992). Intact oleosomes can be recovered from seeds of various oleaginous plants with the use of aqueous solutions, to obtain a natural oil-in-water (o/w) emulsion whose dispersed phase is made by oleosomes (Adams et al., 2012; Bonsegna et al., 2011; Iwanaga et al., 2007; Nikiforidis and Kiosseoglou, 2009; Payne et al., 2014). It has been demonstrated that, during preparation of oilseed rape (OSR) oleosome emulsions, a 0.1 M sodium bicarbonate washing solution has a similar capacity to remove exogenous proteins (proteins which are not an intrinsic part of the oleosome hemi-membrane) to that of a 9 M urea solution (De Chirico et al., 2018). Urea is a very efficient washing agent (Millichip et al., 1996), which produces oleosome emulsions free from exogenous seed proteins without compromising the

stability of the integral oleosins, necessary for oleosome integrity (Lacey et al., 1998).

The o/w emulsions are widely employed in the food industry; several natural and processed foods have characteristics of o/w emulsions (McClements, 2004). Emulsions are normally obtained through emulsification processes, which require energy and the use of emulsifiers (Karbstein and Schubert, 1995). The use of natural oleosome emulsions from oleaginous seed sources in the food industry could overcome the emulsion manufacturing step. Moreover, oleosomes would represent a good alternative to processed foods because they can exhibit high stability and nutritional value as they are not subjected to any refining treatment. Furthermore, the oleosome aqueous recovery process allows a simultaneous co-extraction of proteins which, together with solid residue, could be used for the preparation of protein isolates or concentrates (Nikiforidis et al., 2014). Another important advantage of using natural oil droplets (oleosomes) is the absence of organic solvents, in particular hexane, normally employed for oil extraction and which present environmental issues such as risk of explosions, and health concerns (Nikiforidis et al., 2014).

Plant seeds have been used as sources of oil for a long time, but the

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industrial use of oleosomes has been considered only recently, mainly due to a growing interest towards vegetable alternatives to dairy products. oleosomes present potential food applications, such as dairy-like foods and beverages, salad dressings and sauces, edible films, coatings (Nikiforidis et al., 2014), foams and gels (Dickinson, 2010) or ice creams.

Upon manufacture and storage, o/w emulsions can be subjected to freeze-thawing. In many cases, freezing is employed to preserve the quality and to increase the shelf-life of foods, which are thawed before consumption. Freezing also represents a manufacturing step of ice-creams or other desserts to be consumed as frozen. Regardless the reason of freeze-thawing, this process can destabilise o/w emulsions by inducing modifications to their microstructure. The freeze-thaw stability of both o/w emulsions (Ghosh and Coupland, 2008; Palazolo et al., 2011) and emulsion-based foods (Degner et al., 2014; Shariful et al., 2018) has been investigated given its impact on quality of some foods (ice creams above all). It was observed that o/w emulsions are mostly unstable to freeze-thawing, which represents an issue on the quality of emulsion-based foods. Emulsion instability to freeze-thawing was attributed to the crystallisation of the dispersed phase and/or the continuous phase, or both, depending on the cooling temperature and on the lipid composition of the dispersed phase. Destabilisation caused by crystallisation of the dispersed phase normally occurs through a mechanism of partial coalescence between oil droplets, followed by coalescence upon thawing (Fredrick et al., 2010), which could eventually result in emulsion breakdown and release of the emulsified oil (Tan, 2003).

The effect of cooling temperature, cooling rate and isothermal holding time on the freeze-thaw stability of o/w emulsions has been investigated in several works as those parameters affect the crystallisation profile of the emulsion, in particular that of the dispersed phase (Degner et al., 2014; Magnusson et al., 2011; Vanapalli et al., 2002). When the continuous phase crystallises, ice expansion would promote o/w emulsion instability by exerting pressure on the oil droplets, causing coalescence (Ghosh et al., 2006; Hartel, 2001; McClements, 2004). In this case, the continuous phase volume fraction would affect the emulsion freeze-thaw stability, by regulating the average inter-droplet distance and the probability of physical contact between oil droplets (Cortés-Muñoz et al., 2009; Van Aken, 2002). Furthermore, the continuous phase composition can influence the emulsion stability by affecting the interfacial properties of the dispersed phase. Physico-chemical changes in the aqueous phase, such as increased solute concentration or pH change can be induced by freezing (Demetriades et al., 1997; Ghosh and Coupland, 2008; Thanasukarn et al., 2004; Walstra, 2001). When both the continuous and dispersed phase crystallise, the mechanisms reported above for each emulsion phase could contribute to destabilisation (Cramp et al., 2004; Ghosh and Coupland, 2008; Thanasukarn et al., 2004–2006).

Given the potential application of oleosome emulsions in the food industry, and the lack of study investigating their freeze-thaw stability, it is opportune to evaluate their stability to freeze-thawing. This work aims at assessing the freeze-thaw stability of OSR oleosome emulsions, by investigating the impact of final cooling temperatures (−10, −20, −75, −100 °C), the effect of isothermal holding time at −20 °C, and the role of the continuous phase fraction. Freeze-thaw-induced destabilisation of oleosome emulsions is expected to occur for the same reasons above reported for o/w emulsions. Therefore, the cooling temperatures were selected to gain a better understanding of the impact of continuous and dispersed phase crystallisation on oleosome emulsions instability (or “rupture”). At −10 °C only the aqueous phase of the OSR oleosome emulsion crystallises (although some triacylglycerols in oleosomes may crystallise), whilst at −20 °C or below, both the continuous and dispersed phase would crystallise.

## 2. Materials and methods

### 2.1. Oleosome recovery from OSR seeds

#### 2.1.1. OSR seeds

OSR seeds, variety “Compass” were provided by the University of Nottingham Farm Office (Sutton Bonington Campus, Nr Loughborough, LE12 5RD, UK). The OSR seeds were cleaned with a seed cleaning machine (Haldrup DC-20, Germany) to remove dust and debris and stored in a dry cool environment. Before the seeds were stored, three aliquots of 30 g each were dried in a convection oven (MOV-112F, Sanyo, Japan) at 60 °C until the mass remained constant over time, to determine the seed moisture content. The initial (pre-drying) seed moisture was  $4.5 \pm 0.2\%$  (weight(wt) water/wt seeds).

#### 2.1.2. Recovery of oleosomes bodies from seeds

Oleosomes were recovered from OSR seeds with the method of De Chirico et al. (2018). The seeds were soaked for 16 h in a 0.1 M sodium bicarbonate solution at pH 9.5 (recovery buffer) pre-chilled (4 °C) at a ratio seed-to-buffer of 1:4 (wt/volume(v); 100 g of seeds in 400 mL buffer). At the end of the soaking period, the medium was discarded. The seeds were then blended with a Kenwood kMix, type BLX75, for 60 s at 400 W power (machine half power), in fresh pre-chilled recovery buffer at a ratio seed-to-buffer of 1:7 (based on the original seed weight), and the mix was filtered through three layers of cheesecloth (grade 80, thread count: 40 x 32 threads per square inch) to remove the solid debris and produce a filtrate containing the oleosomes.

The oleosomes were recovered from the filtrate (also called “milk”) by centrifugation (Beckman, Model J2-21 Centrifuge) at 10,000 RCF for 30 min at 4 °C. After centrifugation, the top layer consisting of a creamed emulsion pad containing oleosomes (also called “cream”), was collected. The collected emulsion was designated as crude oil body emulsion (COB). This preparation was subsequently washed to remove the polysaccharide, seed debris and exogenous proteins not covalently bonded to the oleosomes (De Chirico et al., 2018; Tzen et al., 1997); the washing step consisted of re-suspending COB into pre-chilled recovery buffer at emulsion-to-buffer ratio of 1:4 (wt/v), centrifugation (10,000 RCF, 30 min, 4 °C) and collection of the emulsion pad, which was designated as washed oil body emulsion (WOB). In order to reduce the concentration of the exogenous oleosome material (such as exogenous proteins or ions from the recovery buffer), WOB was rinsed with Milli-Q ultrapure water (Millipore) (MQ water) at a cream-to-water ratio of 1:4 (wt/v) and centrifuged (10,000 RCF, 30 min, 4 °C). Sodium azide (final concentration 0.02%, wt/v%) was added to the MQ water to prevent microbial contamination. The collected creamed emulsion was designated as rinsed-washed oil body emulsion (RWOB).

The pH of oleosome emulsions diluted with MQ water to 1:10 ratio, was measured with a Mettler Toledo FE20 pH meter. The pH of RWOB was  $8.96 \pm 0.02$  (for brevity, labelled as pH 9), also defined as original pH as no further pH adjustments were made. Where not otherwise specified, the term RWOB will refer to the emulsion at this pH.

With the aim of investigating the effect of pH on the oleosome stability to freeze-thawing, RWOB at  $\text{pH } 3.04 \pm 0.03$  and  $6.03 \pm 0.03$  (respectively, labelled as pH 3 and 6) were also produced by replacing MQ water in the rinsing step with HCl solutions to the appropriate concentration. The oleosome emulsions were stored at 5 °C and used within 24 h for all further analyses.

### 2.2. Physical disruption of oleosome emulsions: freeze-thawing experiments

The effect of freeze-thawing on the macroscopic stability of the oleosome emulsions was tested. Aliquots of oleosome emulsions (0.5 g in 2 mL centrifuge tubes), stored at 5 °C, were frozen at different conditions (Sections 2.2.2, 2.2.3, and 2.2.4), then thawed in a Sanyo Incubator at 20 °C, for 18 h, followed by centrifugation with a Heraeus™ Fresco™ 21

Microcentrifuge (Thermo Scientific™) at 13,000 RCF, for 5 min at 15 °C. A top layer of free oil appeared and it was collected after addition of hexane to dilute the oil and facilitate the oil recovery. Centrifugation and oil recovery were performed twice. Hexane was removed with a sample concentrator (MiVac, Genevac Ltd, Suffolk, UK) operating at 36 °C for 50 min.

The extent of oleosome destabilisation was determined by quantifying the “oil yield” (or “oiling out”). The oil yield was calculated as the ratio of the amount of oil collected (in g) to the mass of dry cream (in g), as expressed in Equation (1):

$$\text{oil yield (wt\%)} = \frac{\text{oil (g)}}{\text{dry cream (g)}} \times 100\% \quad 1$$

### 2.2.1. Differential scanning calorimetry analysis

Freeze-thaw-induced destabilisation of o/w emulsions is usually caused by the crystallisation of the dispersed and/or continuous phase. Differential Scanning Calorimetry (DSC) analysis was therefore carried out to examine the crystallisation behaviour of RWOBs. A TGA/DSC 3+ Star System (Mettler Toledo) equipped with 40 µL aluminium pans was employed.

The DSC programme consisted of four steps. The emulsions, after 10 min equilibration at 20 °C (step 1), were cooled to −10 °C or −20 °C at 10 °C/min (step 2), then isothermally held at −10 °C or −20 °C, respectively, for 480 min (step 3) and reheated to 20 °C at 10 °C/min (step 4).

### 2.2.2. Freeze-thawing of oleosome emulsions at different pH and cooling times

A PolyScience PP7LR-40 (USA) water bath, set at −20 °C, was used to cool the oleosome emulsions. The samples at 5 °C were plunged into the cooling medium to reach the final temperature of −20 °C, then either immediately thawed once the sample reached −20 °C (0 h isothermal hold), or held at this temperature for 2, 3, 6 or 24 h before thawing. The cooling rate generated by the difference in temperature between sample and cooling medium was approximately −10 °C/min. The emulsion temperature was monitored with a HH309 Datalogger Thermometer (Omega) with continuous recording at a frequency of one point every 2 s. The samples were thawed at 20 °C for 18 h before measuring the oil yield (Equation (1)).

Experiments were carried out for RWOB at pH 9, 6 and 3. Aliquots of RWOB at pH 9 were also freeze-thawed for 2 or 3 cycles before centrifugation and oil collection; the −20 °C isothermal step for those aliquots was 6 h.

Aliquots of RWOB (pH 9) were also isothermally frozen to −10 °C instead of −20 °C and held at that temperature for 6 h before thawing.

**2.2.2.1. Characterisation of the oleosome emulsions at the different pH values.** It is known that the pH could affect the stability of emulsions, consequently their freeze-thaw stability, by modifying the droplet interfacial properties. The pH effect prior to freeze-thawing was examined in terms of particle size distribution (PSD), microstructure, and ζ-potential of RWOBs produced at different pH values (Section 2.1.2) and discussed in relation to their freeze-thaw stability.

The PSD of RWOBs was measured with a LS 13320 laser diffractometer (Beckman-Coulter, USA) using the Mie theory of the scattering of light by spherical particles. The real part of the refractive index was 1.462, which corresponds to the refractive index of OSR oil. The imaginary part represents the ‘attenuation coefficient’ that describes the turbidity of a sample, and it was set to 0.01 as reported in the laser diffractometer guidelines for lightly coloured translucent materials. The PSD was described in terms of the volume frequency distribution as a function of the droplet diameter (µm). Prior to measurement, the emulsions were diluted in recovery buffer at a ratio RWOB-to-buffer of 1:100 (wt/v).

The microstructure of RWOB emulsions was examined using light

microscopy. Prior to examination, the emulsions were diluted into recovery buffer at a ratio RWOB-to-buffer 1:100 (wt/v). A drop of diluted oleosome emulsion, placed on a glass slide and covered with a cover slip, was imaged with a Nikon Eclipse Ci (UK) microscope, at 40× magnification.

The electrical potential at the droplet interface is modified by the pH, and it influences the level of aggregation between oil droplets and their tendency to destabilisation. The ζ-potential of RWOB at different pH values was determined with a method adapted from De Chirico et al. (2018). The oleosome emulsions were diluted in MQ water previously filtered (Corning Vacuum Filtration System, 0.22 µm), to a final concentration of 0.008% (lipid weight basis), then adjusted to pH 3, 6, or 9 with 0.1 M HCl or 0.1 M NaOH solutions. When the pH was stable, the emulsions were injected into the measurement chamber of an electrophoresis light scattering instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA), with the following instrument settings: temperature = 25 °C; refractive index of dispersant = 1.333; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm; voltage = 35 V. The ζ-potential was then determined as a measure of the direction and velocity of the oleosomes moving under the applied electrical field. The ζ-potential values reported are the average and standard deviation of three independent samples from one emulsion replicate at each pH.

### 2.2.3. Freeze thawing of oleosome emulsions at different continuous phase mass fraction

The continuous phase mass fraction may affect the freeze-thaw stability of o/w emulsions, therefore the freeze-thaw stability of oleosome emulsions at different continuous phase mass fractions was tested. Water content of RWOB emulsions was measured and used as an indication of the mass fraction (wt/wt) of the continuous phase. Aliquots of fresh oleosome emulsions were dried at 50 °C for 48 h (until the mass remained constant over time). Water content (wt/wt) was calculated as weight difference between fresh and dried emulsion. The measurements were run in triplicate. The water content varied with extraction, with an average of 0.28 ± 0.02; in some cases we refer to this RWOB as RWOB 0.3.

The RWOB at original water content (i.e., the RWOB water content at the end of the oleosome recovery process, Section 2.1.2) was then gently mixed with bulk continuous phase (see Section 2.2.3.1) to increase the water content to 0.50 (wt/wt; RWOB 0.5) or 0.70 (wt/wt; RWOB 0.7). To evaluate the impact of continuous phase mass fraction on oleosome emulsion stability, RWOB 0.3, RWOB 0.5 and RWOB 0.7 were cooled in water bath at −20 °C for 24 h (Section 2.2.2), then thawed at 20 °C for 18h.

**2.2.3.1. Preparation of the bulk continuous phase (RWOB serum).** RWOB serum was previously purified with a method adapted from De Chirico et al. (2020). An aliquot of serum obtained from the centrifugation step to produce RWOB, was centrifuged (21,000 RCF, 30 min, 4 °C) to remove residual oleosomes. The lower phase was carefully removed with a syringe and needle. This step was performed three times. Eventually, the serum was filtered through a 0.2 µm membrane (Whatman) filter.

### 2.2.4. Freeze-thawing of oleosome emulsions with other cooling methods

The cooling step profile of a freeze-thawing process may induce different extents of destabilisation in o/w emulsions. For this reason, further cooling methods were used to test their effect on the oil yield in freeze-thawed RWOB. The first two methods consisted in cooling RWOB to −75 °C in a freezer, and either holding it at this temperature for the following 24 h before thawing, or, as soon as the emulsion temperature reached −75 °C, transferring it in a commercial freezer at −20 °C and storing it for 22 h before thawing. In the last two methods, RWOB was plunged into liquid nitrogen for 30 s, reaching a final temperature of



–100 °C, then either thawed, or immediately stored in freezer at –20 °C for 22 h and eventually thawed.

### 2.3. Statistical analysis

Experiments were performed in triplicate and averages and standard deviations provided. Statistical analysis was performed by one-way ANOVA (Analysis of Variance) using SPSS version 28.0.1.1 software (IBM, Chicago, USA).

## 3. Results and discussion

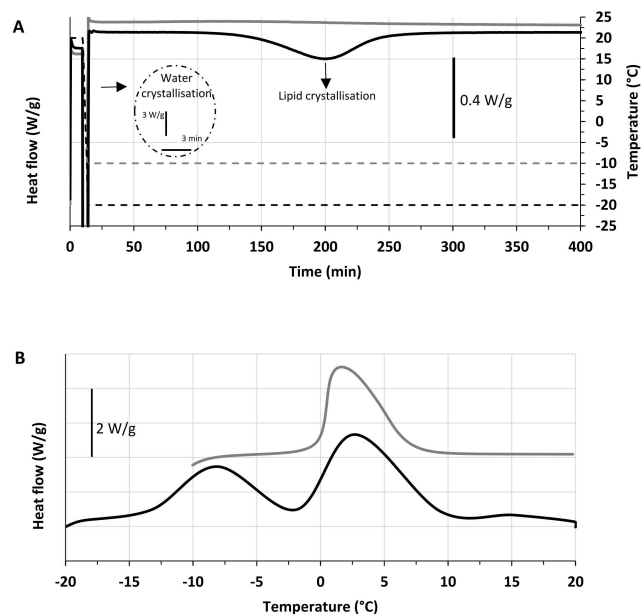
### 3.1. Effect of isothermal holding time on oleosome emulsion stability

The aim of the work presented in this section was to evaluate the freeze-thaw stability of oleosome emulsions (at pH 9) through isothermal holdings at –20 °C (freezing step) followed by re-heating to 20 °C (thawing step) (details in Section 2.2.2). Emulsions were held at the target temperature for up to 24 h followed by centrifugation and inspection for oil release. Oleosome emulsions were partially destabilised after freeze-thaw with –20 °C isothermal cooling temperature, and the extent of oleosome destabilisation increased with isothermal holding time. Data shown in Fig. 1A refer to the effect of isothermal holding time on oil yield.

Samples held for 0 h and 2 h at –20 °C remained stable after freeze-thawing (Fig. 1B), whereas samples held for 3 h showed a gel-like layer at the top (Fig. 1C), which indicated release of oil from ruptured oleosomes, although this oil was not available for collection, as it was entrapped within this layer. Samples that were held at –20 °C for 6 h showed clear oiling off, and the free oil was available for collection (Fig. 1C), although a gel-like layer could be observed in these samples too. Increasing the isothermal time from 6 to 24 h increased oil yield, although to a limited amount, suggesting that most of the oleosome rupture occurred between 2 and 6 h at –20 °C. In Shariful et al. (2018), oil separation in freeze-thawed rapeseed oil mayonnaise occurred through a typical sigmoidal pattern, with initial slow oil separation, followed by a sudden increase in the separation speed and, eventually, a decreased separation speed.

When RWOB was isothermally held for 6 h at –10 °C (Sections 2.2.4 and 3.2), no oil separation was observed, therefore no or very limited oleosome rupture would have occurred. These data suggest that a minimum temperature must be reached for oleosomes to rupture.

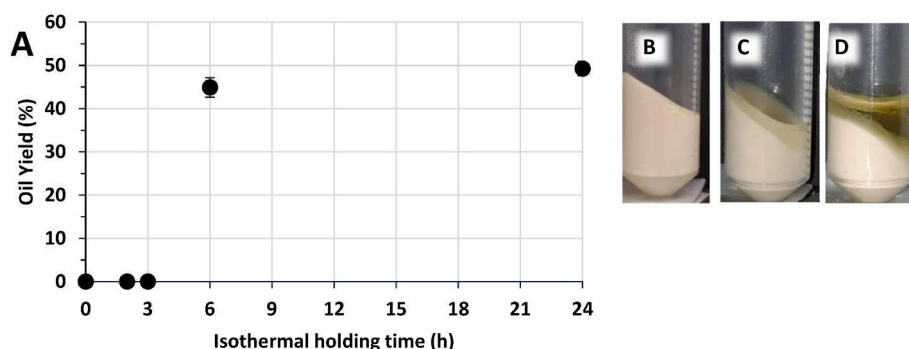
To gain a better insight on RWOB emulsions cooling and phase transition behaviour, and their impact on stability, samples were crystallised at –10 °C and –20 °C for 480 min using a DSC (Fig. 2; also see Section 2.2.1). These programmes were designed to simulate the freeze-thawing process described above. The cooling and isothermal steps in Fig. 2A show two distinct exothermic peaks when the isothermal



**Fig. 2.** RWOB isothermally held at –10 °C (grey line) and –20 °C (black line) (heat flow - continuous line, temperature profile - dashed line). A) Cooling (10 °C/min) and isothermal steps: the peaks at 15 min and 200 min (the latter visible only at –20 °C) represent crystallisation of the continuous and dispersed phase, respectively. The water freezing peak is only partially visible as the y-axis scale is set to facilitate visualisation of the lipid peak. Inset: visualisation of the entire water crystallisation peaks. B) Heating step (10 °C/min) following isothermal freezing at –10 °C (grey line; continuous phase melting onset at  $-0.2 \pm 0.1$  °C) and –20 °C (black line; dispersed and continuous phase melting onset at  $-12.0 \pm 0.0$  °C and  $-0.2 \pm 0.0$  °C, respectively).

holding temperature was –20 °C, with a first, sharp peak occurring upon cooling, and a second, broader thermal transition at 185 min of isothermal hold time (200 min overall time). The first peak and second peak were interpreted as water crystallisation and lipid crystallisation, respectively (Ishibashi et al., 2016; Miyagawa et al., 2016; Shariful et al., 2018).

With an isothermal hold at –10 °C applied for 480 min no exothermic peak associated with the lipid phase was observed, suggesting that this temperature is not sufficient to induce enough oil crystallisation to be detected by the DSC. Data previously discussed in this section showed that no oleosome rupture occurred when holding RWOB at –10 °C for 6 h. Therefore, combination of the data in Figs. 1 and 2 suggest that oleosome rupture is induced by crystallisation of the dispersed phase.



**Fig. 1.** A) Oil yield (wt%) from freeze-thawed RWOB (pH 9), cooled to –20 °C and isothermally held at this temperature for 0 (only cooling step), 2, 3, 6, and 24 h. Respective cooling times above the markers; if not visible, error bars are within the marker. Freeze-thawed and centrifuged RWOB emulsions after 2 h (B), 3 h (C) and 6 h (D) of isothermal hold at –20 °C. In (C), a gel-like layer is visible suggesting initial oleosome rupture, but no oil is recoverable. In (D) recoverable oil following from oleosome rupture is visible.

When the emulsions were reheated from  $-20^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  (Fig. 2B), two clear melting peaks were observed: the peak with onset at  $-12.0^{\circ}\text{C}$  and  $-0.2^{\circ}\text{C}$  were attributed to the dispersed and continuous phase, respectively. For RWOB reheated from  $-10^{\circ}\text{C}$  to  $20^{\circ}\text{C}$ , only the continuous phase melting peak was observed. Oleosomes may possibly rupture through a mechanism of partial coalescence when RWOB emulsion is isothermally held at  $-20^{\circ}\text{C}$  for a sufficient time for the oil to partially crystallise. The amount of oil released after thawing increased upon increasing the isothermal holding time during the freezing step, perhaps due to a higher percentage of oil which crystallised over time (Magnusson et al., 2011; Shariful et al., 2018). Oil yield increased from 44.9% to 49.2% (wt%) from 6 to 24h, suggesting that oleosome destabilisation was limited over that 18h time range.

The role of lipid crystallisation, and partial coalescence of oil droplets, in the destabilisation of rapeseed o/w emulsions has been discussed in literature (Magnusson et al., 2011; Ishibashi et al., 2016; Miyagawa et al., 2016; Shariful et al., 2018). These authors observed emulsion destabilisation following from lipid crystallisation. According to Shariful et al. (2018), the time needed for an emulsion to start destabilising upon cooling is similar to the time required for lipid crystallisation to start. Partial coalescence requires the presence of partially crystalline droplets, as solid fat crystals penetrate from one droplet into the liquid oil phase of adjacent droplets (Boode et al., 1993; Fredrick et al., 2010; McClements, 2004).

Partial coalescence mainly depends on the protrusion of lipid crystals outside the droplet. Lipid crystals formed inside a droplet tend to migrate to the droplet oil-water interface, where the crystals are at an equilibrium position, which determines the crystal contact angle in the aqueous and oil phase. By being at their equilibrium position, the crystal interfacial tension is the lowest at the interface. Usually, lipid crystals are preferentially wetted by the lipid phase, but their edges can protrude out of the droplet by a few tens of nanometres (Boode and Walstra, 1993; Fredrick et al., 2010).

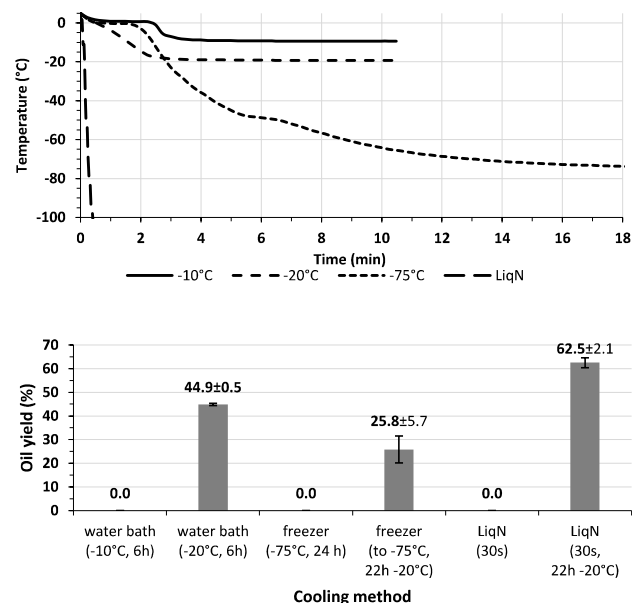
Lipid crystals tend to align tangentially to the surface if they are free to move inside the droplet; however, some of the crystals may be temporarily radially oriented to the surface (Boode et al., 1993) before the network forms, and remain in radial orientation after aggregating to the network and protrude to a distance depending on the contact angle. Presumably, needle-type crystals may protrude farther than small, rounded crystals. Crystals keep growing during network development, and the droplet shrinks as the amount of liquid oil decreases up to a point where it cannot cover the crystals anymore, which protrude (Walstra, 2001). Furthermore, on decreasing the temperature, the volume contraction in the liquid oil is higher than in the solid crystals, contributing to crystal protrusion and to the extent of partial coalescence (Boode and Walstra, 1993). On encountering the surface of other droplets, upon collision or compression, these protruding crystals may pierce the interfacial layer of the other droplets and grow within them if liquid oil is available.

Summary of this section is that lipid crystallisation in RWOB at pH 9 isothermally held at  $-20^{\circ}\text{C}$  occurs after water crystallisation and causes RWOB destabilisation that is apparent by the release of oil seen after thawing.

### 3.2. Effect of cooling method on oleosome emulsion stability

The results of Section 3.1 suggest that oleosomes are destabilised by partial coalescence, which implies a condition of partial crystallinity. Modifying the cooling method to induce more rapid lipid crystallisation could therefore affect RWOB freeze-thaw stability. Considering this premise, the main aim of this section was to test the effect of different cooling methods on the freeze-thaw stability of RWOB.

The temperature-time profiles of RWOBs (pH 9) exposed to different cooling methods and temperatures are shown in Fig. 3A, and the respective oil yields at the end of the freeze-thawing cycles are shown in Fig. 3B. All RWOBs were thawed at  $20^{\circ}\text{C}$  for 18 h.



**Fig. 3.** A) Temperature-time profile of RWOB (pH 9) exposed to four crystallising temperatures (initial temperature  $5^{\circ}\text{C}$ ). B) Oil yield (wt%) from RWOB isothermally held for 6 h in water bath at  $-10^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ , in freezer at  $-75^{\circ}\text{C}$  for 24h, cooled in freezer to  $-75^{\circ}\text{C}$  then held at  $-20^{\circ}\text{C}$  for 22 h, in liquid nitrogen (LiqN) for 30 s, or in liquid nitrogen for 30 s followed by 22h in freezer at  $-20^{\circ}\text{C}$ . In all cooling methods, samples were isothermally cooled to the respective final temperatures, prior to the isothermal hold. Cooling methods are described in Sections 2.2.2 and 2.2.4.

Since the cooling media temperatures and type of device and media were different, the cooling rates experienced by the RWOB changed (Fig. 3A). For RWOB cooled in ethylene glycol-water bath, the cooling rate when the final temperature was  $-20^{\circ}\text{C}$  was higher than that at  $-10^{\circ}\text{C}$ . The temperature-time profile shows a plateau at  $0^{\circ}\text{C}$  for RWOB cooled to  $-10^{\circ}\text{C}$ , indicating crystallisation of the continuous phase. Such a plateau is not observed for RWOB cooled to  $-20^{\circ}\text{C}$ , suggesting a more efficient heat exchange due to the higher cooling rate.

RWOB freezer-cooled to  $-75^{\circ}\text{C}$  also showed the plateau at  $0^{\circ}\text{C}$ , despite the much lower final temperature than that in water bath ( $-20^{\circ}\text{C}$ ). This suggests that the heat exchange is more efficient with the water-ethylene glycol mixture bath, rather than with the air present in the freezer chamber. The cooling curve slope of the  $-75^{\circ}\text{C}$  freezer increased steadily after water crystallisation, and a second, less pronounced plateau was observed at about  $-48^{\circ}\text{C}$  which was attributed to the oil crystallisation in RWOB. The liquid nitrogen method induced a very high cooling rate in the sample, with the sample temperature dropping from  $5^{\circ}\text{C}$  to about  $-100^{\circ}\text{C}$  in 30 s (with no plateau observed) due to the very low cooling medium temperature ( $-196^{\circ}\text{C}$ ) and its liquid form, which would increase the capacity of absorbing heat from the sample.

As shown in Fig. 3B, the emulsion isothermally held at  $-10^{\circ}\text{C}$  remained visibly intact, with no oil release at the end of the freeze-thaw cycle, whilst the emulsion at  $-20^{\circ}\text{C}$  ruptured. As discussed in Section 3.1, oleosome rupture is caused by lipid crystallisation, which does not occur at  $-10^{\circ}\text{C}$ . Similarly, RWOB held for 24 h at  $-75^{\circ}\text{C}$  remained stable after thawing, but in this case lipid crystallisation occurred. An explanation for this behaviour can be provided recalling that oleosome rupture would occur at  $-20^{\circ}\text{C}$  through partial coalescence (Section 3.1), whereas cooling to  $-75^{\circ}\text{C}$  would result in complete lipid crystallisation in a time range too short to allow partial coalescence. In fact, DSC data show how lipid crystallisation at  $-20^{\circ}\text{C}$  (Fig. 2B) occurred over a time range of about 200 min, whereas the cooling curve of RWOB

cooled to  $-75^{\circ}\text{C}$  (Fig. 3A) suggests that lipid crystallisation occurred in about 1 min during the plateau at  $-48^{\circ}\text{C}$  (overall cooling time 8 min), with an average cooling rate in the segment  $-20^{\circ}\text{C}$  to  $-75^{\circ}\text{C}$  of  $-3.3^{\circ}\text{C}/\text{min}$ . Similarly, Vanapalli et al. (2002) observed freeze-thaw stability in an emulsion whose lipid phase crystallised in a shorter time scale, whereas when lipid crystallisation was slower, partial coalescence could occur. In Vanapalli et al. (2002) the different freeze-thaw stability depended on lipid composition differences between the emulsions, as the different emulsions experienced the same cooling process. In our study, the oleosome emulsions all had the same lipid composition, and their different freeze-thaw stability was attributed to variations in the applied cooling rate and target temperature. Moreover, the lipid crystals formed during cooling to  $-75^{\circ}\text{C}$  would be smaller and perhaps less destabilising than those formed at  $-20^{\circ}\text{C}$ , due to the faster crystallisation at  $-75^{\circ}\text{C}$ . The larger crystals formed at  $-20^{\circ}\text{C}$  may protrude more than smaller crystals, increasing the probability of penetrating other droplets. Besides, the network made of larger crystals has larger pores, which contain liquid oil, thus increasing the permeability of the fat crystal network (Fredrick et al., 2010).

Similarly, RWOB cooled in liquid nitrogen for 30 s and then thawed, remained stable after centrifugation, showing only a very thin oily layer at the top, again due to the absence of partial coalescence and small crystal size caused by fast lipid crystallisation. Moreover, slow crystallisation in isothermal at  $-20^{\circ}\text{C}$  may have caused more stable polymorphs. In fact, more metastable polymorphs are formed at higher cooling rate as fast crystallisation does not give time for triacylglycerols to arrange into lamellar structures (Campos et al., 2002). The relationship between polymorphism and crystal morphology was reported by some authors (Sato, 2001; Arima et al., 2007), but not confirmed by others (Kellens et al., 1992; Rousseau, 2000; Ishibashi et al., 2016). Nevertheless, it is possible that the more stable polymorphs were related to crystals with more destabilising properties, whereas metastable polymorphs would have formed by the high cooling rate generated by liquid nitrogen-cooling, decreasing the extent of droplet rupture.

However, when RWOB was cooled in liquid nitrogen then immediately transferred and stored in a freezer at  $-20^{\circ}\text{C}$  for 22 h prior to thawing, the highest oil yield among the freeze-thawed samples was observed. Similarly, RWOB cooled to  $-75^{\circ}\text{C}$  (cooling step length of 18 min, Fig. 3A) then immediately transferred in freezer at  $-20^{\circ}\text{C}$  and stored for 22 h was also destabilised, but less than when cooled with liquid nitrogen then stored at  $-20^{\circ}\text{C}$ . Both experiments indicate that if RWOB temperature is raised from  $-100^{\circ}\text{C}$  (experiment in liquid nitrogen) or  $-75^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$ , rupture occurs. Further DSC work (data not shown) showed that oil in RWOB cooled to  $-60^{\circ}\text{C}$  starts to melt at  $-30^{\circ}\text{C}$ , thus a condition of partial crystallinity may be reached when RWOB is re-heated to  $-20^{\circ}\text{C}$  from  $-100^{\circ}\text{C}$  or  $-75^{\circ}\text{C}$  and cause partial coalescence. Crystals could melt, re-form and further grow during the 22 h storage in freezer. Recrystallisation is a phenomenon by which smaller crystals convert to larger crystals over time (Tan et al., 2021), driven by the thermodynamic difference in equilibrium behaviour based on crystal size (Hartel, 2013). This increase of crystal size due to recrystallisation could have been the cause of the higher extent of oleosome rupture upon 22 h storage at  $-20^{\circ}\text{C}$ .

Marefati et al. (2013) also observed higher destabilisation in Pickering emulsions (triacylglycerol droplets stabilised by starch granules) frozen to  $-196^{\circ}\text{C}$  with liquid nitrogen and stored in freezer overnight, than in o/w emulsions cooled directly in freezer at  $-18^{\circ}\text{C}$ , suggesting that flash freezing in liquid nitrogen is a harsher process than that in freezer. Liquid nitrogen cooling would have caused displacement of the starch granules at the interface, and promoted partial coalescence caused by crystal protrusion. According to Marefati et al. (2013), the higher degree of cooling (to  $-196^{\circ}\text{C}$  for liquid nitrogen vs  $-18^{\circ}\text{C}$  in the freezer) would have induced more fat to crystallise, therefore more protrusions, leading to partial coalescence. However, since it appears less likely for partial coalescence to occur at very high cooling rates, as a condition of partial crystallinity is difficult to be met (Boode et al.,

1993), we may propose that rupture would occur following a similar mechanism to that occurring in RWOB. In fact, Marefati et al. (2013) stored the samples in a freezer after cooling in liquid nitrogen, and it may rather be this second step that increased the emulsion destabilisation. Similarly, in this current work we did not observe oleosome destabilisation simply by cooling in liquid nitrogen, but destabilisation occurred when liquid nitrogen cooling was paired with storage in freezer at  $-20^{\circ}\text{C}$ .

It is interesting to note that a potentially similar mechanism, can occur in other types of natural emulsified systems such as human milk. It was in fact observed that human milk fat globules, frozen at  $-18^{\circ}\text{C}$ , were more destabilised (higher PSD) when thawed at  $4^{\circ}\text{C}$  for 10 h, rather than at  $45^{\circ}\text{C}$  for 1 min (Zhang et al., 2022). The longer thawing time at  $4^{\circ}\text{C}$  was proposed to cause more damage to the globules. Since human milk fat (Lopez et al., 2013) and cow milk fat (Tomaszewska-Gras, 2013) are partially crystalline at  $4^{\circ}\text{C}$ , it is reasonable to believe that milk destabilisation in Zhang et al. (2022) occurred by partial coalescence, after partial melting of the solid milk fat from  $-18^{\circ}\text{C}$  (freezing step temperature) to  $4^{\circ}\text{C}$  (thawing step temperature). Conversely, thawing at  $45^{\circ}\text{C}$  caused reasonably complete melting of the milk globule fat, thus no destabilising effect of fat crystals was observed.

Experiments reported in this section suggest that the time required for the oil to crystallise in oleosomes has a crucial role in determining the phenomenon of partial coalescence and related instability to freeze-thawing. In order to have a deeper understanding of the rupture mechanism occurring in freeze-thawed emulsions, studying the changes occurring at the oleosome interface upon cooling with different methods may be considered as future work.

### 3.3. Effect of continuous phase mass fraction on oleosome emulsion stability

In Section 3.1, it was proposed that freeze-thawed RWOB destabilise through partial coalescence, induced by crystallisation of the dispersed phase. Oil droplets must therefore be sufficiently close for lipid crystals from one droplet to penetrate other droplets. The emulsion water content regulates the inter-droplet distance and may affect the extent of droplet destabilisation upon freeze-thawing.

The effect of water content on the degree of oleosome destabilisation was examined by comparing the oil yield from freeze-thawed RWOB emulsions (pH 9) which had increasing water content, from the original value ( $0.28 \pm 0.02$ ) to 0.50 and 0.70 (wt/wt; Section 2.2.3). As shown in Fig. 4, about 50% (wt%) of oil was recovered from RWOB at the original water content, whereas no oil was recoverable at 0.50 and 0.70 water content, suggesting reduced oleosome destabilisation at higher water content.

The lower emulsion rupture at higher water content could be due to the increased inter-droplet distance, by which oleosomes are less likely to be in physical contact with each other and undergo partial

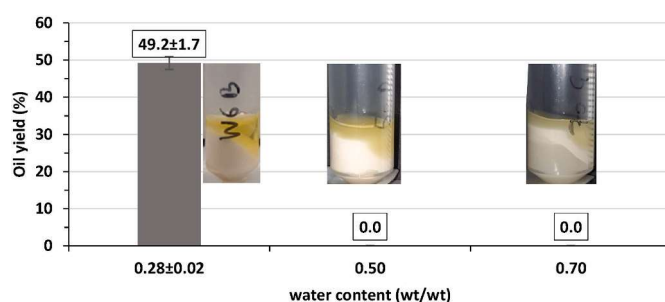


Fig. 4. Oil yield (wt% of RWOB dry mass) from freeze-thawed RWOB (pH 9) emulsions at  $0.28 \pm 0.02$ , 0.50 and 0.70 water content (wt/wt), isothermally cooled to  $-20^{\circ}\text{C}$  for 24 h.



coalescence. This was also observed in several types of emulsions (Cortés-Muñoz et al., 2009; Palazolo et al., 2011; van Aken, 2002; Vanapalli et al., 2002).

We must consider that released oil is also observed in the emulsions at 0.50 and 0.70 (wt/wt) water content, suggesting oleosome rupture to a certain extent, but the oil released could not be quantified as the oil was entrapped in a gel-like layer formed at the top of the freeze-thawed RWOB after centrifugation. The nature of such a layer is not clear yet (probably a network made of oil, water, and surfactants – mainly liberated phospholipids and oleosins). The gel-like layer was also observed in freeze-thawed RWOB at original water content after centrifugation and oil collection, but it was thinner (average thickness approximately 1 mm), thus the entrapped oil would be only a minor fraction of the total released oil. Due to the presence of such a layer, in which oil was entrapped, oil yield was thus always under-estimated (albeit to a much lower extent at the original water content), as the amount of oil contained within the layer could not be quantified. Furthermore, hexane addition in the freeze-thawed emulsion did not allow collection of the oil trapped in the gel, suggesting the water continuous nature of the layer. The relative thickness of the layer, seemed therefore to increase at higher water content of the emulsion. Further work may be required to quantify the extent of oleosome rupture within this layer.

In conclusion, increasing the relative dilution in RWOB reduces the oil yield after freeze-thawing, which could depend on both a reduced extent of oleosome destabilisation and the entrapment of higher amount of oil in a gel-like layer.

3.4. Effect of pH on the destabilisation of oleosome emulsions by freeze-thawing

The characteristics of the interfacial layer (e.g., emulsifier type and concentration) surrounding the oil droplets in o/w emulsions are important to determine the droplet stability to freeze-thawing. The emulsion pH can affect the droplet interfacial properties and their resistance to partial coalescence upon freeze-thawing, by modifying the droplet electrostatic charge at the interface or by modifying the conformation of interfacial proteins in protein-stabilised emulsion droplets. For this reason, the effect of RWOB pH on the degree of oleosome rupture at different isothermal holding times is investigated in this section. In Fig. 5, the oil yield in freeze-thawed RWOB at pH 9, 6 and 3 (Section 2.2.2) is shown. It can be observed how, at 6 h and 24 h of isothermal hold at  $-20\text{ }^{\circ}\text{C}$ , the oil yield increases by lowering the pH.

In Section 3.3, we reported a possible effect of water content on the freeze-thaw stability of oleosome emulsions. The water content was similar for the three emulsions (inset table, Fig. 5), nevertheless the emulsions were more destabilised by lowering the pH, thus suggesting the destabilising effect of lowering the pH. The increasing oil yield at longer isotherm time suggests the role of lipid crystallisation for oleosome rupture for both RWOB at pH 6 and 9. However, the oil yield at pH 6 was significantly higher than at pH 9, which could be explained by a reduced inter-droplet distance caused by a less negative  $\zeta$ -potential, which promoted partial coalescence.

The gel-like layer at pH 6 is observed at 6 h and 24 h isothermal hold, but its thickness is reduced if compared with that at pH 9, although the two emulsions had similar water content. It was already mentioned in Section 3.3, that the gel-like layer thickness depends on the water content, but also the pH contributes to determine its thickness. It is possible that the lower pH has modified the structure of integral and/or residual exogenous proteins and reduced their water holding capacity, resulting in a thinner layer. The reduced water holding capacity was also indicated by the presence of a much larger water layer pooled at the bottom of the centrifuged emulsions at pH 6 (and pH 3) (Fig. 5C and D) than that at pH 9 (Fig. 5B).

Unlike the emulsions at pH 9 and 6, RWOB pH 3 showed extensive oleosome rupture (oil yield  $>80\%$ ) already after the cooling step (0 h isothermal hold), suggesting that water crystallisation is sufficient to rupture oleosomes, and that lipid crystallisation is not necessary to induce destabilisation. Furthermore, even without freeze-thawing, RWOB at pH 3 is unstable and free oil is visible within four days of storage at  $5\text{ }^{\circ}\text{C}$ . Therefore, freezing only accelerated oleosome rupture, probably due to ice crystals compressing the droplets and/or piercing the interface. The reduced oleosome stability at  $\text{pH} < 6$  is also reported in Tzen et al. (1992), who observed coalescence at pH below 5; peanut oleosomes extracted at pH 4 resulted partially destabilised, with oil release (Rhee et al., 1972).

A possible explanation of the high instability in RWOB pH 3 is a rearrangement of the oleosome interface due to the low pH, as hexane can penetrate fresh oleosomes (unlike at pH 9 and pH 6), and oil is visibly released in hexane within 2 h. Lowering the oleosome emulsion pH to 3 may have irreversibly altered the oleosome interfacial architecture, leading to a loss of stability. Exposure of oleosomes to hexane is normally used as a diagnostic method to test the oleosome integrity (Tzen et al., 1997). It is possible that hexane penetrates the oleosomes through specific patches generated by the low pH, such as fractures or interfacial areas with altered organisation.

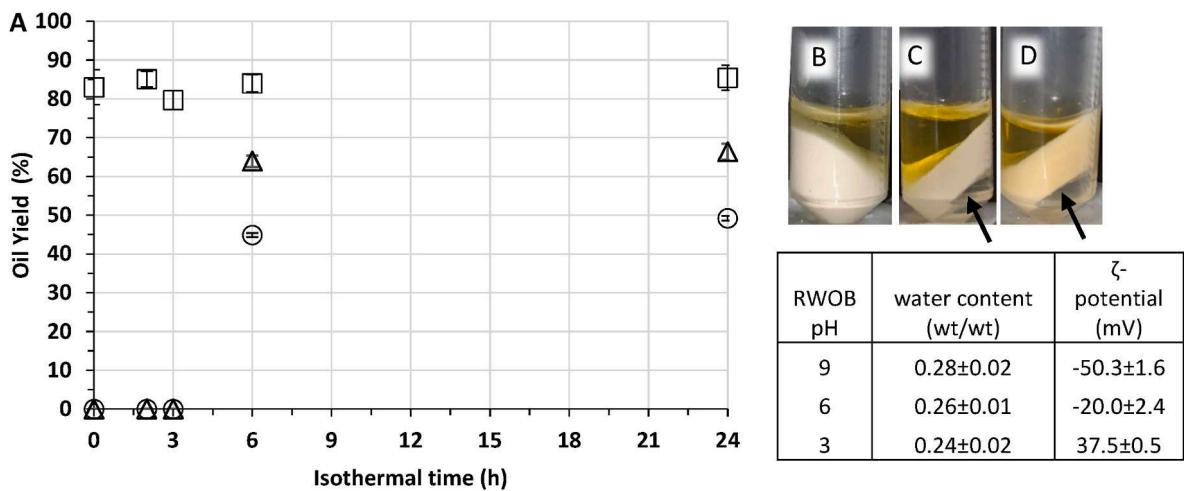


Fig. 5. A) Oil yield from freeze-thawed RWOB emulsions at pH 9 (circle), 6 (triangle) and 3 (square). RWOBs were isothermally held at  $-20\text{ }^{\circ}\text{C}$  for 0 (only cooling step), 2, 3, 6, and 24 h. If not visible, error bars are within the marker. B, C, D) Images of freeze-thawed RWOB emulsions at pH 9 (B) and pH 6 (C) after 6 h of isothermal hold at  $-20\text{ }^{\circ}\text{C}$ , and (D) at pH 3 after 0 h (only cooling); black arrows in (C) and (D) indicate the pooled water. Inset table contains compiled values of water content and  $\zeta$ -potential for each emulsion.



The emulsion aggregation progressively increased with lowering the pH, turning from a creamy consistency at the original pH into a more solid-like consistency at pH 3. This increased aggregation may depend in part on the water content, slightly decreasing at lower pH, but mainly on the altered interfacial properties of the oleosomes at lower pH.

The RWOB  $\zeta$ -potential was measured at pH 9, 6 and 3 (inset Table in Fig. 5). The  $\zeta$ -potential accounts for the surface charge of the oleosomes, which in turns determines the extent of droplet repulsion. The  $\zeta$ -potential values went from negative to positive upon adjusting the oleosome pH from alkaline to acid, and this trend was also observed in other studies with oleosome emulsions washed with sodium bicarbonate 0.1 M (De Chirico et al., 2018; Romero-Guzmán et al., 2020). The  $\zeta$ -potential at pH 9 was highly negative ( $-50.3 \pm 1.6$  mV); at this value oleosomes would experience electrostatic repulsion and remain physically separated, although during crystallisation of the continuous phase, ice would apply pressure on the oleosomes and overcome their natural resistance to flocculation. When the pH was lowered to 6, the  $\zeta$ -potential decreased to a less negative value ( $-20 \pm 2.4$  mV), which may have provided a less effective oleosome electrostatic repulsion allowing more physical interaction between oleosomes, explaining the increased emulsion thickness and the higher oil yield after freezing of the lipid phase at this pH. At pH 3, the  $\zeta$ -potential was  $+37.5 \pm 0.5$  mV; such a value would suggest electrostatic repulsion between oleosomes, consequently reduced aggregation as the oleosomes would be physically separated. However, this is not the case, as it was previously reported that the emulsion at pH 3 was the most solid-like, and the increased aggregation may depend on the aforementioned conformational changes in the structural proteins (and in part in the remaining exogenous proteins), which could interact with each other and create a network. We may point out that oleosome aggregation could have occurred while measuring  $\zeta$ -potential at pH 3; however, due to the very low oleosome concentration (0.008%; Section 2.2.2.1) used for the  $\zeta$ -potential measurement, it is likely that aggregation did not occur.

Changes in the emulsion microstructure induced by different pH values are also suggested by the PSD of RWOBs (Fig. 6). Prior to the measurement, the emulsions were re-dispersed into recovery buffer at pH 9.5 at a ratio of RWOB-to-buffer 1:100 (wt/v); this was done to facilitate oleosome re-dispersion by creating an alkaline environment. Due to the high dilution ratio, the pH of the re-dispersed emulsions equalised that of the buffer (pH 9.5). Despite the increase in pH, RWOB PSD at pH 6 and especially at pH 3 was higher than that at pH 9. Hence adjusting the pH to alkaline values in those emulsions was not sufficient to restore the properties of the initial RWOB thus suggesting that oleosomes at pH 6 and pH 3 must have experienced irreversible changes at the oleosome interface determining the observed instability. Such changes are also suggested by the presence of aggregates visible in the micrographs (Section 2.2.2.1) of RWOB pH 3, shown in Supplementary Fig. 1.

The results related to the freeze-thaw stability of oleosomes at different pH suggest that the interfacial layer has a role in the oleosome

resistance to rupture by freeze-thawing. The low pH may modify the oleosin conformation, in particular the two arms, with the N- and/or C-terminus no longer lying flat on the interface and playing a protective role, allowing hexane penetration. To the best of our knowledge, there seems to be a lack of studies related to the oleosin configuration in oleosomes at different pH values, although Qi et al. (2017) reported that soybean oleosin secondary structure experiences a decrease in alpha helix structures from pH 9 to pH 3, due to protonation, and a concomitant increase in beta sheet. The authors did not report destabilisation of oleosomes at pH 3, whereas in our work destabilisation was evident, perhaps due to a different behaviour of rapeseed oleosomes with respect to soybean oleosomes.

The rearrangement at low pH may be also influenced by a possible protonation of a fraction of the phospholipids interacting with the oleosins. At pH 3, it is possible that a fraction of the oleosome interfacial phospholipid heads is positively charged. The pKa of the amino-groups in phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, and the carboxylic-group in phosphatidylserine are  $>11$  and  $>5$ , respectively; therefore, they would be protonated at pH 3. The pKa of the phospholipid phosphate group is about 3 (except for phosphatidylcholine, which has pKa of about 1.4) (Tsui et al., 1986; Marsh, 2013), suggesting that a fraction of the phospholipids is likely to be positively charged at pH 3. Repulsive electrostatic forces between phospholipid heads and the interior face of the oleosin C- and N-terminal domains may contribute to a change of its original configuration, reducing the oleosin ability to cover the oleosome surface. As reported by Nikiforidis et al. (2013), the absence of polar lipids in purified oleosins might influence the intermolecular interactions and the tertiary structure of the protein molecule. Similarly, in RWOB pH 3, the oleosin-stabilising role of phospholipids may be lower, and the oleosin anchorage into the phospholipid monolayer would rely more on the hydrophobic interaction with the phospholipid tails. Moreover, the electrostatic repulsion between the fraction of positively charged phospholipids and the inner face of the oleosin C- and N-terminals may increase the distance between the oleosin terminals and from the phospholipid surface, which resulted less covered by the oleosins. In such a condition, the steric hindrance effect by oleosins would be reduced, promoting oil release.

To summarise, the pH in the continuous phase modifies oleosome  $\zeta$ -potential, affects the consistency of RWOB, and oleosome stability to freeze-thaw. The RWOB at pH 3 was unstable per se, and freeze-thawing only accelerated oleosome rupture. Lowering the pH from 9 to 6 or 3 increased irreversibly the PSD of oleosome emulsions, which could not be reprecipitated by increasing the RWOB pH back to 9, suggesting that oleosomes had been destabilised via coalescence. Considering the results of Sections 3.2 and 3.4, a conclusive observation that could be made is that both the crystallisation profile of the dispersed phase and the resistance of the interfacial layer should be considered when oleosomes undergo freeze-thawing, either if freeze-thaw stability is desired, or if droplet destabilisation is desired for future use.

### 3.5. Effect of number of freeze-thaw cycles on oleosome emulsion stability

Another factor which could affect the freeze-thaw stability of oleosomes is the number of freeze-thawing cycles applied. Data in Fig. 7 show the oil yield values from RWOB (pH 9) which underwent 1, 2 or 3 freeze-thaw cycles before centrifugation and oil collection (Section 2.2.2). This experiment was carried out only for RWOB pH 9, as RWOB pH 3 was unstable per se, and RWOB pH 6 also had a degree of instability, as shown by its higher PSD compared with RWOB pH 9. Therefore, further destabilisation of RWOB pH 3 and 6 may have at least partially depended on their intrinsic instability rather than on the additive freeze-thaw cycles.

Increasing the number of freeze-thawing cycles from one to two caused an increase in the oil yield, whereas no further increase in the oil yield was observed with a further cycle. The oil released from the first cycle was 44.9% of the total oil in the oleosome emulsion, whereas the

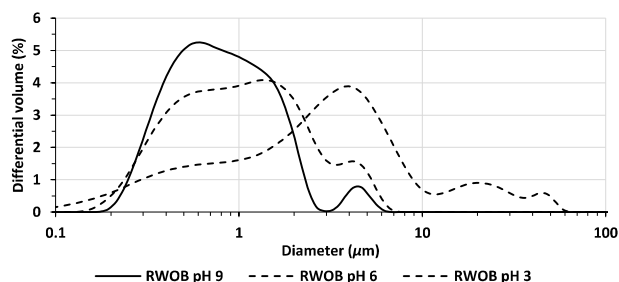
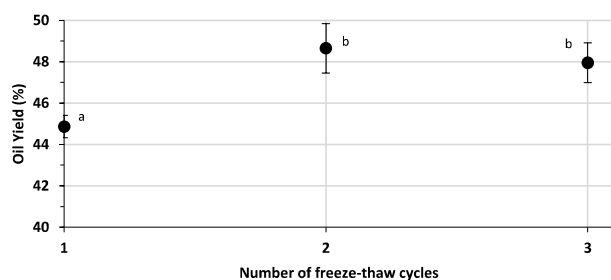


Fig. 6. Oleosome size distribution measured from RWOB pH 9, 6 and 3 redispersed at pH 9.5 at a ratio emulsion-to-buffer of 1:100. The values of pH reported in the legend are referred to the undiluted original emulsions.



**Fig. 7.** Oil yield (wt%) from RWOB (pH 9) freeze-thawed for 1, 2 or 3 cycles prior to centrifugation and oil recovery. For each freeze-thawing cycle, emulsions were isothermally cooled to  $-20^{\circ}\text{C}$  and held at this temperature for 6 h, then thawed at  $20^{\circ}\text{C}$  for 18 h. Different letters indicate statistically different samples as established by ANOVA ( $P < 0.05$ ).

oil released from the second or third cycle was approximately 48% of the total oil. Therefore, most of the oleosome rupture occurred after one cycle at those specific cooling conditions. The reason for this may be that smaller oleosomes are more resistant to freeze-thawing, due to the higher surface curvature, which may affect the bending energy of the interface, thus the free energy for the coalescence transition state (Kabalinov and Wennerström, 1996; Ghosh and Coupland, 2008). Hence, increasing the number of freeze-thaw cycles does not cause further oleosome rupture as the remaining oleosomes may be resistant to those specific cooling conditions (target cooling temperature, cooling rate, thermal exchange with cooling medium). A general higher stability of smaller oleosomes was reported by Boulard et al. (2015) and Yang et al. (2020), perhaps provided by the higher protein-to-oil ratio compared with larger oleosomes (Tzen et al., 1993), and by the higher curvature which would make them less prone to coalescence. Measuring PSD of the freeze-thawed RWOB would provide useful information about the size of the oleosomes remaining after each freeze-thawing cycle; however, as the freeze-thawed RWOB PSD is affected by the released oil, at the moment this technique cannot be used to identify the size of the remaining intact oleosomes.

Another aspect to consider is that dilution occurs in the emulsion upon thawing as the oil released from ruptured oleosomes pools to form larger oil drops, leading to an increase in relative dilution in the residual, unbroken emulsion. As reported in Section 3.3, emulsion stability to freeze-thawing may increase at higher water content, therefore further oleosome destabilisation after cycle 1 may be mitigated by the higher relative water content in the residual emulsion.

Other authors tested the stability of o/w emulsions at increasing number of freeze-thaw cycles. Vanapalli et al. (2002) observed progressively higher oil release in an o/w emulsion of confectionery coating fat (cocoa butter substitute 'high trans') and polyoxyethylene sorbitan monolaurate (Tween 20), with the number of freeze-thaw cycles increasing from 1 to 4; the destabilisation also progressively increased with higher dispersed phase mass fraction. The authors suggested that the higher emulsion destabilisation would have been caused by a greater number of droplet collisions, facilitated by the higher dispersed volume fraction, and promoted by further freezing cycles. Magnusson et al. (2011) reported that mayonnaise-type emulsions after five freeze-thaw cycles were more destabilised, in terms of increased droplet particle size, than after one single cycle.

The above studies reported that increasing the freeze-thaw cycle number generally leads to higher destabilisation, whereas RWOB seems to maintain a certain stability after the first freeze-thawing cycle. This suggests that the effect of freeze-thaw cycle number would also depend on the emulsion composition and by the cooling profile adopted.

#### 4. Conclusions

The integrity of oleosomes recovered from OSR seeds is affected by freezing and thawing, in the most extreme cases resulting in the rupture of oleosomes and release of free oil. The degree of rupture is affected by the emulsion characteristics, namely the continuous phase pH (higher oleosome destabilisation upon decreasing pH, when the freezing temperature was  $-20^{\circ}\text{C}$ ), and the concentration of the oleosome dispersed phase (more destabilised at higher emulsion concentration).

Other important parameters affecting the oleosome stability were the rate and extent of freezing regimes. After 2 h of isothermal holding at  $-20^{\circ}\text{C}$  followed by thawing, RWOB at pH 9 and 6 were not destabilised, whereas initial destabilisation occurred after freezing for 3 h at  $-20^{\circ}\text{C}$ , then thawing. As shown by the DSC data, lipid crystallisation starts after 2 h at  $-20^{\circ}\text{C}$ , suggesting its role for oleosome destabilisation. In the case of RWOB at pH 3, this instead was destabilised after a few minutes cooling, when only the aqueous phase crystallised.

When the emulsions were cooled in a freezer to  $-75^{\circ}\text{C}$  for 18 min (time needed to reach  $-75^{\circ}\text{C}$ ) or in liquid nitrogen for 30 s (time needed to reach  $-100^{\circ}\text{C}$ ), then thawed, they remained stable. Conversely, if they were immediately stored to  $-20^{\circ}\text{C}$  after reaching  $-75^{\circ}\text{C}$  or  $-100^{\circ}\text{C}$ , they destabilised. Increasing the number of freeze-thaw cycles (cooling temperature  $-20^{\circ}\text{C}$ ) to 2 led to 8% increase in oil yield, but no further increase after 3 cycles.

Besides the freezing profile, also the thawing profile can affect the oleosome stability as observed in the extensive oleosome rupture when oleosomes frozen with liquid nitrogen were stored at  $-20^{\circ}\text{C}$ , before complete thawing at  $20^{\circ}\text{C}$ .

#### CRedit authorship contribution statement

**Filippo Bramante:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vincenzo di Bari:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Gary Adams:** Writing – review & editing, Supervision, Methodology. **Frederic Beaudoin:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Gustav Waschatko:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Ralf Jakobi:** Supervision, Methodology. **Nils Billecke:** Supervision. **David A. Gray:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, 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.jfoodeng.2025.112471>.

#### Data availability

Data will be made available on request.

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