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Assessment of rapeseed oil body (oleosome) lipolytic activity as an effective predictor of emulsion purity and stability.

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Abstract

The lipolytic activity in oil body creams as affected by recovery and washing protocols was investigated. The effect of thermal treatment on the hydrolytic activity and physical stability of fresh and aged (up to 30 days) oil body emulsions was studied. The use of alkaline pH solutions (9.5) to soak and grind rapeseeds were more effective reducing the contamination of oil body material from seed proteins/enzymes, compared with neutral pHs. Soaking and grinding seeds with a $NaHCO_3$ solution (0.1 M, pH 9.5) yielded oil bodies with a similar composition to those prepared in urea (9 M); however, the physical stability over storage was compromised due to the presence of hydrolytic enzymes. Heating a dispersion of oil bodies for 6 mins at 95°C did not alter the physical properties of oil bodies and significantly reduced lipolytic activity (>90% enzyme inactivation), resulting in a stable emulsion.

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1. Introduction

The aqueous grinding of oil crop seeds allows to recover oil bodies (oleosomes) in the form of a natural oil-in-water emulsion (Adams et al., 2012; Bonsegna et al., 2011; Iwanaga et al., 2007; Nikiforidis & Kiosseoglou, 2009; Payne, Lad, Foster, Khosla, & Gray, 2014). Seed oil body (OB) size is variable among plant species (0.2-2.5 μm of diameter), consisting of one triacylglycerol (TAG) core, stabilised by proteins inserted into a phospholipid monolayer (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). Unlike other cellular membranes, the OB surface consists of a monolayer (half unit membrane), and those in seeds contain unique proteins called oleosins (Frandsen, Mundy, & Tzen, 2001; Huang, 1996; Tzen, Lie, & Huang, 1992), whose quantity has been demonstrated to be correlated to the OB size (Jolivet et al., 2013; Siloto, 2006; Vandana & Bhatla, 2006). OBs are formed by vesciculation from the endoplasmic reticulum (ER) of the cell. Newly synthetized TAGs accumulate between the two leaflets of the ER membrane leading to its swelling and, when the vesicle reaches a critical size, buds off completely (Bewley, Bradford, Hilhorst, & Nonogaki, 2013).

Oleosins consist of two amphipathic domains at the N- and C-terminal exposed to the cytosol, and a central hydrophobic domain inserted into the TAG matrix (Huang, 1992). Oleosins, providing steric hindrance and electrostatic repulsion, helps the OBs to maintain their individuality over seed maturation, drying (Tzen & Huang, 1992) and following rehydration. However, the actual role of those integral proteins over germination has remained speculative among researchers. This process starts with TAG hydrolysis which is mediated by lipases at the OB surface, and yields glycerol molecules and free fatty acids (FA). It has been suggested that oleosins

(together with caleosins, another group of integral protein in OBs) may play a role in facilitating the access of lipases to its substrate (Lin, Wimer, & Huang, 1983; Lin & Huang, 1983), as this enzyme can act only upon binding to a membrane (Allen & Tao, 2007). Besides, it has also been suggested that the OB surface forms a barrier from lipolytic action and that a phospholipase A_2 needs to degrade the phospholipid monolayer prior to TAG hydrolysis (Gupta & Bhatla, 2007; May, Preisig-Müller, Höhne, Gnau, & Kindl, 1998). In a different work (Sadeghipour & Bhatla, 2002) it was shown that the proteolysis of oleosins takes place at the onset of TAG mobilization in sunflower seeds, but this may not be a necessary step for the lipolysis (Beisson et al., 2001). More work is required to understand which are the factors playing a major role in TAG hydrolysis. Moreover, to the best of the authors' knowledge, no one has measured the hydrolytic activity in purified OB extract, nor determined the minimal processing parameters to stabilise the recovered material against the general hydrolytic activity. Oleosins are hydrolysed rapidly during post-germinative growth in many plant species, including maize, rape, sesame and sunflower seed, with the concomitant conversion of triacylglycerols (TAGs) into fatty acids (FAs) (Murphy, Cummins, & Kang, 1989a; Sadeghipour & Bhatla, 2002). In a similar process, during the storage of OBs, lipase, protease and phospholipase will inevitably lead to the degradation of the recovered OB cream. However, the amount and activity of those enzymes in OB extracts, as well as other seed proteins, may depend on a number of factors, including the grinding medium used. We recently demonstrated that when preparing OB creams from oilseed rape, 0.1 M of sodium bicarbonate solutions ($NaHCO_3$) removes exogenous proteins (proteins not considered to be an intrinsic part of the OB structure) to a similar extent as 9 M urea (De Chirico, di Bari, Foster, & Gray, 2018). What was not established was whether this degree of washing

removed enough loosely-associated enzymes to limit enzyme-induced deterioration of OBs during storage. If enzyme carry-over during the initial process of OB recovery can be reduced, then this will lower the thermal treatment required to stabilise the cream. Thermal denaturation of seed enzymes in OB extracts has not been investigated in detail in literature, and the parameters used in this process are not consistent among authors. In fact, thermal treatment may vary between 10 to 30 minutes, at temperatures ranging between 70 to 100 °C (Chen, McClements, Gray, & Decker, 2012; Iwanaga et al., 2007; Karkani, Nenadis, Nikiforidis, & Kiosseoglou, 2013; Naziri et al., 2017; Nikiforidis, Donsouzi, & Kiosseoglou, 2016). Clearly, a better understanding of the minimal thermal treatment required to enhance OB stability against enzymatic degradation needs to be developed.

The aim of this work was to test the impact of OB grinding and washing media on lipolytic activity, and to establish, using this enzymatic activity as marker, the minimal parameters to stabilise OBs against hydrolytic damage. Lipolytic enzymes isoforms may be located in different compartments of the seed, depending on the species (Hills & Murphy, 1988; Lin & Huang, 1983; Theimer & Rosnitschek, 1978). For example, in rapeseed, lipase activity is usually found in both OBs and soluble cell fraction after density gradient separation of the homogenised seed, while soy seeds do not have any lipolytic activity associated to the OB (Hills & Murphy, 1988). Lipolytic activity was chosen as a marker because: (i) enzymes with lipolytic activity are physically associated with recovered OBs from oilseed rape seeds (Lin & Huang, 1983; Rosnitschek & Theimer, 1980; Theimer & Rosnitschek, 1978) (ii) their thermal tolerance is relatively high (Ponne et al., 1996) and (iii) lipolytic enzymes in oilseed rape seed, if not OBs, are well characterised.

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2. Materials and Methods

2.1 Materials

The chemicals used were conforming to quality specifications by the Committee on Analytical Reagents of the American Chemical Society (ACS grade) or higher and sourced from Sigma-Aldrich Co. (St. Louis, MO, USA). Ultrapure water was used to prepare all buffers (Nanopure Infinity system, Barnstead International, IA). Oilseed rape seeds (*Brassica napus L.*, variety DK Exalte) were provided by a local farm following the 2017 summer harves, and stored at 20°C and rH 50% until use.

2.2 Oil Body Recovery

Oil bodies from oilseed rape were isolated and purified following the method of De Chirico *et al.* (2018), with the following modifications (S.I. 1). Seeds (200 g) were soaked in $NaHCO_3$ pH 9.5 (0.1 M, adjusted using a 0.1 M NaOH), or ultrapure water (dH_2O) at ratio of 1:4 (w/v) at 4°C for 16 h and the soaking medium was then discarded. The soaked seeds were ground in the same pre-chilled (4°C) media used for soaking at ratio (dry seed weight based) of 1:7 (w/v) in a Kenwood blender (BLX52) at full power (800 W) for 90 sec. The dispersion was filtered through three layers of cheesecloth (grade 80, thread count: 40 x 32 threads per square inch) and transferred in 400 mL tubes, centrifuged at 10,000 g for 30 min at 4°C (Beckman J2-21 centrifuge, fixed rotor JA-10). The upper layer (crude OB fraction – COB) was isolated using a spatula and drained on filter paper (Whatman, grade 1). The COB preparation was dispersed in washing solution (0.1 M $NaHCO_3$, pH 9.5, or dH_2O, 1:4 w/v) and centrifuged (10,000 g, 30 min at 4°C). The fat pad was isolated using a

spatula, drained on filter paper (Whatman, grade 1), and designated as “WOB I”. Each cream was then suspended in dH_2O (15% w/v lipid weight basis) and centrifuged (10,000g, 30 min at 4°C). The cream layer was collected using a spatula, drained on filter paper (Whatman, grade 1) and designated as “WOB II”. All creams were stored at 4°C until further characterisation.

2.3 Thermal treatment of oil body emulsions

WOB I creams were re-suspended in dH_2O (15% w/v lipid weight basis, S.I. 1) and oil body emulsions (5 mL) were pipetted into glass tubes (8 mm inner diameter, 15 cm length), flashed with nitrogen and closed with a cap. Prior to the treatment, the samples were equilibrated at 40 °C (3 min) to shorten and standardise the come-up time (35-40 sec). The thermal treatment consisted of heating emulsions to 95°C for up to 6 min, in a circulating thermostatic water bath (Grant instruments, Cambridge, UK). A thermocouple, connected to a data logger, was inserted at the centre of the tube to record the emulsion time-temperature history during the treatment. Following the thermal treatment, the samples were cooled in ice and centrifuged (10,000 g, 30 min at 4°C). The cream layer was collected using a spatula, drained on filter paper (Whatman, grade 1) and designed as “thermally treated WOB II” (S.I.1).

2.4 Lipolytic activity assessment

2.4.1 Preparation of enzyme solutions from oil body creams

Freshly extracted WOBII creams and thermally treated WOBII (1 g) were placed in a 50 mL conical tube and 20 mL of cold acetone (4 °C) added. Each tube

was frozen in liquid nitrogen, placed in ice, and the oil bodies broken using an Ultra Turrax (T18, Ika, Oxford) at 20,000 rpm for 1 min. Samples were centrifuged (5,000 g, 10 min, 3°C) and the supernatant (containing the oil) collected. This step was repeated twice. The residual pellets were completely dried under nitrogen until constant weight. Dry pellets were then suspended in sodium phosphate buffer (50 mM, pH 7) with the addition of 0.1% w/v Triton X-100 and agitated at 4°C for 6 h. The samples were filtered (0.45 μm) and the clarified enzyme solution was used for lipase assay on the same day.

2.4.2 Preparation of enzyme solutions from oil seed rape seeds

Lipase was extracted as described by Ponne et al. (1996) with the following modifications. Dry (internal moisture content of 8%) or soaked (16 h, in dH_2O or 0.1 M $NaHCO_3$, pH 9.5) rapeseed seeds (5 g) were crushed using a cool mortar and pestle and then transferred into a conical tube. Cold acetone (4 °C, 20 mL) was added, the conical tube was placed in ice, and the seeds were mixed for 1 minute using an Ultra Turrax (T18) at 20,000 rpm. The samples were centrifuged (5,000 g, 10 min, 4°C) and the oil phase removed. This step was repeated twice, and residues of acetone removed by nitrogen gas. The pellet was re-suspended in sodium phosphate buffer (50 mM, pH 7) with 0.1 % Triton X-100 and incubated for 6 hours (4 °C) on a rotary shaker. The mixture was filtered using cheesecloth, and the crude extract was centrifuged at 21,000 g (4 °C). The water phase was collected and used on the same day for the lipase assay.

2.4.3 Clarification of serum phases from residual oil bodies

The serum phases were collected after centrifugation of untreated and thermally treated WOB I emulsions and was centrifuged (21,000 g, 1 h, 4°C) to remove oil body contamination. The lower phase was carefully removed with a syringe and needle. This step was repeated three times. Finally, the serum was passed through a 0.2 μm membrane and used for lipolytic assay the same day.

2.4.4 Lipolytic assay

Lipase activity was measured using a modified protocol of Ruiz et al. (2004). The non-coloured substrate p-NPL was dissolved in 2-propanol at a final concentration of 20 mM by sonication for 3 minutes. The substrate was diluted at a final concentration of 2 mM in sodium phosphate buffer- Triton X-100, with gentle agitation until a clear solution was formed. Enzyme solution (1 mL, 50mM sodium phosphate buffer, pH 7) was mixed with 1 mL of substrate mixture to obtain a 2 mL final reaction mixture (1 mM p-NPL, 5% v/v 2-propanol, 0.6% w/v Triton X-100, 50 mM sodium phosphate buffer), which was incubated at 37°C for 2 h. At the end of the incubation time, the samples were cooled in ice for 1 minute to stop the reaction and the absorbance at 405 nm was measured. The blanks, corresponding to the absorbance of the reaction mixture without enzyme solution, were determined and subtracted from the total absorbance. The net absorbance was compared to a standard curve built using commercial lipase. One enzymatic activity unit (U) is expressed as the enzymatic activity that released 1 μmol of p-NP from p-NPL under the assay condition. This value was normalised by protein content.

208

209 ### 2.6 Protein quantification and analysis by SDS-PAGE

210 Protein extracts as recovered from Section 2.4 were assayed on the same
211 day for protein content using BCA assay (Smith et al., 1985). On performing the
212 analysis, samples were diluted to obtain absorbance values within the range of the
213 standard curve. For compositional analysis, an appropriate amount of protein extract
214 collected was mixed with an equal volume of sample buffer (Laemmli buffer - Biorad,
215 UK) + 5% w/v β-mercaptoethanol, and heated at 95 °C for 5 min then cooled on ice.
216 Proteins were resolved by SDS-PAGE using 4–15% w/v polyacrylamide gels (Mini-
217 Protean TGX Gels, 10-well, 50 *μ*L, Bio-Rad, Hercules, USA); gels were positioned
218 within a SE 600 BioRad separation unit and suspended in tank buffer (25 mM Tris,
219 250 mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was run at 100 V for 40 min.
220 After electrophoresis, the gels were fixated and stained (1 h) using Coomassie
221 brilliant blue R-250 (0.1% w/v Coomassie, 50% v/v ethanol, 10% v/v acetic acid) and
222 de-stained with water. Gels were imaged using Bio-Rad Gel Doc XR System
223 (Hercules, USA) .

224

225 ### 2.7 Particle size analysis of oil body emulsions

226 The particle size of oil body dispersions was measured with an LS 13320
227 laser diffractometer from Beckman-Coulter (FL, USA) using the Mie theory of the
228 scattering of light by spherical particles. The samples were diluted as appropriate
229 prior to measurement. The real part of the refractive index used for the calculation of
230 oil body size was 1.462, corresponding to the refractive index of rapeseed oil. The

231 imaginary part, corresponding to the attenuation coefficient that describes the
232 turbidity of a sample, was set to 0.01 according to the laser diffractometer guidelines
233 for lightly coloured translucent materials. For the description of particle size
234 distribution (PSD), the volume frequency distribution (%/μm) and the volume mean
235 diameter ($D_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$) have been reported.

236

237 **2.8 Zeta potential analysis of oil body emulsions**

238 A series of oil body emulsions were prepared in ultrapure filtered (0.2 μm)
239 water at a concentration of 0.008% (lipid weight basis) and the pH adjusted between
240 3 and 10 using 0.1 M HCl or 0.1 M NaOH. After stabilisation of the pH, the emulsions
241 were injected into the measurement chamber of the particle electrophoresis
242 instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA). The
243 instrument settings used were: temperature = 25 °C; refractive index of dispersant =
244 1.333; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of
245 dispersant = 79.0; electrode spacing = 50.0 mm; voltage = 35 V. The ζ-potential was
246 then determined by measuring the direction and velocity of the oil bodies moving
247 under the applied electrical field. The ζ-potential value was reported as the average
248 and standard deviation of three independent samples from each emulsion replicate,
249 with one reading taken per sample.

250

251 **2.9. Preparation of oil body emulsions for stability test**

252 Oil body emulsions were prepared suspending the WOB II creams (untreated
253 or thermally treated at 95 °C, up to 6 minutes) in dH_2O (10% w/v lipid weight basis)

using a vortex at maximum speed (1 min). Sodium Azide (0.02 mM) was present in all emulsions to avoid microbial spoilage. The particle size and zeta potential were measured at a time over 30 days storing the samples at 20°C.

2.9 Light microscopy of fresh and aged oil body emulsions

The microstructure of oil body dispersions was investigated using light microscopy (EVOS, UK). A small drop of oil body emulsion was placed on a glass slide, covered with a cover slide and imaged at a magnification of 40×.

2.10 Confocal microscopy of fresh and aged oil body emulsions

Oil body emulsions were stained to visualise the protein and oil fractions. Nile Red (Sigma Aldrich, 0.01%), was used to label lipids, and Fast Green FCF (Merck 0.01%) was used to bind to structural proteins. Microstructural Imaging Images of the emulsion sample was acquired using CLSM. Samples were mounted on a glass slide and examined using a Zeiss LSM880 Laser Scanning Microscope. A band pass filter between 550-625 nm was selected for the detection of nile red when excited at 488nm, while a band pass filter between 640-700 nm for the detection of fast green FCF when excited at 633nm. Images were acquired using an x63 oil objective, with argon and HeNe laser, respectively. An overlay of the two channels, to the same area, gave the final image of the network seen in the samples, showing in red the lipid, and in green the protein structural component.

2.11 Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed by one-way ANOVA (ANalysis Of Variance) using SPSS version 13.0 software (IBM, Chicago, USA). Assessment of significance difference was based on a 95% confident limit ($P < 0.05$).

3. Results

3.1 Effect of recovery medium on the carry-over of hydrolytic activity

Recent works have focused on the physicochemical characterization of OBs from oil crop seeds as affected by extraction media, demonstrating the alkaline solutions (pH>8) to be particularly effective against the carry-over of exogenous seed material (Chen & Ono, 2010; De Chirico et al., 2018; Zhao, Chen, Chen, Kong, & Hua, 2016). However, despite the efforts made, some enzymes were still found in purified OB creams (Katavic, Agrawal, Hajduch, Harris, & Thelen, 2006; Zhao et al., 2016). Although it has been discussed that the presence of exogenous proteins may play a role in preserving structural stability (Bettini, Santino, Giancane, & Valli, 2014; Nikiforidis, Karkani, & Kiosseoglou, 2011) leading to enhanced coalescence over time (De Chirico et al., 2018). These findings suggest that the quantification of proteins may not be a reliable indicator of hydrolytic activity, therefore, a different approach should be applied.

Lipolytic activity has been associated with recovered OBs (Hills & Murphy, 1988; Lin & Huang, 1983), but it may be possible to remove these enzymes by washing the OB preparation with acqueous media. It was hypothesised that a reduction in lipolytic activity (due to the protocol for the recovery and washing of OB cream) would also correspond to a reduction of other hydrolytic activities (tested in Sections 3.4 and 3.5). In this set of experiments, high protein content OBs, recovered using dH_2O, were assayed for lipolytic activity and compared with low protein content OBs, recovered using $NaHCO_3$ solution (pH 9.5, 0.1 M), to establish the impact of seed soaking, grinding medium and washing protocol used on the carry-over of lipolytic activity in the OB preparation. As a reference of the purest preparation of OBs, COB

recovered in $NaHCO_3$ (0.1 M) were washed in urea (9 M) and rinsed in water as described by De Chirico *et al.* (2018). The colorimetric method to measure lipase activity using p-NPL (Ruiz et al., 2004) was adopted (Section 2.4.4).

Lipolytic activity was assayed in proteins extracted from dry, soaked (16 h, dH_2O or $NaHCO_3$) oilseed rape seeds and from OB creams recovered in each step of the extraction protocol (as described in Section 2.2). As it can be seen in Table 1, some lipolytic activity was already present in mature seeds, which showed an increase of about 38% after soaking (16 h) using either dH_2O or $NaHCO_3$ (0.1 M, pH 9.5). However, the type of media (and the pH) did not have any influence ($P< 0.05$). Some reports showed that lipolytic activity is generally absent in dry seeds of different crops, rising only on the onset of germination (Huang, 1992), except for castor beans which have shown active acid lipase in the dormant seed (Ory, 1969; Ory, Angelo, & Altshul, 1960). The presence of active enzymes at this stage of maturity has been a subject of controversy among researchers. Indeed, in contrast with Huang (1992) and Lin and Huang (1983), Hoppe and Theimer (1997) were able to detect low lipase activity in mature seeds of rape, which is in line with the findings in Table 1.The increase in activity over soaking may suggest the initiation of the germination process, which involves synthesis de novo of enzymes (Bewley et al., 2013).

After grinding of seeds using either dH_2O or 0.1 M $NaHCO_3$ (pH 9.5), the enzymatic activity in the crude material (COB) showed a dependency on the type of solution used (Table 1). When soaked seeds were ground in dH_2O, the activity in COB was about two-fold higher than those recovered in $NaHCO_3$. Washing these COB samples with the corresponding solution (same as soaking and grinding), then rinsed in dH_2O (to produce WOB II) caused a similar reduction (about 13%) in the total

328 lipolytic activity in each set of OB samples, probably due to the solubilisation of the
329 enzyme in the serum phase after centrifugation. The small reduction in lipolytic
330 activity over washing protocol in OBs recovered using $NaHCO_3$ (Table 1), reveals
331 that this trend did not follow the same decay as seen for the protein content of the
332 corresponding OB sample (-40%). The weak correlation between these data sets
333 (Table 1, *R*= 0.39), suggests that different factors may play an important role, such
334 as the presence of more than one isoform of lipase, each with a different strength of
335 association with the OB, or by the presence of other molecules which may have
336 affected the activity. The presence of lipolytic enzymes with distinct properties (Hills
337 & Murphy, 1988) may imply that these have a different degree of association with the
338 OBs, which may be used to separate those lipolytic enzymes by controlling the type
339 of solution used over recovery. In fact, Table 1 shows that urea-washed OBs had the
340 lowest activity in the recovered cream. Despite the similarity in protein content
341 between Urea-WOB and $NaHCO_3$-WOB (Table 1), the lipolytic activity was
342 significantly higher in the latter, which could suggest that either cofactors or lipase
343 enzymes are more effectively removed with urea (9 M) that with $NaHCO_3$ solution, or
344 urea has a stronger capacity to cause a loss of function of the enzyme by
345 denaturation. The low residual activity in urea-WOB is in line with the findings of De
346 Chirico *et al.* (2018) showing OB preparations stable up to 1 month at 20 °C even
347 without thermal treatment. Considering these findings, it can be confirmed that the
348 measurement of enzymatic activity is a more accurate methodology than protein
349 content for the assessment of OB purity from germinating enzymes. It can be seen
350 from these results that the carry-over of enzymes (as well as of other seed material)
351 is affected by recovery and/or washing solutions, as initially suggested by Zhao
352 (2016) and De Chirico *et al*. (2018). Given the presence of residual activity in the

washed product, a thermal treatment seems to be a necessary step to assure OB stability against lipolytic activity over time. The OB recovered in $NaHCO_3$ will be considered in the next sections.

3.2 Effect of thermal treatment on lipolytic activity

As previously discussed (Section 3.1), lipolytic enzymes are present in OB extracts, either as intrinsic or passively associated components. The degree of this association is not known, but it appears to be strong as it was shown to remain with the OB fraction after applying washing protocols (Table 1). The recalcitrant nature of OB-related lipolytic enzymes to washing could be used to measure the effectiveness of any thermal treatment applied to a preparation of OBs. One possible explanation for the reduction of lipase activity in OB preparations could be the loss of the enzyme into the serum phase during washing and centrifugation steps (Section 3.1). It was hypothesised that the solubilisation of the enzyme into the continuous phase may be enhanced by heating the OB emulsion, leading to a further reduction of hydrolytic activity present in the final cream. By this way, the application of thermal treatment (95°C) on WOB I OB material (before the last centrifugation to recover WOB II, S.I. 1), could be an effective methodology in reducing lipolytic activity. The effect of heating on emulsion stability is discussed in Section 3.3.

In this set of experiments, lipolytic activity and protein content were measured in protein extracts from the cream (wet weight basis) and serum phases of WOB II thermally treated samples. The lipolytic activity results were normalised to the protein content in the parent WOB I cream, assuming that over thermal treatment and subsequent centrifugation the protein mass balance (WOB I = WOB II + serum

phase) was maintained. Heating the OB emulsions (95°C), followed by centrifugation, had a significant effect on both protein content and lipolytic activity. It can be seen from Fig.1A that lipase activity decreased sharply from 0.05 ±0.01 U to 0.02 ±0.005 U over the first minute of holding emulsions at 95°C. Afterwards, the decay in activity was slower, reaching a reduction of up to 90% of the initial activity after 6 min. Protein content showed a decrease from 22.29 ±1.29 mg in the untreated sample to 18.47 ±0.82 mg already over the first 30 seconds (Fig. 1C). Extending the thermal treatment for up to 6 min, the protein content was further reduced to 15.44 ±0.61 mg. To establish whether the reduction in lipolytic activity in the OB cream was due to physical removal or thermal inactivation, an aliquot of serum phase (1 mL) recovered after centrifugation at each thermal treatment time point, was assayed for protein content and lipolytic activity, and the result shown in Fig. 1B and 1D. Over the thermal treatment, the activity in the serum phase (Fig. 1B) increased from 0.023 ±0.001 U in the untreated to 0.055 ±0.005 U after 1 minute of heat treatment. However, the activity decreased linearly after 1 min reaching 0.026 ±0.007 U at 6 min. The total protein content in the serum phase (Fig. 1D) increased from 1.24 ±0.26 mg to 5.98 ±0.3 mg over the first minute of treatment time, to achieve 7.35 ±0.29 mg at the end of thermal treatment (6 min). These results suggest that the reduction of lipolytic activity in the thermally treated WOB II samples is due to a combination of different phenomena. The decrease in lipolytic activity in the OB cream over the first minute of thermal treatment (Fig. 1A), which resulted in a 65% reduction of the initial activity, was attributed by the authors to two phenomena: (I) thermal inactivation of the lipolytic enzymes, (II) separation of the enzyme from lipid-rich phase (i.e. the cream). This latter phenomenon was speculatively explained hypothesising physical detachment of the lipolytic enzymes from the oil body surface

occurring during thermal treatment. This detachment resulted in the increase in lipolytic activity and total protein content in the serum phase (Fig. 1B and D, respectively). Although the number and types of bonds between lipolytic enzymes and OB surface have not been elucidated and this was outside the scope of this work, our data suggest that the lipolytic enzymes can withstand short thermal treatments and longer heat exposure times are required to achieve denaturation in both the cream and the serum.

To the best of the authors' knowledge, there are only few works on the enzymatic characterization of OB extracts (Allen & Tao, 2007; Hills & Murphy, 1988; Hoppe & Theimer, 1997; Lin & Huang, 1983; Murphy, Cummins, & Kang, 1989b), but none of them focused on the development of a recovery method aimed at the removal of the carried over enzymes. In this section, it was shown to which extend a thermal treatment at 95 °C can reduce the lipolytic activity. In Section 3.4, the suitability of lipolytic activity assessment for the development of minimal conditions to stabilise OBs will be tested.

3.3. Effect of thermal treatment on particle size and ζ-potential of OB emulsions.

If thermal treatment is employed to inactivate enzymes that could cause the deterioration of OB quality, it is vital that the applied heating does not lead to the physical destabilisation of the OB dispersion. The purpose of these experiments was to examine the effect of high temperature on OBs extracted from rapeseed.

Untreated emulsions recovered in 0.1 M $NaHCO_3$ (pH 9.5) had a bimodal size distribution ranging between 0.2 and 6 μm, with major peak at 1 μm (S.I. 2A). The ζ-potential changes from +55 ±4 mV to -65 ±2.5 mV as the pH increases from 3 to 10 (pI 6.5). Upon heat treatment (95°C, up to 6 min), no significant change in particle size (S.I. 2A) was observed. However, a slight change in ζ-potential occurred at pH 6 (S.I. 2B) with a 6 minutes thermal treatment, probably due to the loss of exogenous proteins at the interface (Fig. 1). Moreover, there was no evidence of aggregation, flocculation or phase separation in the heated samples (data not shown). These results suggest that the process did not significantly affect the surface charge and integrity of OBs. Similar findings were seen in OBs from hazelnut and soy bean (Nikiforidis et al., 2016), where a thermal treatment at 98°C for 30 minutes, applied before the last step of centrifugation to recover OB cream, did not appear to have any appreciable effect on the particle size and surface charge. The good thermal stability is presumably due to the T-like structure of oleosin molecule, which penetrates through the phospholipid layer into the TAG core (Huang, 1992; Tzen & Huang, 1992) remaining tightly associated to the non-polar matrix during heating. However, in other reports (Chen et al., 2012; Iwanaga et al., 2007) there appeared to be a decrese in ζ-potential at temperatures higher than 60 °C.

3.4. Effect of thermal treatment on the physical stability of aged emulsions

The aim of these experiments was to examine the effect of thermal inactivation of the carried over enzymes, on the storage stability of OBs extracted from rape seeds using 0.1M $NaHCO_3$ (pH 9.5). Particle size measurements over storage, supported by light microscopy, can give important information on the stability of the emulsion

448 and on the effectiveness of the process. Untreated (Fig. 2A, reported as reference)
449 and thermally treated OB emulsions (Fig. 2B and 2C, for 1 and 6 min. treatment,
450 respectively), were stored at 20°C for 30 days. Moreover, confocal imaging (Fig. 3)
451 of fluorescently stained OBs was performed at the start and end of the trial, so
452 structural information could be gained.

453 At the start of the storage, size measurements and confocal imaging (Fig. 3) showed
454 that the OBs were small ($D_{4,3}$= 1.2 ± 0.05 µm), discrete organelles stabilised by a
455 layer of proteins (in green, Fig. 3A) surrounding the oil core (in red). Over the time
456 course, untreated emulsions (Fig. 2A) showed poor storage stability, with the first
457 signs of size increase already over the first seven days of storage (20 °C).
458 Microscopy analysis (SI 3B) showed some aggregated droplets (indicated by
459 arrows), which could have affected the particle size distribution over the first days.
460 However, between 7 and 30 days, the effects of enzymatic hydrolysis are visible to a
461 greater extent, with enhanced size increase and presence of free oil (SI 3D, 3E and
462 3F). Moreover, confocal images (Fig. 3B) clearly show after 30 days droplets with
463 altered morphology and disordered protein aggregates (in green, indicated by an
464 arrow), which are probably the result of the proteolytic activity (as confirmed later in
465 this section).

466 Thermal treatment (95 °C) had a significant impact on the storage stability of OBs
467 (Fig. 2B and 2C). Particle size measurements showed a slight size increase over the
468 first 7 days of storage of particles at high diameters for both thermally treated
469 emulsions, but to a much lesser degree than the untreated samples. The OBs
470 treated for 6 minutes remained stable and had a $D_{4,3}$ of 1.4 ± 0.1 μm after 30 days;
471 the samples treated only for 1 minute showed a further increase in size between 14

472 and 30 days of storage ($D_{4,3}$ = 1.7 ± 0.15 μm), probably due to some residual
473 hydrolytic activity visible only at long term storage. Although thermally treated
474 emulsions were creaming after 3 weeks (data not shown), the OBs remained as
475 discrete droplets, with no sign of aggregation or altered morphology (Fig. 3C, 3D and
476 SI 4, SI 5), suggesting that the structure of the OB was preserved.

477 Matsui et al. (1999) initially hypothesised that the proteolysis of the oil body protein
478 coat would be the first step required for the oil mobilization, by the activity of a
479 cytosolic thiol-protease detected in germinating sunflower seed found to be
480 associated to the surface of recovered oil bodies (Sadeghipour & Bhatla, 2002;
481 Vandana & Bhatla, 2006). To test the presence of proteolytic activity over storage,
482 protein extracts from fresh (day 0) and aged (day 30) oil body emulsions were
483 analysed by SDS-PAGE (Fig. 4). At the end of the storage trial, the protein extracts
484 on SDS-PAGE from untreated OB samples (Fig. 4, lane 2), showed proteolytic
485 activity on oleosins, resulting in a series of peptides at the low molecular weight
486 range (10-15 kDa). Heated emulsions (Fig 4, lane 3 and 4), retained a protein
487 composition similar to the fresh sample (lane 1), revealing that the oleosin isoforms
488 (indicated by arrows) were not affected by enzymes. Bearing in mind that the
489 addition of NaN_3 should limit microbial spoilage, the primary cause of the poor OBs
490 stability ex-vivo (Fig. 3A), as well as in-vivo (Hoppe & Theimer, 1997), seems to be
491 related to the carried over enzymes in the extract. In untreated emulsions, the
492 instability may be triggered by oleosin proteolysis (Fig. 4), which would cleave the
493 hydrophilic domains (N- and C- terminus). Consequently, the electrical charge
494 repulsions and steric hindrance which prevents OBs from coalescence (Tzen et al.,
495 1993, 1992) will not be based on the exposed oleosin domains, but mainly on the
496 zwitterionic properties of the exposed interfacial phospholipid layer. Altered surface

chemistry is likely to reduce physical and electrostatic protection, making OBs more prone to coalescence.

These results demonstrate that the thermal treatments enhanced the stability of OBs; heat treatment of oil body emulsions that reduces the lipolytic activity by 90% also disables proteolytic activity enough to retain oil body integrity. To gain a deeper understanding of the colloidal stability of aged emulsions, ζ-potential was measured (Section 3.5).

3.5. Zeta potential of aged emulsions

To better understand the destabilising phenomena of aged emulsions and how the enzymes can affect the surface charge, the ζ-potential of fresh and aged OBs was measured over 30 days at pH values from 3 to 10 (Fig. 5).

As previously mentioned (Section 3.3), all the samples showed a zeta potential changing from about +55 ± 4.5 mV at pH 3 to -60 ± 2.5 mV at pH 10 reaching zero between 6.3 and 6.5, which is typical behaviour for protein-covered OBs (Tzen et al., 1993, 1992). As expected, ζ-potential profiles of the untreated samples changed significantly over time. While ζ-potential is similar at the extreme of pH values (3, 8, 9 and 10), a shift towards more negative ones was seen around the pH region of 4 to 7. Moreover, OB preparations showed pI values at more acidic pHs (5.7, 5.1 and 5.0 for 7, 14 and 30 days of storage, respectively). These changes over time were diminished in a temperature/time-dependent fashion, suggesting that a loss of hydrolytic enzyme activity preserved the surface chemistry (and so the charge) over storage. Most of the changes happened in the first 7 days of storage, reducing the pI

520 at about 6. However, after 30 days the pI of the 1 min treated samples was further
521 reduced to 5.5, while the 6 min treated samples did not show further significant
522 change. With a reduction of 90% of the total lipolytic activity, the changes in the zeta
523 potential are quite limited, and only confined at pH values of 5, 6 and 7.

524 It is likely that the OB-associated proteolytic activity is responsible for most of the
525 changes that are seen in the ζ-potential of untreated emulsions. However, lipolytic
526 activities may be also considered. The hydrolysis of oleosin exposes the charged
527 hydrophilic heads of the PLs to the continuous phase, and the lipolytic activity
528 releases negatively charged fatty acids to the exterior. As the protection of the OBs
529 provided by oleosins is lost, the high negative charges seem not to be enough to
530 overcome the various attractive forces (e.g., van der Waals and hydrophobic),
531 leading to coalescence, as shown in Fig. 2A. The limited changes in zeta potential
532 for a thermally treated emulsion are probably due to the reduced enzymatic activity,
533 as also reported by Chen et al (2012). However, using a short-time thermal
534 treatment (e.g. 1 min), the emulsion displayed a higher negative charged surface at
535 pH 6 than the 6 min treatment (-18.3 mV and -3.8 mV respectively) after 30 days
536 storage at 20°C, probably due to the residual hydrolytic activity.

537

538 **4. Conclusions**

539 The findings in this work demonstrate that lipolytic enzymes are found to be
540 associated to the surface of OBs, despite the efforts in purifying the OB extract. This
541 study shows how this activity is affected by seed soaking time, the chemical nature
542 of the recovery medium, and the nature of the washing protocol applied. A crude OB
543 preparation recovered after soaking and grinding seed in $NaHCO_3$, had a significantly

lower lipolytic activity than the one recovered using dH_2O, probably due to the higher capacity of alkaline pHs to solubilise exogenous proteins. The washing of this crude OB material did not lead to a complete purification from seed enzymes, destabilising the emulsion over time. For this reason, thermal treatment is necessary to reduce enzyme activity further. Storage studies showed that proteolytic activity is the main factor that leads to changes of droplet particle size and zeta potential, by weakening the emulsification system relegating the phospholipid heads to stabilise the OBs. A short-time thermal treatment (95°C, at times shorter than 1 minute) of the milk (re-suspended WOB I cream), and subsequent centrifugation, is an efficient way to reduce the number of exogenous proteins reducing up to 65% of the total lipolytic activity in the recovered cream. Longer thermal treatments (>1 min) were necessary to enhance the thermal denaturation of proteolytic and lipolytic enzymes. In this work, it was proved that the assessment of lipolytic activity as a marker for OB purity and/or thermal process efficiency, could be a new approach for the enhancement of OB stability.

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728

729 **Captions**

730 **Table 1**: Lipolytic activity and protein content (expressed as gram of protein per
731 gram of dry material, i.e. dry weight basis (dwb)) of oil bodies. Low protein content oil
732 bodies were recovered soaking and grinding seeds in $NaHCO_3$ (COB), then washed
733 in the same media (WOB I) and rinsed in dH_2O (WOB II). High protein content
734 samples were recovered using only dH_2O. Urea-WOB were produced washing
735 COBs (recovered with $NaHCO_3$) with urea solution (9 M) for the first wash (WOB I),
736 then rinsed in dH_2O to produce WOB II.

737

738 **Figure 1:** Effect of thermal treatment on lipolytic activity (left) and protein content
739 (right) in cream (Panel A/C) and serum (Panel B/D) phases from OBs recovered
740 using $NaHCO_3$. Results are expressed as enzymatic units (U) normalised by protein
741 content in 1 g of WOB I cream and total protein (mg) in 1 g of freshly extracted OB
742 material (wet weight basis).

743

744 **Figure 2:** Panel A: Particle size distributions of oil bodies recovered and washed in
745 $NaHCO_3$ (0.1 M, pH 9.5) over a period of 30 days of storage (20 °C). Measurements
746 were taken at day (D) 0/ 3/ 7/ 14/ 30. Panel B-C: Particle size distribution of thermally
747 treated (95 °C) oil body emulsions for 1 (B) and 6 minutes (C). NaN_3 was added to
748 avoid microbial spoilage.

749

750 **Figure 3:** Confocal images of fresh (Panel A, $D_{4,3}$= 1.2±0.05 μm) and aged (day 30)
751 untreated (Panel B, $D_{4,3}$= 3.7±0.5 μm) and thermally treated (95 °C) oil body
752 emulsions for 1 (Panel C, $D_{4,3}$= 1.7±0.15 μm) and 6 minutes (Panel D, $D_{4,3}$= 1.4±0.1
753 μm). White arrows show OBs with altered morphology. Green: protein; Red: oil.
754 Arrows show oil bodies with altered droplet structure. Scalebar represent 5 μm.

755

756 **Figure 4**: SDS-PAGE analysis of protein extracts from fresh (day 0, lane 1) and
757 aged (day 30, lane 2-4) oil body emulsions. Lane 2: untreated oil body emulsions.

Lane 3 and 4: thermally treated oil body emulsions (95 °C) for 1 and 6 minutes, respectively. Arrows indicate oleosin molecules.

Figure 5: Zeta potential of untreated (Panel A) and thermally treated (95 °C, 1 and 6 minutes for panel B and C, respectively) oil body emulsion at day 0/ 7/ 14/ 30 of storage at 20 °C. Zeta potential at day 0 is shown as reference. NaN_3 was added to avoid microbial spoilage.

***Declaration of Interest Statement**

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***Credit Author Statement**

Simone De Chirico: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Vincenzo di Bari:** Conceptualization, Methodology, Investigation, Writing - Review & Editing. **María Juliana Romero Guzmán:** Conceptualization, Methodology, Writing - Review & Editing. **Costas Nikiforidis:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Tim Foster:** Conceptualization, Methodology, Funding acquisition. **David Gray:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration.

Table1

Activity (U/g protein)	Dry Seeds	Soaked Seeds (16 hrs)	COB	WOB I	WOB II
dH_2O	68.9±5.1	95.2±4.8	177.5±4.9	154.5±7.9	153.2±8.2
$NaHCO_3$		99.1±3.3	87.1±2.7	83.6±1.7	76.1±2.3
Urea-WOB	-	-	-	-	13.5±0.5

Protein content (g/g dwb)	Dry Seeds	Soaked Seeds (16 hrs)	COB	WOB I	WOB II
dH_2O	0.2±0.02	0.19±0.03	0.098±0.010	0.078±0.008	0.065±0.005
$NaHCO_3$		0.20±0.05	0.045±0.003	0.033±0.003	0.027±0.002
Urea-WOB	-	-	-	-	0.025±0.001

Figure1

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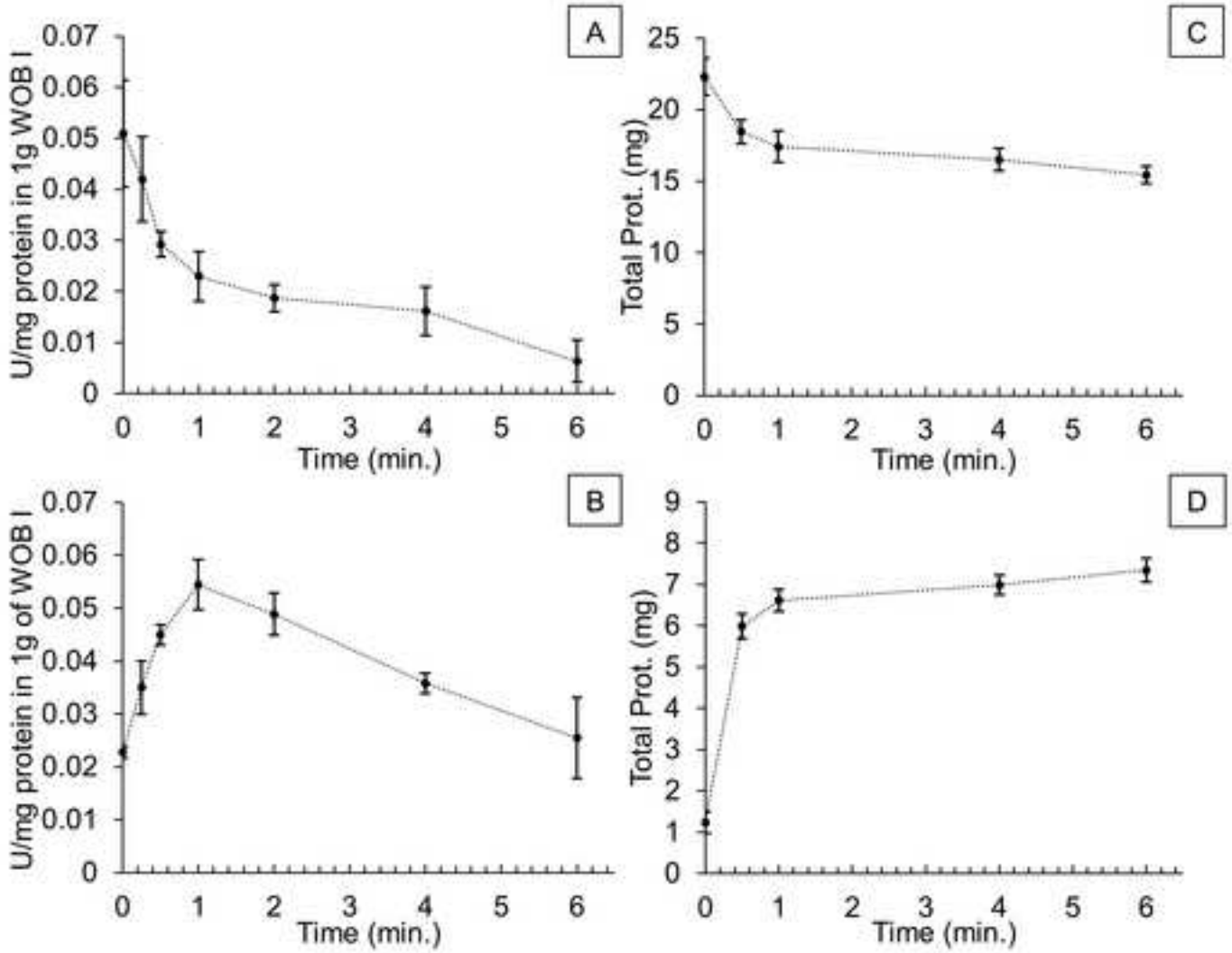

Figure2

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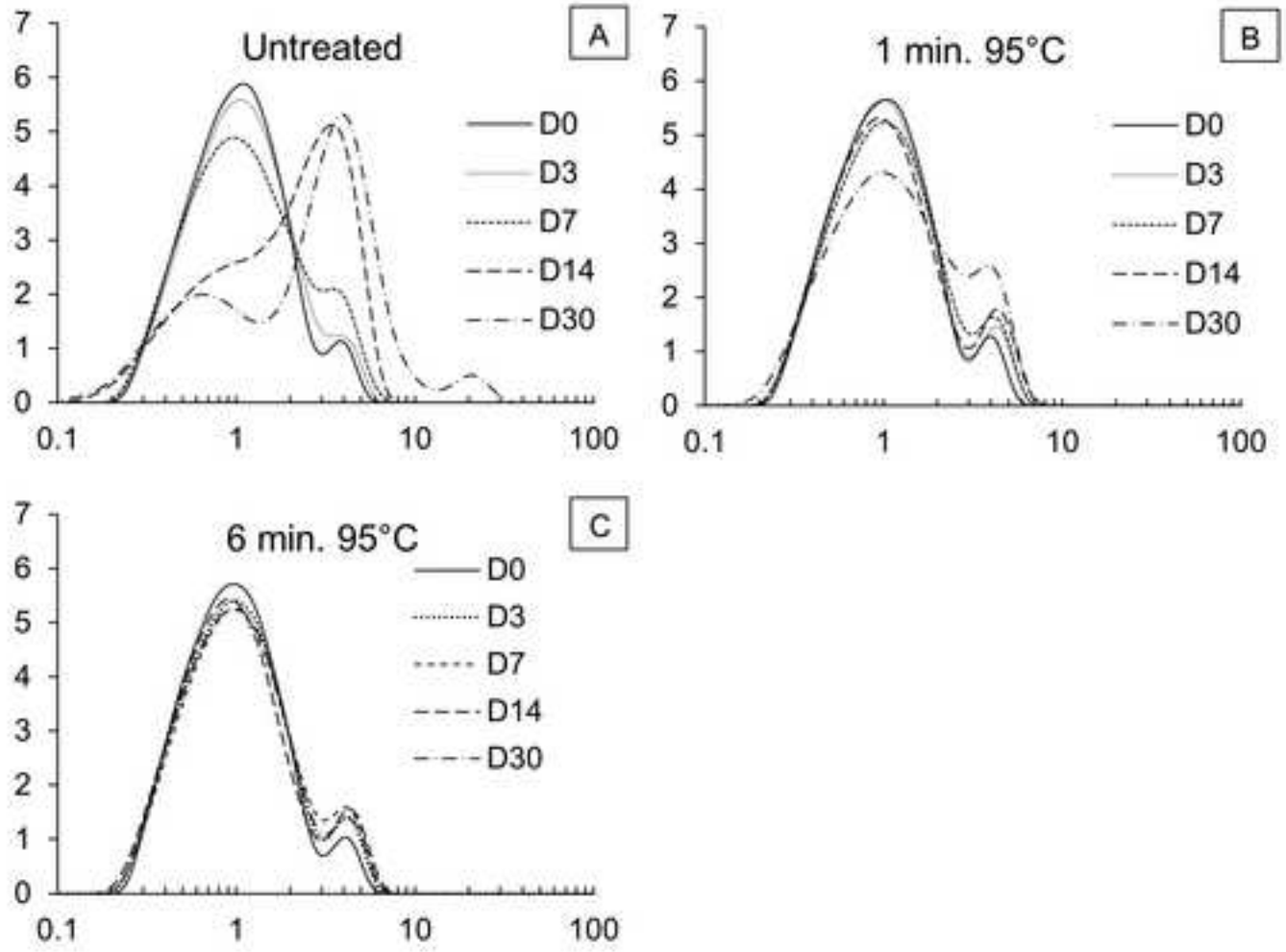

Figure3

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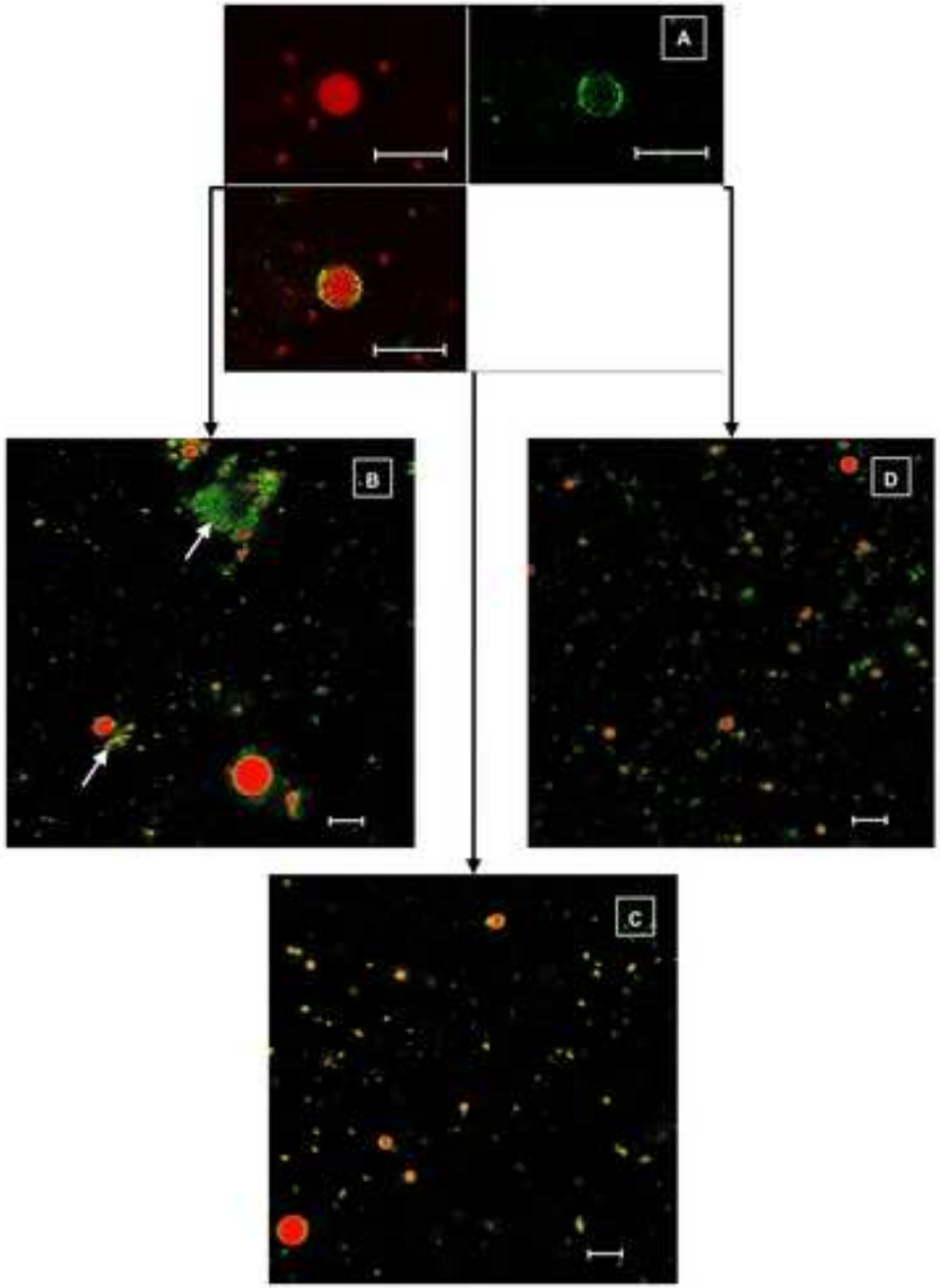

Figure4

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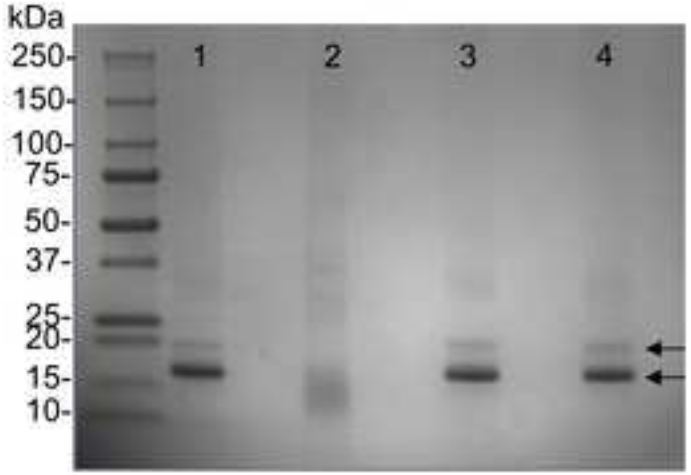

Figure5

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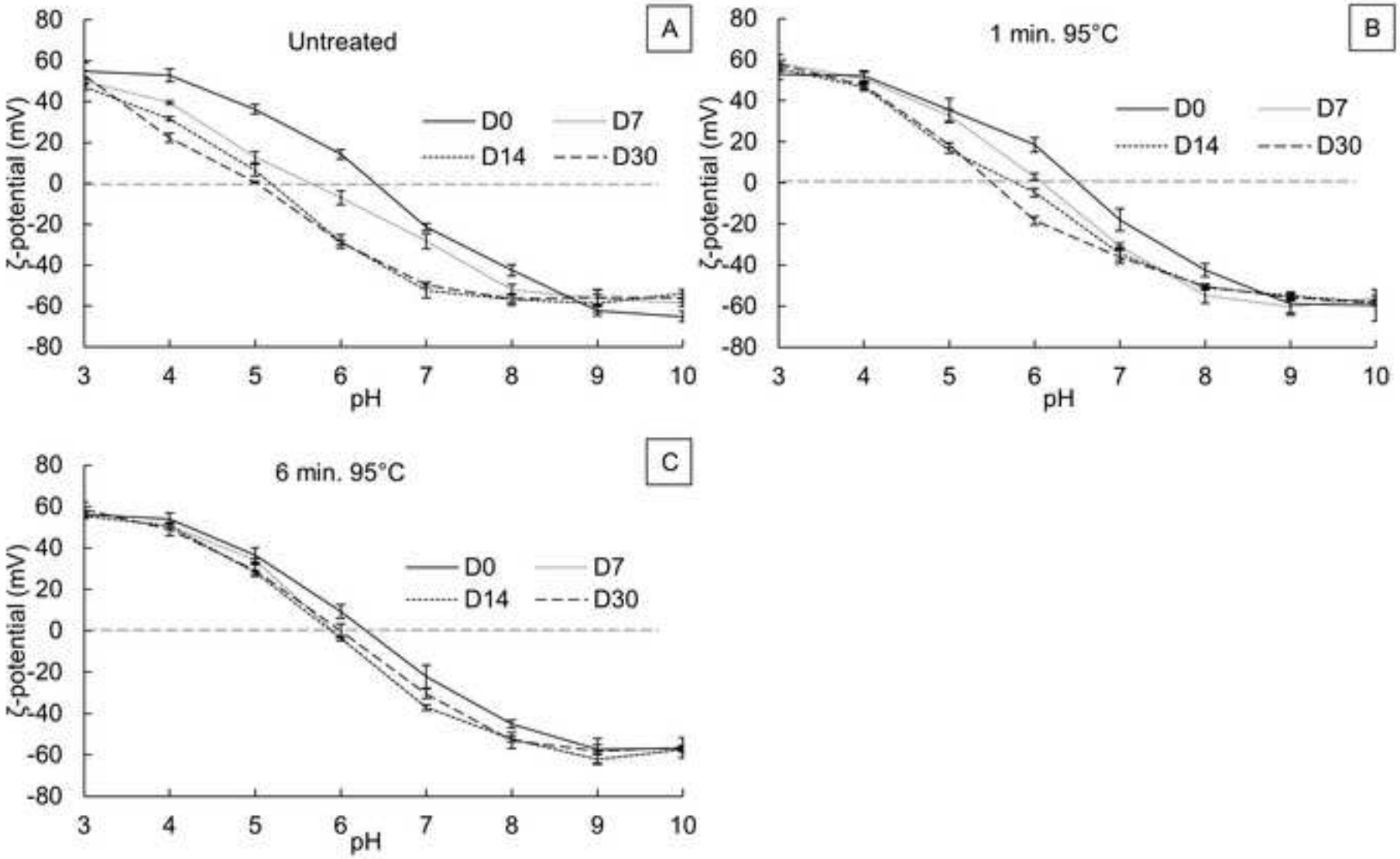
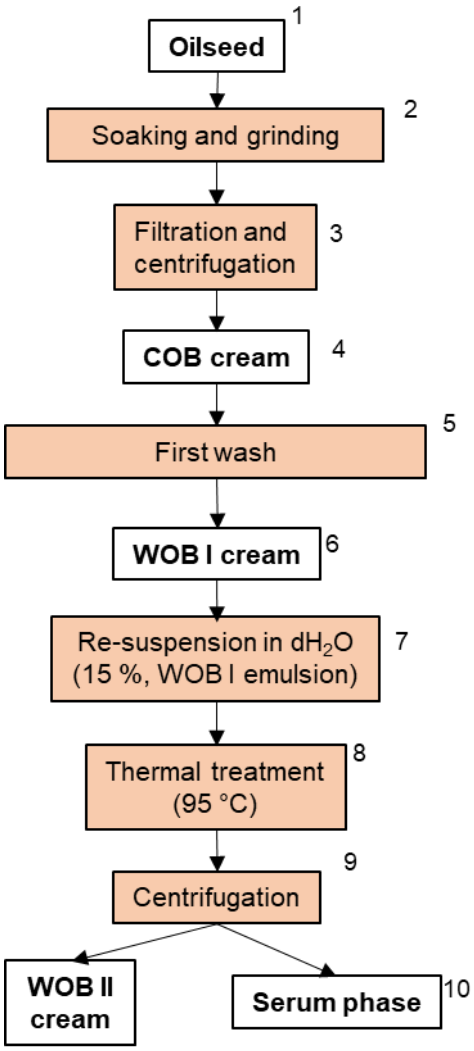
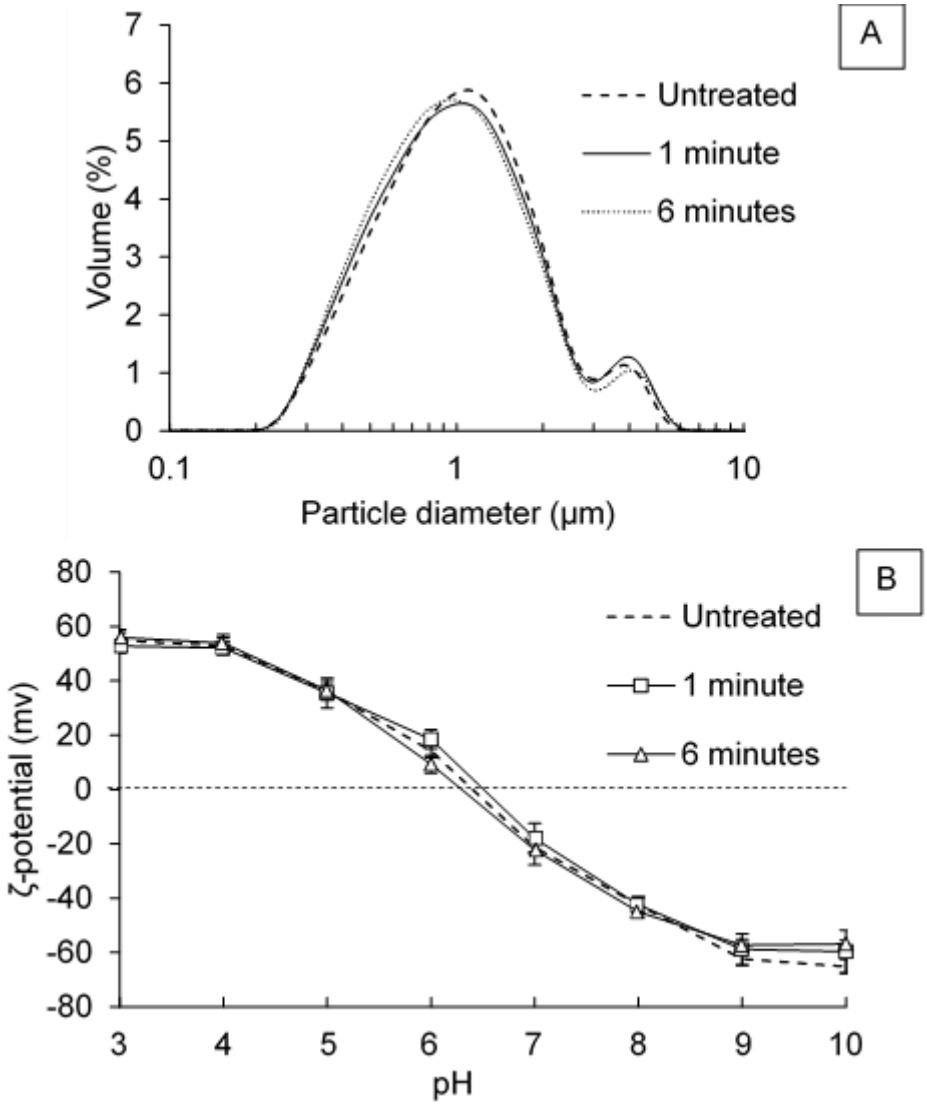


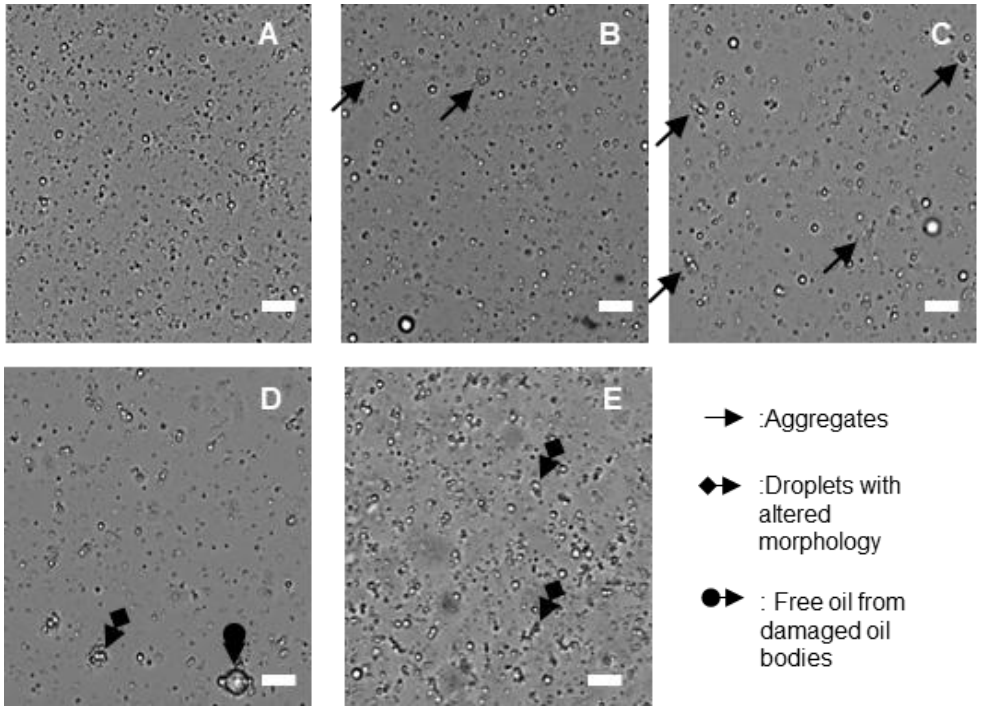
Supplementary Images



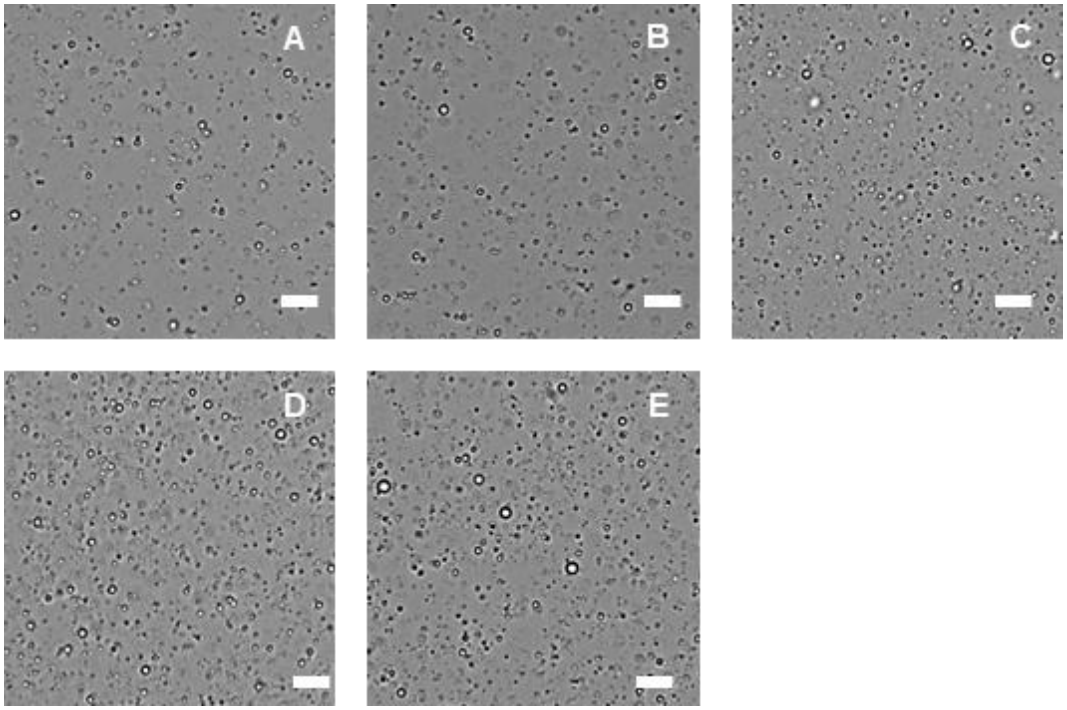
Supplementary Image 1: Protocol for the recovery and thermal treatment of oil bodies from rapeseeds. COB were recovered soaking and grinding oilseeds either in dH_2O or $NaHCO_3$ (pH 9.5, 0.1M). After the first washing cycle in either dH_2O, $NaHCO_3$ (pH 9.5, 0.1M), or urea (9M), "WOB I" creams were recovered. Each sample was re-suspended in dH_2O (producing WOB I emulsions, 15 % oil weight basis) and, where specified, were thermally treated at 95 °C. After cooling and a centrifugation step, "WOB II" cream and a serum phase were collected.



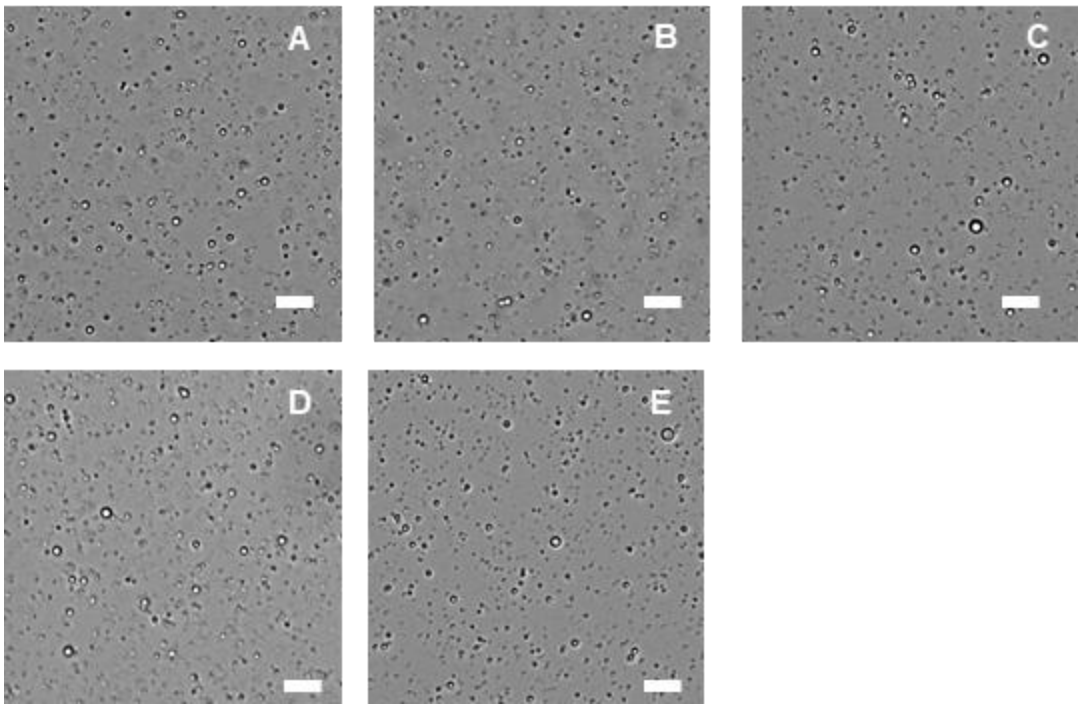
Supplementary Image 2: Particle size (A) and ζ-potential (B) of untreated and thermally treated (1 and 6 minutes, 95°C) of fresh oil body emulsions. WOB I cream was re-suspended in dH_2O (15% lipid basis emulsion) and thermally treated. After cooling in ice and centrifugation, the WOB II cream was recovered and analysed to obtain these results.



Supplementary Image 3: Microscopy images of thermally untreated oil body emulsions over storage at 20 °C. Pictures taken at day 0/ 3/ 7/ 14/ 30,corresponding to micrographs A/ B/ C/ D/ E, respectively. Arrows indicate aggregates or droplet with altered morphology (details in the image legend). Scalebar represents 5 µm.



Supplementary Image 4: Microscopy images of thermally treated oil body emulsions (95 °C, 1 minute) over storage at 20 °C. Pictures taken at day 0/ 3/ 7/ 14/ 30, corresponding to micrographs A/ B/ C/ D/ E, respectively. Scalebar represents 5 µm.



Supplementary Image 5: Microscopy images of thermally treated oil body emulsions (95 °C, 6 minutes) over storage at 20 °C. Pictures taken at day 0/ 3/ 7/ 14/ 30, corresponding to micrographs A/ B/ C/ D/ E, respectively. Scalebar represents 5 µm.