Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com

Enhancing the recovery of oilseed rape seed oil bodies (oleosomes) using bicarbonate-based soaking and grinding media

Simone De Chirico, Vincenzo di Bari, Tim Foster, David Gray*

Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

ARTICLE INFO

Keywords:
Emulsions
Oil bodies
Rapeseed
Sodium bicarbonate solution
Alkaline pH
Exogenous proteins
Storage stability

ABSTRACT

An aqueous process for the recovery of oil bodies from rapeseed using sodium bicarbonate-based soaking and grinding media (pH 9.5) was investigated. The effect of the ratio between seed and mass of media during grinding and molarity of the medium used on oil body integrity, purity and storage stability have been studied. The grinding of seeds in solution at a ratio of 1:7 (w/w) significantly improved the quality of oil body suspension to a size more in-line with that seen in vivo (average $D_{4,3}$ of 1.19 μm). The purity and the composition of the recovered oil bodies depends on the molarity of medium used; the use of a sodium bicarbonate solution (pH 9.5, 0.1 M) in the grinding and washing steps produced oil body preparations with the same purity as that resulting from washing a crude preparation with 9 M urea. The resultant emulsion had improved physical stability over a storage period of one month.

1. Introduction

Oil bodies (OBs, denoted often also as oleosomes or lipid bodies) provide the seed with energy during germination; they are generally between 0.2 and 2.5 μm in diameter (Tzen & Huang, 1992). When viewed under an electron microscope, an electron-opaque matrix of triacylglycerols (TAGs) surrounded by one electron-dense layer of phospholipids (Tzen & Huang, 1992; Yatsu & Jacks, 1972) with embedded surface active proteins (*e.g.* oleosin) can be seen (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). The oleosin contains three basic structural domains: an amphipathic domain present at the N-terminal and C-terminal, residing on the OB surface ensuring stability *via* steric hindrance and electronegative repulsion, and a highly hydrophobic domain penetrating into the TAG matrix (Frandsen, Mundy, & Tzen, 2001; Murphy & Cummins, 1989; Qu & Huang, 1990). The unique structure of the oleosin is thought to prevent coalescence of OBs both in the cytosol of oilseed cells and *ex vivo* (Tzen & Huang, 1992).

The conventional process of oil extraction from oleaginous seeds using organic solvents and the subsequent oil refining has a high environmental impact, and it is hazardous due to flammability and risk of explosion. During this process, OBs are destroyed as the neutral oil (triacylglycerol) partitions into the organic solvent. The production of oil from vegetable sources is estimated to be approximately 150 million tonnes per annum, of which 30% is processed into emulsions using high energy homogenisation and the addition of emulsifiers/surface active agents (Gunstone, 2004). Many natural and processed foods are considered either partly or wholly as oil-in-water emulsion (Mcclements, 2004). When OBs are released (recovered) from the cells of oilseeds into an aqueous medium (a relatively benign wet milling process) they may form a stable oil-in-water emulsion, depending on the solution implied. Compared with conventional oilseed processing, the recovery of OBs as a natural source of pre-emulsified oil, to be used in a range of food applications, is likely to reduce the impact on the environment. OBs have been recovered from a number of different seeds, such as pumpkin (Adams et al., 2012), soybean (Chen, Mcclements, Gray, & Decker, 2012; Chen & Ono, 2010; Iwanaga, Gray, Decker, Weiss, & Mcclements, 2008), and maize (Nikiforidis & Kiosseoglou, 2009). Despite the differences between the authors, the key points for the extraction of OBs are similar, comprising: disruption of the seed matrix in aqueous media; collection of a "crude" OB cream after centrifugation and washing of the cream. The latter stage is aimed to remove the polysaccharide, seed debris and exogenous proteins that are not covalently bonded to the OB (Tzen, Peng, Cheng, Chen, & Chiu, 1997), using buffers, detergents or chaotropic agents, to obtain a purified OB cream free from exogenous material. As detailed by Millichip

* Corresponding author.
Email addresses: simone.dechirico@nottingham.ac.uk (S. De Chirico); vincenzo.dibari@nottingham.ac.uk (V. di Bari); tim.foster@nottingham.ac.uk (T. Foster); david.gray@nottingham.ac.uk (D. Gray)

https://doi.org/10.1016/j.foodchem.2017.09.008
Received 1 June 2017; Received in revised form 26 July 2017; Accepted 3 September 2017
Available online xxx
0308-8146/

et al. (1996) urea (9 M) is a rigorous washing agent that produces an OB preparation free from exogenous seed proteins without destabilising the integral oleosin proteins necessary to retain OB integrity (Lacey, Wellner, Beaudoin, Napier, & Shewry, 1998).

Some studies have identified enzymes associated with recovered oil bodies (Chen & Ono, 2010; Chen, Zhao, Cao, Kong, & Hua, 2014; Karkani, Nenadis, Nikiforidis, & Kiosseoglou, 2013; Zhao, Chen, Chen, Kong, & Hua, 2016). The carry-over of enzyme activity (proteolytic or lipolytic) could cause destabilisation of OBs ex-vivo. The removal of these enzymes would therefore reduce the temperature and time required for effective biochemical stabilisation of the OB preparation. Urea is not a food grade material, so cannot be used to wash OBs and remove enzymes. However, as the enzymes contain multiple charged groups, they can be solubilised in salt solutions, and their solubility can increase with increasing salt concentration, in a phenomenon called "salting-in" (Arakawa & Timasheff, 1985). We hypothesised that a salt such as sodium bicarbonate, could be a suitable food grade replacement of the urea. The aim of this work was to test the ability of a sodium bicarbonate solution (pH 9.5) to recover intact OBs with reduced carry-over of exogenous proteins. The effect of changing the molarity of the bicarbonate solution, and the ratio between seed mass and mass of the medium during grinding were tested.

2. Materials and methods

Oilseed rape seeds (variety Quartz) were sourced from a local farm at time of harvest (2015), and stored at 20 °C and rH 50% until use. All chemicals and reagents were purchased from Sigma-Aldrich Trading Co., Ltd. of analytical reagent grade or higher. Precision Plus Protein Dual Color Standard (Bio-Rad, USA) was used as protein marker. Ultrapure water (Nanopure Infinity system, Barnstead International, IA) was used for all media.

2.1. Oil body recovery and purification

OBs were isolated from oilseed rape seeds and purified according to the method of Tzen et al. (1997) with the following modifications. Seeds (200 g) were soaked in sodium bicarbonate pH 9.5 (0.01, 0.1 or 0.3 M, adjusted with 0.1 M NaOH) or water, at ratio of 1:4 (w/w) at 4 °C for 16 h (unpublished data) and the soaking medium was then discarded. The soaked seeds were then ground in the same medium type as used on soaking (4 °C) at ratios (dry seed weight based) of 1:4 (w/w) or 1:7 (w/w) in a Kenwood blender (BLX52) at full power for 90 s. After grinding, the slurry was left stirring for 24 h at 4 °C, to improve the yield of oil bodies, and then filtered through three layers of cheesecloth. The filtrate was transferred in 400 mL tubes and centrifuged at 10000*g* for 30 min at 4 °C using a Beckman J2-21 centrifuge, fixed rotor JA-10. The upper layer was isolated using a spatula, drained on filter paper (Whatman, grade 5) and called crude oil body fraction (COB). Sodium azide (0.02 mM) was added to all media to avoid microbial spoilage. The COB preparation was dispersed in washing solution (0.1 M $NaHCO_3$, pH 9.5 or 9 M urea, 1:4 w/w) and centrifuged (10000*g*, 30 min). To remove any residue of the washing solution, the fat pad was isolated using a spatula, drained on filter paper, suspended in water (1:4 w/w) and centrifuged (10000*g*, 30 min). After centrifugation, the cream layer was collected and designated as urea-washed oil body cream (Urea-WOB) or sodium bicarbonate washed oil body cream ($NaHCO_3$-WOB). All creams were stored at 4 °C and analyses were completed within 24 h.

2.2. Particle size measurement

The particle size of OB suspensions was measured with a LS 13320 laser diffractometer (Beckman-Coulter, USA) using the Mie theory of the scattering of light by spherical particles. The samples were diluted as appropriate prior to measurement. The real part of the refractive index, was 1.462, corresponding to the refractive index of rapeseed oil. The imaginary part, corresponding to the 'attenuation coefficient' that describes the turbidity of a sample, was set to 0.01 according to the laser diffractometer guidelines for lightly coloured translucent materials. For the description of particle size distribution (PSD), the volume frequency distribution (%/μm) and the volume mean diameter ($D_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$) have been reported. The fraction of intact droplets (≤2.5 μm in diameter), expressed as percentage, was found using the LS 13320 software from the cumulative volume frequency distribution curve.

2.3. Preparation of oil body emulsions for stability test

Oil body emulsions were prepared suspending the washed cream in water (10% 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 $D_{4,3}$ was measured at time 0, 7, 14, 21 and 28 days storing the samples at 20 °C.

2.4. Lipid extraction

COB or WOB creams (approximately 0.2 g) were dried in a vacuum oven (40 °C, −900 mbar) for at least 48 h until constant mass. Dry matter was ground (mini-bead beater, 3450 rpm) for 2 min in isooctane (1 ml) to extract the oil (Gray, Payne, Mcclements, Decker, & Lad, 2010). Samples were centrifuged (Thermo Heraeus Fresco 21) at 17,000*g* for 5 min at 4 °C and the upper phase was removed with a pipette; this process was repeated three times and the recovered lipids dried under nitrogen. The resultant pellet after delipidation was used for protein extraction and analysis as described in Section 2.6.

2.5. Protein content

The proteins were extracted from delipidated dry material (as detailed in Section 2.4) using 2% sodium dodecyl sulfate solution (SDS, 1 ml) and incubated for 30 min at 60 °C in a water bath. Protein extracts were vortexed for 1 min and centrifuged at 17000*g* (5 min, 4 °C). The supernatant was collected and assayed on the same day for protein content using BCA assay (Smith et al., 1985). On performing the analysis, samples were diluted to obtain absorbance values within the range of the standard curve.

2.6. SDS-PAGE

To compare the degree of exogenous protein carried over in the extracts, protein samples were appropriately diluted in 2% SDS solution normalised by the amount of oleosin. 20 μL of protein extract was then mixed with an equal volume of sample buffer (Laemmli buffer (Bio-rad, UK) + 5% β-mercaptoethanol), heated at 95 °C for 5 min and then cooled on ice. Proteins were resolved by SDS-PAGE using 4–15% polyacrylamide gels (Mini-Protean TGX Gels, 10-well, 50 μL, Bio-Rad, Hercules, USA); gels were positioned within a SE 600 BioRad separation unit and suspended in tank buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was run at 100 V for 40 min. After electrophoresis, the gels were washed three times (10 min, H_2O). The gel

was then stained using comassie brilliant blue R-250 (1 h) and de-stained with de-staining solution (2 h). Gels were imaged using Bio-Rad Gel Doc XR System.

2.7. Zeta potential measurement

A series of OB emulsions were prepared in ultrapure filtered (0.2 μm Millex syringe filter unit, Millipore) water at a concentration of 0.008% (lipid weight basis) and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH solutions. After stabilization of the pH, the emulsions were injected into the measurement chamber of the particle electrophoresis instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA). The instrument settings used were: 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 by measuring the direction and velocity of the OBs moving under the applied electrical field. The ζ-potential value was reported as the average and standard deviation of two independent samples from each emulsion replicate, with two readings taken per sample.

2.8. Light microscopy

The microstructure of OB emulsions 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×.

2.9. Statistical analysis

All extraction experiments were carried out at least in triplicate and analyses were made on at least three emulsion preparations. Statistical analysis was performed by one-way ANOVA using SPSS 22. Assessment of significance difference was based on a 95% confident limit ($P < 0.05$).

3. Results and discussion

3.1. Effect of the seed:medium ratio and type of media during grinding on recovered OB integrity

The viscosity of a system with dispersed solids is reduced on increasing the volume of the medium (Austin & Trass, 1997), which may lead to a reduction in the forces experienced by OBs during their recovery, and to a reduction in the concentration of seed particulates that might collide with and damage OBs. In this set of experiments, seeds soaked in water or sodium bicarbonate (0.1 M, pH 9.5) for 16 h (54% weight increase, unpublished data) were ground using two ratios of seed mass to mass of media to investigate the effect of seed loading during grinding on OBs integrity. Based on preliminary data, a 1:4 and 1:7 ratio (w/w), was selected.

To evaluate whether a specific seed-to-medium ratio would damage the OBs, the crude oil bodies (COBs) were exposed to urea (9 M) to remove the exogenous seed proteins that could be adsorbed at the interface of damaged droplets, temporarily stabilising them. After exposing the cream to urea, intact OBs will remain small discrete entities (Tzen et al., 1997), whereas hybrids (damaged OBs with altered surface chemistry) will rapidly destabilise and coalesce forming larger droplets. No change in the size after wash of COBs with urea (9 M) has been used in this study as a diagnostic method for the detection of intact OBs.

Fig. 1 shows the particle size distribution of urea-WOBs extracted using water and sodium bicarbonate (0.1 M, pH 9.5) at both seed-to-medium ratios. At high solid loading ratios (i.e. 1:4 ratio w/w) the OB particle size distribution (PSD) showed a broad distribution after grinding seed in water and sodium bicarbonate, with a major peak at 12 and 6 μm, respectively. The presence of peaks at diameters above 3 μm is due to coalescence of droplets as confirmed by optical images (Fig. 1A-B). In a more diluted seed grinding system (1:7 ratio w/w) (Fig. 1) smaller droplets were observed after grinding seed in either water or sodium bicarbonate media then washed with urea; this confirmed that the low solid load of the grinding media improved OB integrity. With a low solid loading during grinding, the volume mean diameter ($D_{4,3}$) of urea-WOB extracted in water (3.6 μm) was larger than the urea-WOB extracted in sodium bicarbonate (1.2 μm). The presence of OBs with higher diameter after grinding seeds in water, suggests that an alkaline pH plays an important role in the maintenance of the droplet integrity, as it is discussed in Section 3.2. The results shown here suggest that the optimal grinding condition to recover intact OBs is the sodium bicarbonate solution at 1:7 ratio, as the lower density of seed solids in this grinding medium reduced the damage to OBs.

3.2. Effect of sodium bicarbonate solution strength during seed grinding on recovered oil body integrity

The charged surface of OBs held in an aqueous solution at certain pH values, can cause electrostatic repulsion that promotes the maintenance of droplets as discrete organelles, leading to an increased stability of the emulsion against aggregation (Iwanaga et al., 2008). However, at pH values close to the isoelectric point (5.5–6.6), the overall charge is zero, and in absence of repulsion forces, aggregation occurs (Tzen & Huang, 1992; Tzen et al., 1993; White et al., 2008). We hypothesized that if the pH of the grinding solution was far from OB pI, coalescence could be reduced. Therefore, in this set of experiments, the buffering capacity of the extraction media (to intend as the ability to hold the pH value far from the pI during grinding), and its effect on the recovered OB integrity, was tested. Sodium bicarbonate solutions with increasing molarity (0.01 M, 0.1 M, 0.3 M) were prepared and compared to water and the PSDs of the recovered OB was analysed. Subsequently, all COB preparations were exposed to urea (9 M) to reveal the presence of hybrids (as detailed in Section 3.1), and the following PSD changes compared to COB distributions were used to assess droplet integrity. The COBs recovered in sodium bicarbonate solution showed similar droplet size (Fig. 2A) for all molarities considered. However, when water was used as extraction media, the PSD showed a similar size range but with higher percentage for bigger droplets, due to the formation of aggregates (Fig. 2C). The buffering capacity of the system changes using different molarities of the medium, reaching a pH value following seeds grinding of 8.5, 7.0 and 6.0 for 0.1 M and 0.01 M sodium bicarbonate, and water, respectively. At higher buffering capacity (0.3 M), the solution is able to hold the pH value at 9.5. After exposure to urea (urea-WOB, Fig. 2B), the quality of OBs recovered using sodium bicarbonate (at all concentrations tested), improved considerably with those recovered using water. When grinding seeds in water, the pH value of the media is within the pI of OBs (5.5–6.6), neutralising the surface charge of the droplet and causing the formation of aggregates, which may enhance coalescence during the grinding process. As a result, a remarkable droplet size shift to bigger droplets occurs when water is used (Fig. 2B-C-G). A similar size shift was registered for 0.01 M solution, probably due to the weak repulsion forces during grinding, as the pH value was close to the pI (described in Section 3.4) that were not enough to avoid the OBs to coalesce (Fig. 2B-D-H). Among the different molarities of sodium bicarbonate used, only the 0.1 M did not show any shift to particles of higher diameter after urea exposure, and the recovered droplets maintained their singularity (Fig. 2B-E-I). However, despite the pH being far from the pI using

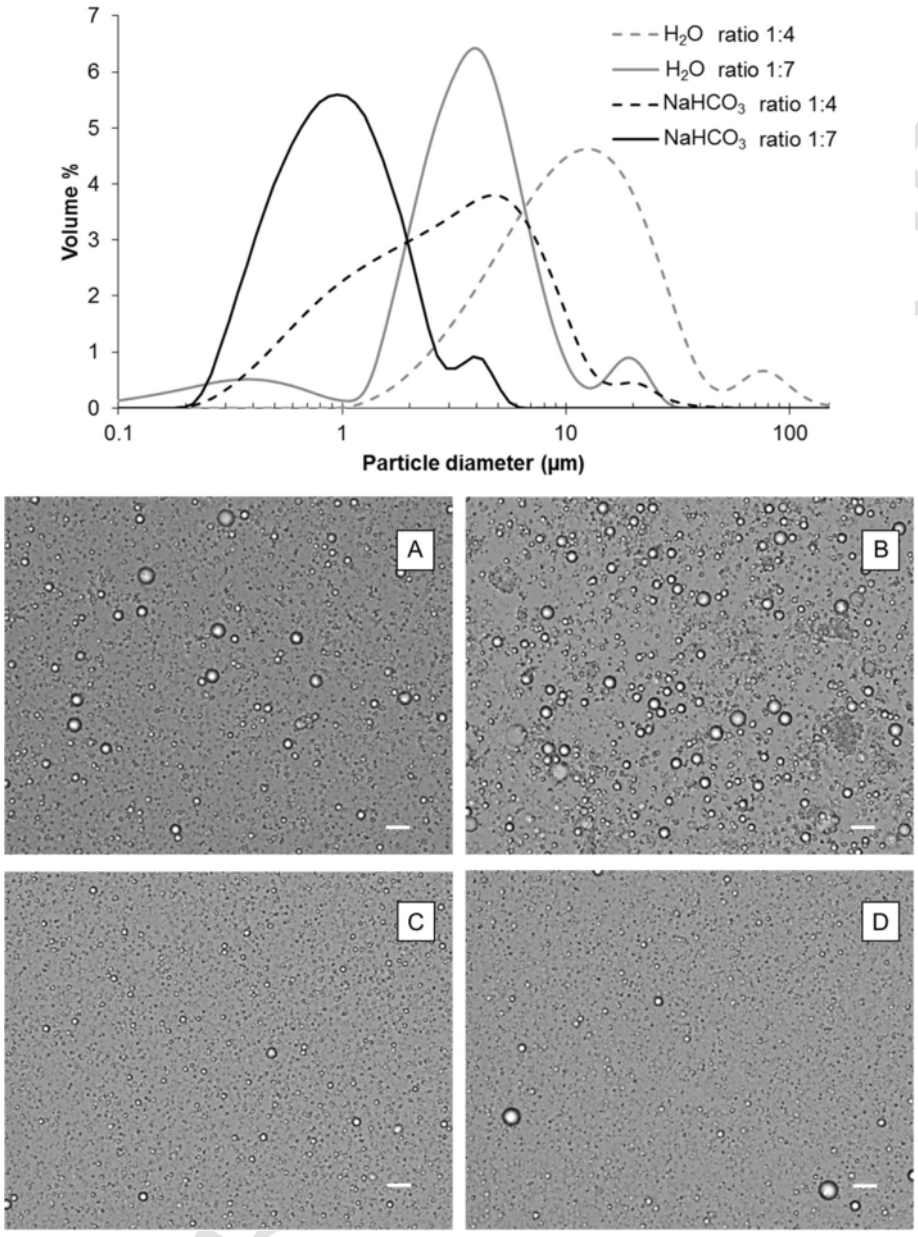

Fig. 1. Above: Particle size distribution of OB extracted in 0.1 M $NaHCO_3$ and then exposed to urea (9 M) (urea-WOB) to reveal the presence of hybrids. Dashed and solid lines are for cream recovered at ratio seed-to-grinding medium mass of 1:4 and 1:7, respectively, using H_2O (grey lines) and $NaHCO_3$ (black lines). Below are shown optical images of Urea-WOB extracted using different ratios seed mass to mass of grinding media (w/w). Panel A and B: urea-WOB extracted at 1:4 ratio in $NaHCO_3$ and H_2O, respectively. Panel C and B: urea-WOB extracted at 1:7 ratio in $NaHCO_3$ and H_2O, respectively. Scale bar represent 10 μm.

higher molarities of the sodium bicarbonate solution (0.3 M), the PSD of urea-WOB showed the presence of coalesced droplets (Fig. 2B-F-J).

Those results demonstrate that holding the pH value above 8.5, the OB surface may create enough repulsion forces that prevent the aggregation of droplets during the grinding step, limiting the coalescence. Moreover, the alkaline pH value of the system (≥8.5) is a suboptimal environment for rapeseed enzymes, such as lipases which optimum pH value is 5 (Hoppe & Theimer, 1997), resulting in the inhibition or delay of their activity on the OB over the extraction process. However, the prolonged exposure to high alkaline pH (>8.5) and high salt concentration (0.3 M), may have contributed to reduce the number of salt bridges between deprotonated oleosin branches and phospholipid heads (Cao et al., 2015), facilitating coalescence of OB droplets during grinding. Those results suggest that the optimum salt concentration to extract intact OBs is the 0.1 M, as the buffering capacity of the medium is able to hold the pH value far from the pI, without destabilising the integral proteins. Using this solution, over 90% of OBs were within the range of intact droplets (<2.5 μm) (Tzen et al., 1993), while the rest of the population at higher diameters can be attributed to limited coalescence.

3.3. Protein carry-over in crude oil body emulsions (COB)

In addition to the integral protein of OBs (e.g. oleosins), many seed exogenous protein molecules (located elsewhere in the cell) may bind to the OB surface during their recovery (Tang, Ono, & Mikami, 1997). As mentioned above, the protein solubility is influenced by the nature of the grinding medium: ionic strength, type of solvent, pH, temperature (Zayas, 1997). The effect of grinding medium (water and sodium bicarbonate at different molarities) on the carry-over of exogenous pro-

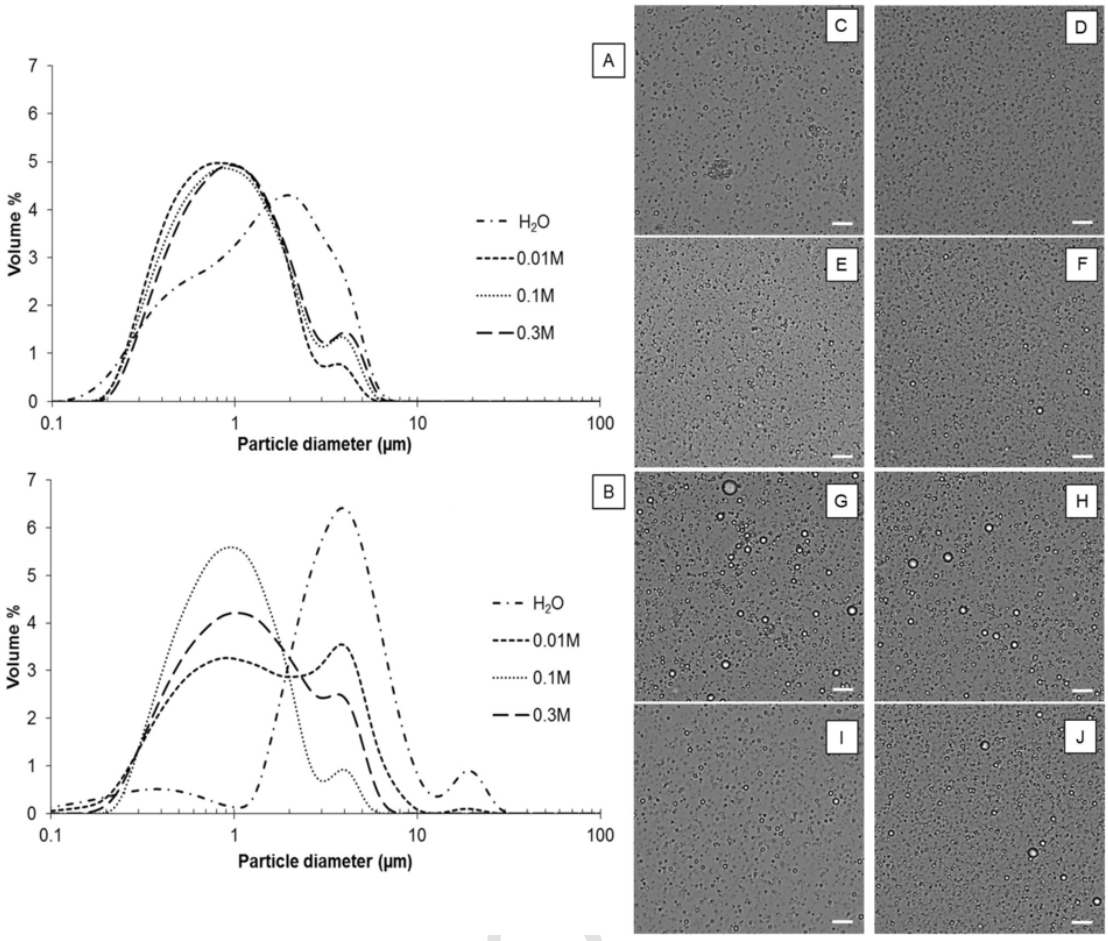

Fig. 2. A) Particle size distribution and microscopy images (Panel C, D, E, F) of crude oil bodies (COB) recovered on grinding oilseed rape seeds (1:7 seed:grinding medium) in H_2O, or $NaHCO_3$ pH 9.5, at molarities of 0.01, 0.1, 0.3, respectively. B) Particle size distribution and microscopy images (Panel G, H, I, J) of the same COB preparations exposed to urea (9 M) to reveal the presence of hybrids. Scale bar represent 10 μm.

teins in the COB extract was tested. Fig. 3A shows the protein content (as% of dry matter) of COB and WOB extracts. The COB recovered in water had the highest fraction of total protein (9.8 ± 0.74%), which decreased to 8.5 ± 0.27%, 4.5 ± 0.17% and 3.8 ± 0.25% when using 0.01, 0.1 and 0.3 M sodium bicarbonate, respectively. After exposure to urea (9 M), the washed preparation recovered using water or sodium bicarbonate had a protein content of 2.60 ± 0.05%. This value is in line with the one reported by (Tzen et al., 1993) in different varieties of seeds including rapeseed (0.59–3.46%, dry basis), confirming that the urea-WOB represent the purest preparation of OBs. In order to confirm the washing effect of the bicarbonate solution, protein samples of COBs and WOB were analysed by SDS-PAGE (Fig. 3B). Lane 2, corresponding to the protein extract of OBs recovered in water, showed several bands of exogenous proteins (at higher molecular weight of the oleosin, 18 kDa) carried over in the extract. By increasing the molarity of the sodium bicarbonate solution (lanes 3, 4 and 5), the majority of bands at high molecular weight lost intensity, suggesting that they had been removed from the OB emulsion.

The removal of exogenous proteins in the presence of sodium bicarbonate is probably due to a combination of pH and salt concentration effect. As detailed by Wanasundara and Mcintosh (2013), at a pH value between 3 and 5 the solubility of *Brassicaceae* oilseed proteins is the lowest, but increases at values that are above and below this range. Therefore, the trend of protein content in Fig. 3A is related to the pH value during grinding as discussed in Section 3.2. Similar results were reported by Chen and Ono (2010), where by neutral pH extraction the surface of oil bodies from soybean was characterised by the presence of several proteins and enzymes, like lipoxygenase, β-amylase, γ-conglycinin, and some not well known proteins; while at alkaline values (up to pH 11) a washing effect (removal of exogenous proteins) occurred. In addition to this, the increase in salt concentration, causes an increase in protein solubility (Arakawa & Timasheff, 1985). pH and salt concentration in our system may be working in synergy to remove exogenous proteins without bringing the system to high pH values.

The different composition and quantity of protein on the surface affects the OB properties. Therefore, the surface potential in the different emulsions was measured.

3.4. ζ-potential of COB preparations

The ζ-potential is a measure of the surface potential of a colloidal system, measured when exposed to an oscillating voltage across a measurement cell. In this study ζ-potential was used to predict how the protein carry over affects the droplet stability at different pH values, due to the type of extraction solution, as detailed in Section 3.2. The extracted COBs were suspended in ultra-pure water and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH. The surface charge of COBs (Fig. 4) moved from positive to negative values when the pH was increased, which is typical for protein-covered oil droplets (Guzey & Mcclements, 2006). The charge was negative (< -40 mV) with a similar ζ-potential for all emulsions at pH from 8 to 10, but differences were observed at pH's 3 to 8, due to the different protein com-

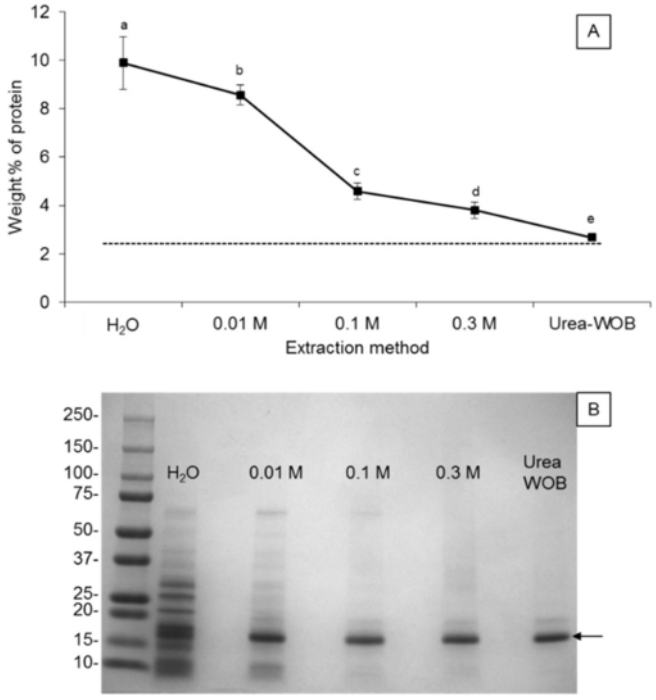

Fig. 3. A) Protein content of COB recovered using H_2O or $NaHCO_3$ (0.01, 0.1, 0.3 M) and urea-WOB (from COB recovered in 0.1 M $NaHCO_3$), expressed as percentage based on dry weight. Values are means ± SD, with P value <0.05. B) SDS-PAGE of protein extract from COB extracted with H_2O or $NaHCO_3$ (0.01, 0.1, 0.3 M) and of urea-WOB. Oleosin band is indicated by an arrow.

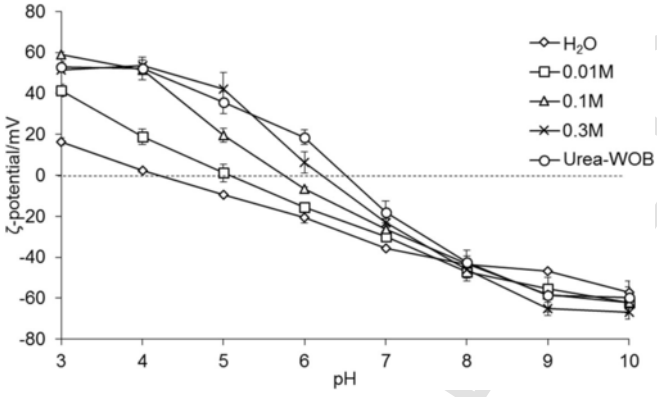

Fig. 4. ζ-potential of COB extracted from oilseed rape using H_2O (♦) and $NaHCO_3$ at molarity of 0.01 (■), 0.1 (▲), 0.3 (x). Urea-WOB (○, from COB recovered in 0.1 M $NaHCO_3$) is shown as reference, representing the purest preparation.

position at the interface of the extracted OBs (Fig. 3B). As detailed in Section 3.3, the use of sodium bicarbonate solution system, led to a purification of the OB extract from exogenous protein, increasing the molarity of the salt solution used reaching values close to the urea-WOB. However, among all emulsions tested, only recovering the OB in sodium bicarbonate at a molarity of 0.3, the ζ-potential had a comparable trend to the urea-WOB ($P < 0.05$) in all pH considered, suggesting that the two emulsions had similar proteins at the interface. Similar findings have been registered in Payne, Lad, Foster, Khosla, and Gray (2014), where the differences in ζ-potential was also attributed to proteins that become associated with the surface of the oil bodies, altering also the pI of the organelle *ex vivo*. When the surface charge reaches the neutrality, the ζ-potential value is equal to zero, which corresponds to the pI of the droplets. The pI of COBs depended on the extraction medium used, ranging from 4.2 using water to 5.0, 5.7 and 6.2 for COBs extracted with, respectively, sodium bicarbonate at molarities of 0.01, 0.1 and 0.3. The pI values of the cleanest preparations form exogenous protein (urea-WOB and COBs extracted in 0.3 M sodium bicarbonate) are higher than the ones registered in other studies on different seeds (Chen et al., 2012; Iwanaga et al., 2007; Nikiforidis & Kiosseoglou, 2009), which may be due to the different methodology used, but in line with the work of Tzen, Lie, and Huang (1992) and Tzen et al. (1993) on rape seeds.

The ζ-potential can be used for evaluation of colloidal interaction, classifying the emulsion stability depending on its value (Patel & Agrawal, 2011). The presence of high amount of exogenous proteins for COBs recovered in water and 0.01 M sodium bicarbonate, results in a shift towards more acidic pH values of the medium (as detailed in Section 3.3) and a decay of ζ-potential. As mentioned in Section 3.2, the pH values for those emulsions were 6 and 7, corresponding to ζ-potential values lower than -30 mV (Fig. 4), considered the minimal value for highly stable emulsions. However, using sodium bicarbonate 0.1 M, the droplets had a higher ζ-potential (around −50 mV) at the corresponding pH of the solution after grinding, confirming this salt concentration as the optimum for the recovery of intact OBs.

3.5. Effect of washing intact COB preparations on OB storage quality

The presence of active exogenous and endogenous enzymes associated to the OB interface depends on the pH of extraction, on the number of washing steps, and on any heat treatment applied to increase the shelf life of the material (Chen et al., 2014; Zhao et al., 2016). However, the removal of exogenous proteins during the extraction using appropriate media would eliminate the need of long holding time at high temperature for enzyme denaturation. As detailed by Millichip et al. (1996), the exposure of OBs to 9 M urea resulted in the progressive removal of contaminating proteins, leaving only the integral ones.

This section examined whether it would be possible to reach the same purity of the urea-WOB from crude extract of intact COB (extracted using a 0.1 M sodium bicarbonate solution, as detailed in Section 3.2), using the same solution as a washing medium, and its effect on the storage stability. If sodium bicarbonate were to be used in an industrial process, then it may be necessary to carry out a final water wash to remove the salt to meet recommended levels of sodium in food and ingredients. For this reason, the COB preparations were washed in urea (9 M) or 0.1 M sodium bicarbonate (pH 9.5) and subsequently rinsed in water to remove traces of the washing solution. The sequential washing in 0.1 M sodium bicarbonate and water, removed the majority of exogenous proteins from the COB preparation (final concentration of 2.69 ± 0.21% dry matter), reaching the same content of the urea WOB (2.60 ± 0.05%). The removal of exogenous protein from the 0.1 M COB extract had an effect on the surface charge of the washed droplets (Fig. 5). Compared to the COB preparation, the PSD of the washed OB in sodium bicarbonate (0.1 M) and subsequently washed in water, did not show any size increase (data not shown) and it displayed a zeta potential trend very similar to the urea-WOB preparation ($P < 0.05$).

Although the peripheral proteins could prevent the lipid oxidation of oil bodies (Karkani et al., 2013), the presence of enzymes already active during the imbibition process (Theimer & Rosnitschek, 1978), can cause the OB structure breakdown. The use of the sodium bicarbonate washing solution can remove exogenous proteins in a concentration dependent fashion, reaching a comparable purity to the urea-washed extract, considered the purest preparation. We hypothesized that a reduction in protein content extracting and washing the OBs using 0.1 M sodium bicarbonate, would correspond to the removal of active germinating enzymes from the extract, and so the extension of the droplet integrity over storage. For this reason, OB emulsions (10% lipid weight

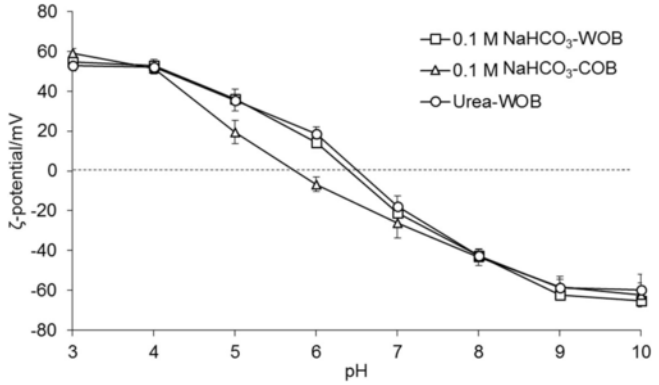

Fig. 5. Effect of washing COB using sodium bicarbonate solution on ζ-potential. COB were recovered and washed using 0.1 M $NaHCO_3$, pH 9.5 (□). Urea washed oil body (urea-WOB, ○) and crude oil body recovered in 0.1 M $NaHCO_3$ (0.1 M COB, Δ) are shown as references, representing the unwashed cream and the purest preparation, respectively.

basis) with a different content in protein, were kept at room temperature (20 °C) and the particle size was evaluated over a period of four weeks. NaN_3 (0.02 mM) was added in all preparations to avoid microbial growth (Lichstein & Soule, 1944).

In Fig. 6 it is shown the $D_{4,3}$ of COB emulsion extracted using only water, rich in peripheral proteins and the urea-WOB, practically free from their presence as detailed in Section 3.3. With a lower protein content compared to the water recovered COB, the preparation extracted and washed in sodium bicarbonate represent the optimised extraction process, with a similar protein composition than the urea-WOB. Over the time course, unwashed COB samples recovered using water were the least stable, with clear membrane disruption and reduced number of droplets after four weeks (Fig. 6B). Significant improvement in the physical stability of OB on storage were achieved by grinding seed in 0.1 M sodium bicarbonate, and even more on washing this material with the same solution. The most stable samples were the ones washed in urea (9 M) without showing altered droplet structure (Fig. 6E) nor a considerable size increase. Despite the small difference in $D_{4,3}$ between the washed emulsions, the one recovered and washed in sodium bicarbonate showed a loss of oil body structure (Fig. 6D, indicated by arrows), suggesting a slight enzymatic activity over the storage trial.

Different enzymes such as lipase (Lin, Moreau, & Huang, 1982; Lin, Wimer, & Huang, 1983), phospholipase (May, Preisig-Müller, Höhne, Gnau, & Kindl, 1998), protease (Sadeghipour & Bhatla, 2002), have been localised in the proximity of the OB over the germination process. The presence of those enzymes in protein-rich extracts, leads to the rapid destabilization of the OB structure over time. Purifying the OB using the correct combination of pH and buffer, limits the carry-over of active germinating enzymes, leading to a better stability of the emulsion over time (Fig. 6).

4. Conclusion

The findings of this work demonstrate that different methodologies adopted in grinding affect the integrity and the characteristics of the recovered oil body emulsion, such as particle size, protein content and ζ-potential. This study shows that the use of a medium with a high alkalinity overcomes the buffering capacity of the seed material in solution; using a low solid-to-media load (1:7 w/w), and a concentration of 0.1 M of the sodium bicarbonate, the system appears to maintain the integrity of OB droplets by creating enough repulsion forces to avoid coalescence. The differences in the purity of the OB preparations is a result of the ability of the alkaline medium to interfere with the bonds

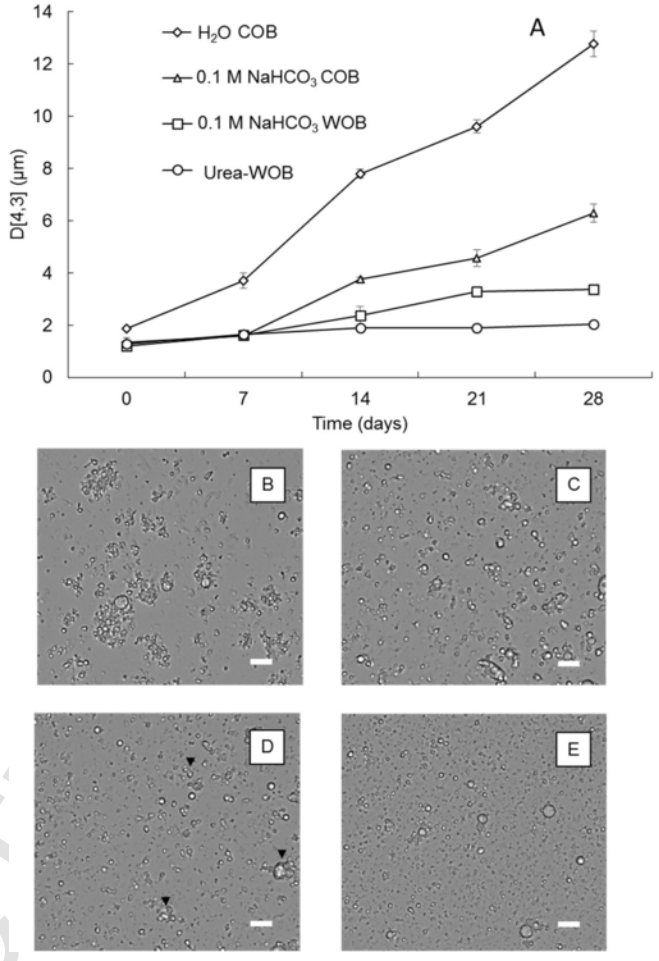

Fig. 6. A) Mean volume diameter ($D_{4,3}$) over four weeks of COB extracted with H_2O (◇) and 0.1 M $NaHCO_3$ (Δ), and WOB using 0.1 M $NaHCO_3$ (□) or Urea 9 M (○). Optical images after 30 days of storage of COB extracted using H_2O (B), sodium bicarbonate (0.1 M, panel C), and WOB extracted in 0.1 M $NaHCO_3$ and washed using 0.1 M $NaHCO_3$ (D) or urea 9 M (E). Scale bar represent 10 μm.

that connect exogenous proteins with the surface of OBs. However, the exposure of OBs to high pH values and salt concentration, may promote coalescence due to deprotonation of N- and C-terminus of oleosin, decreasing the number of salt bridges with the phospholipid. Over the 90% of OBs recovered after soaking the seeds for 16 h (then grinding using 0.1 M sodium bicarbonate at a ratio of 1:7 (w/w), and washed in the same solution) showed an equal protein composition and surface potential as the purest OB preparation (urea-WOB). Moreover, the removal of exogenous proteins retained the physical stability of oil bodies probably due to the removal of hydrolytic enzymes, which may lead to a reduction of time and temperature required for biochemical stabilization. These results offer an important contribution on the route for the development of a method to extract intact and pure OBs from oilseed rape seed suitable for the food industry.

Acknowledgments

This research was funded through the EPSRC Centre for Innovative Manufacturing in Food.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Adams, G.G., Imran, S., Wang, S., Mohammad, A., Kok, M.S., Gray, D.A., et al., 2012. Extraction, isolation and characterisation of oil bodies from pumpkin seeds for therapeutic use. Food Chemistry 134, 1919–1925.

Arakawa, T., Timasheff, S.N., 1985. Theory of protein solubility. Methods in Enzymology 114, 49–77.

Austin, L. G. & Trass, O. 1997. Size Reduction of Solids Crushing and Grinding Equipment. In: Fayed, M. & Otten, L. (eds.) Handbook of Powder Science & Technology. Springer, US.

Cao, Y., Zhao, L., Ying, Y., Kong, X., Hua, Y., Chen, Y., 2015. The characterization of soybean oil body integral oleosin isoforms and the effects of alkaline pH on them. Food Chemistry 177, 288–294.

Chen, B.C., Mcclements, D.J., Gray, D.A., Decker, E.A., 2012. Physical and oxidative stability of pre-emulsified oil bodies extracted from soybeans. Food Chemistry 132, 1514–1520.

Chen, Y., Ono, T., 2010. Simple extraction method of non-allergenic intact soybean oil bodies that are thermally stable in an aqueous medium. Journal of Agricultural and Food Chemistry 58, 7402–7407.

Chen, Y., Zhao, L., Cao, Y., Kong, X., Hua, Y., 2014. Oleosins (24 and 18 kDa) are hydrolyzed not only in extracted soybean oil bodies but also in soybean germination. Journal of Agricultural and Food Chemistry 62, 956–965.

Frandsen, G.I., Mundy, J., Tzen, J.T.C., 2001. Oil bodies and their associated proteins, oleosin and caleosin. Physiologia Plantarum 112, 301–307.

Gray, D.A., Payne, G., Mcclements, D.J., Decker, E.A., Lad, M., 2010. Oxidative stability of Echium plantagineum seed oil bodies. European Journal of Lipid Science and Technology 112, 741–749.

Gunstone, F. D. 2004. The Chemistry of Oils and Fats: Sources, Composition, Properties, and Uses, Blackwell Pub.

Guzey, D., Mcclements, D.J., 2006. Formation, stability and properties of multilayer emulsions for application in the food industry. Advances in Colloid and Interface Science 128–130, 227–248.

Hoppe, A., Theimer, R.R., 1997. Rapeseed lipase-pH dependent specificity for native lipid body autolysis and lipolysis of artificial oil droplets. Journal of Plant Physiology 151, 390–398.

Iwanaga, D., Gray, D., Decker, E.A., Weiss, J., Mcclements, D.J., 2008. Stabilization of soybean oil bodies using protective pectin coatings formed by electrostatic deposition. Journal of Agricultural and Food Chemistry 56, 2240–2245.

Iwanaga, D., Gray, D.A., Fisk, I.D., Decker, E.A., Weiss, J., Mcclements, D.J., 2007. Extraction and characterization of oil bodies from soy beans: a natural source of pre-emulsified soybean oil. Journal of Agricultural and Food Chemistry 55, 8711–8716.

Karkani, O.A., Nenadis, N., Nikiforidis, C.V., Kiosseoglou, V., 2013. Effect of recovery methods on the oxidative and physical stability of oil body emulsions. Food Chemistry 139, 640–648.

Lacey, D. J., Wellner, N., Beaudoin, F., Napier, J. A. & Shewry, P. R. 1998. Secondary structure of oleosins in oil bodies isolated from seeds of safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.). Biochemical Journal, 334 (Pt 2), 469–77.

Lichstein, H.C., Soule, M.H., 1944. Studies of the effect of sodium azide on microbic growth and respiration: I. The action of sodium azide on microbic growth. Journal of Bacteriology 47, 221–230.

Lin, Y.H., Moreau, R.A., Huang, A.H.C., 1982. Involvement of glyoxysomal lipase in the hydrolysis of storage triacylglycerols in the cotyledons of soybean seedlings. Plant Physiology 70, 108–112.

Lin, Y.-H., Wimer, L.T., Huang, A.H.C., 1983. Lipase in the lipid bodies of Corn Scutella during seedling growth. Plant Physiology 73, 460–463.

May, C., Preisig-Müller, R., Höhne, M., Gnau, P., Kindl, H., 1998. A phospholipase A2 is transiently synthesized during seed germination and localized to lipid bodies. Biochimica et Biophysica Acta (BBA) – Lipids and Lipid Metabolism 1393, 267–276.

Mcclements, D.J., 2004. Food Emulsions: Principles, Practices, and Techniques, 2nd ed. Taylor & Francis.

Millichip, M., Tatham, A.S., Jackson, F., Griffiths, G., Shewry, P.R., Stobart, A.K., 1996. Purification and characterization of oil-bodies (oleosomes) and oil-body boundary proteins (oleosins) from the developing cotyledons of sunflower (Helianthus annuus L). Biochemical Journal 314, 333–337.

Murphy, D.J., Cummins, I., 1989. Seed oil-bodies – isolation, composition and role of oil-body apolipoproteins. Phytochemistry 28, 2063–2069.

Nikiforidis, C.V., Kiosseoglou, V., 2009. Aqueous extraction of oil bodies from maize germ (Zea mays) and characterization of the resulting natural oil-in-water emulsion. Journal of Agricultural and Food Chemistry 57, 5591–5596.

Patel, V.R., Agrawal, Y.K., 2011. Nanosuspension: An approach to enhance solubility of drugs. Journal of Advances Pharmaceutical Technology Research 2, 81–87.

Payne, G., Lad, M., Foster, T., Khosla, A., Gray, D., 2014. Composition and properties of the surface of oil bodies recovered from Echium plantagineum. Colloids and Surfaces B: Biointerfaces 116, 88–92.

Qu, R., Huang, A.H.C., 1990. Oleosin Kd-18 on the surface of oil bodies in Maize – Genomic and Cdna sequences and the deduced protein-structure. Journal of Biological Chemistry 265, 2238–2243.

Sadeghipour, H.R., Bhatla, S.C., 2002. Differential sensitivity of oleosins to proteolysis during oil body mobilization in sunflower seedlings. Plant Cell Physiology 43, 1117–1126.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., et al., 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76–85.

Tang, G.S., Ono, T., Mikami, M., 1997. Interaction between protein and lipid in soybean milk at elevated temperature. Journal of Agricultural and Food Chemistry 45, 4601–4605.

Theimer, R.R., Rosnitschek, I., 1978. Development and intracellular localization of lipase activity in rapessed (Brassica napus L.) cotyledons. Planta 139, 249–256.

Tzen, J.T.C., Cao, Y.Z., Laurent, P., Ratnayake, C., Huang, A.H.C., 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiology 101, 267–276.

Tzen, J.T.C., Huang, A.H.C., 1992. Surface-structure and properties of plant seed oil bodies. Journal of Cell Biology 117, 327–335.

Tzen, J.T.C., Lie, G.C., Huang, A.H.C., 1992. Characterization of the charged components and their topology on the surface of plant seed oil bodies. Journal of Biological Chemistry 267, 15626–15634.

Tzen, J.T.C., Peng, C.C., Cheng, D.J., Chen, E.C.F., Chiu, J.M.H., 1997. A new method for seed oil body purification and examination of oil body integrity following germination. Journal of Biochemistry 121, 762–768.

Wanasundara, J. P. D. & Mcintosh, T. C. 2013. Process of aqueous protein extraction from Brassicaceae oilseeds. US8557963 B2.

White, D.A., Fisk, I.D., Mitchell, J.R., Wolf, B., Hill, S.E., Gray, D.A., 2008. Sunflower-seed oil body emulsions: Rheology and stability assessment of a natural emulsion. Food Hydrocolloids 22, 1224–1232.

Yatsu, L.Y., Jacks, T.J., 1972. Spherosome membranes: half unit-membranes. Plant Physiology 49, 937–943.

Zayas, J., 1997. Solubility of Proteins. Functionality of Proteins in Food. Springer Berlin Heidelberg.

Zhao, L., Chen, Y., Chen, Y., Kong, X., Hua, Y., 2016. Effects of pH on protein components of extracted oil bodies from diverse plant seeds and endogenous protease-induced oleosin hydrolysis. Food Chemistry 200, 125–133.