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Influence of soaking time on the mechanical properties of rapeseed and their effect on oleosome extraction

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ABSTRACT

Oleosomes (or oil-bodies) are the oil storage structures of plant seeds. We typically extract oil by disrupting the oleosomes through mechanical-pressing of dry seeds and by using organic solvents. Nevertheless, it is possible to extract whole oleosomes, by breaking pre-soaked seeds. A key point to avoid oleosome damage seems to be the soaking step. Hence the aim of this work was to understand the effect of soaking time on the mechanical properties of rapeseed and its effect on oleosome extraction. The results showed that the diffusion of water through the seeds was negatively associated to the mechanical strength of the seeds. This effect occurs in 3 stages and reaches a plateau at 8 h. The extraction of oleosomes and proteins kept constant for all different treatments. However, the duration of the soaking step influences oleosome integrity, as oleosomes extracted after shorter soaking times (<8 h) yielded bigger oleosomes with extraneous proteins bounded to them. After 8 h soaking time, the mechanical properties of the seeds kept constant and oleosomes of native size and with oleosome endogenous proteins were recovered. Therefore, a minimum soaking time of 8 h is required to achieve the extraction of intact oleosomes.

1. Introduction

Oleosomes, also known as oil bodies, are the oil storage structures present in seeds such as rapeseeds (Jolivet et al., 2009). They are composed of a core of triglycerides surrounded by a monolayer of phospholipids and proteins that render their outer surface hydrophilic (Maurer et al., 2013). Their hydrophilic interface allows their extraction using water instead of an organic solvent (Nikiforidis et al., 2014). Moreover, due to their particular structure, oleosomes have the potential to replace the synthetic oil droplets in food emulsions, pharma and cosmetics (Nikiforidis, 2019).

To extract oleosomes is necessary to soak the seeds, which it is known as the step for conditioning the seeds before the extraction. More specifically, it is believed that the mechanical properties of the material are being softened and prepared for the cell-lysis. It is expected that soaking the seeds could aid the aqueous media to solubilize the cellular material (Nikiforidis and Kiosseoglou, 2009) and to weak the cellular structure to reduce the stresses experienced by the oleosomes during their extraction (Chigwedere et al., 2019; De Chirico et al., 2018; Sayar et al., 2001).

Recently it was reported that when using a twin-screw press to perform the aqueous extraction, solubilization of cellular material played a less important role during the extraction. Even when extractions were performed with less favorable conditions (i.e. low liquid to solids ratio and water at neutral pH), the oleosome and protein extraction yields were like those obtained using optimal conditions (i.e. high liquid to solid ratio and alkaline conditions) (Romero-Guzmán et al., 2020a). This could mean that soaking time could also play a less important

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role during aqueous extraction, when using twin-screw press. However, besides its relevancy, no mechanistically study was performed before to understand the exact influence of soaking time on the extraction yield nor on the extracted oleosome characteristics. This lack of information hinders the feasibility of the process as the proposed times for the extraction are arbitrary lasting between 16–24 h and no rules have been proposed to objectively select a correct time to perform this step (Campbell and Glatz, 2009; De Chirico et al., 2018; Iwanaga et al., 2007; Nikiforidis and Kiosseoglou, 2009; Yoshie-Stark et al., 2008).

Therefore, being soaking of the seeds such an important step for the oleosome extraction (Carter and Manthey, 2019; Chigwedere et al., 2019; Waschatko et al., 2016; Yiu et al., 1982), the aim of this work was to obtain a better understanding on the effect of soaking time on the overall performance of oleosome extraction process using twin-screw press.

2. Materials and methods

2.1. Materials

Rapeseeds were purchased from a seed producing company. Deionized water was obtained from a Milli-Q purification system (Merck Millipore, USA). KCl was bought from Merck (Merck, Germany). Petroleum ether p.a. was obtained from Fisher Scientific (Baker Fisher Scientific, USA). All other chemicals were obtained in analytical grade from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Water soaking experimental design

Batches of 20 rapeseeds were soaked in 20 mL of water (pH7) for 0, 1, 4, 6, 8, 16 and 24 h. This step was conducted at 4 °C to prevent germination to begin (Beisson et al., 2001; Simon et al., 1976). The rapeseeds were taken out of the water. The adhering water was gently removed with a piece of paper before their weight was determined. Immediately after weighting, a compression test was done on the seeds.

2.3. Seed compression test

A compression test was performed with the TA.XT plus C Texture Analyzer (Stable Micro Systems, UK), operating with the Exponent Connect software, using a 4 mm DIA cylinder stainless probe. After having been weighed, each seed was placed under the probe. The force (N) necessary to break the seeds is displayed. For each soaking time, 10 different seeds were used.

2.4. Cryo-SEM sample preparation and imaging

Three to four steeped seeds were placed together into the slit of a copper stub and fixated with carbon glue (Leit-C, Neubauer Chemikalien, Germany). The stub was attached to a cryo-sample holder, put into the cryo-sample loading system (VCT 100, Leica, Vienna, Austria) and simultaneously frozen in liquid nitrogen. The frozen sample holder was transferred to the cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto the sample stage at −92 °C. The frozen seeds were frozen fractured longitudinally and freeze–dried for about 7 min at −92 °C and 5.10–6 torr. After sputter coating with 20 nm tungsten at the same temperature, the sample holder was cryo-shielded and transferred into the field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, The Netherlands) onto the sample stage at −120 °C and analyzed with SE detection at 2 kV and 6.3 pA.

2.5. Extraction of oleosomes and recovery of oleosome-rich creams

All the extractions were performed in batches of 100 g of seeds. Prior to the extraction, the seeds were soaked 1:1 ratio using a solution of either 0.1 M $NaHCO_3$ adjusted to pH 9.5 or 0.2 M KCl adjusted to pH 7 or H_2O at pH 7, for 1, 4, 8, 16 or 24 h at 4 °C. The pH was adjusted with a solution of 1.0 M NaOH for the alkaline solution and a solution of 0.1 M NaOH for the neutral solution. The pre-soaked seeds were processed with an Angel 7500 extractor (lab-scale twin-screw press), maintaining the seeds to solution ratio of 1:1 wt.% from the soaking step. From this extraction, two streams were recovered: (1) the press cake, which was collected at the end of the press and (2) a concentrated slurry (first extract), which exuded from small holes along the device. The first extract was diluted with $NaHCO_3$ 0.1 mol/L solution at pH 9.5, KCl 0.2 mol/L solution at pH 7, or H_2O at pH 7, to a solid to liquid ration of 1:4. The oleosomes were recovered from this dilute via centrifugation at 3,000 *g* and 4 °C for 15 min. An oleosome rich cream layer was recovered and drained from the rest of the fluid using filter paper. The collected oleosome cream was subsequently dispersed in the same solution as was used for the dilution after the primary extraction (to 1:4 wt./v). A second centrifugation followed at 10,000 *g*, 4 °C for 30 min (Sorval Lynx 4000 Centrifuge, Thermo Scientific USA). This cycle of recovery and dilution was performed twice. The oleosome rich cream obtained after the third centrifugation was then again drained from the fluid using filter paper and dispersed in ultra-pure water at 1:4 wt./v and centrifuged at 10,000 *g*, 4 °C for 30 min. This obtained cream was then collected and analyzed for its composition and physical properties.

2.6. Characterization of the solid residue after the extraction (cake) and the creams (concentrated oleosomes)

2.6.1. Moisture content

One gram of sample was dried with a Moisture Analyzer (Leicester, UK) at 90 °C until constant weight. The drying time varied from 10 to 40 min, depending on the sample. The moisture content was determined by the weight difference between the initial weight and the weight of the dehydrated sample, relative to the weight of the original sample.

2.6.2. Lipid content

The lipid content was determined in duplicates by Soxhlet extraction method with petroleum ether (B-811 Buchi Extractor, Switzerland). The oleosome extraction yield was calculated based on the difference between lipid content remaining in the cake and the initial oil content in the seeds (36 wt.%). This yield was directly taken as oleosome yield as most of the oil inside of oil-rich seeds is present in this form (98 %) (Hu et al., 2009; Tzen et al., 1993). Therefore other researches have followed a similar approach (Matsakidou et al., 2015; Nikiforidis and Kiosseoglou, 2009).

2.6.3. Protein content

The protein content of dry and defatted samples was calculated by determining the amount of Nitrogen in the samples using the Dumas method and using a conversion factor of 5.5 as suggested in literature (Lindeboom and Wanasundara, 2007) (Nitrogen analyser, FlashEA 112 series, Thermo Scientific, Interscience, The Netherlands). The protein extraction yield was calculated based on the difference between pro-

tein content remaining in the cake and the initial protein (18 wt.%) content in the seeds. The measurements were done in triplicates.

2.7. Particle size distribution determination

The particle size of the creams was measured with static laser light scattering (Malvern Master Sizer 3000, Malvern Instruments, UK). The oil refractive index used was 1.43. To perform the analysis the oleosome cream was first dissolved in ultrapure water at a 1:10 (wt./v.). Each measurement was done in triplicate and expressed with volumetric particle size distributions.

2.8. Protein profile characterization

The protein profile was analyzed qualitatively with SDS polyacrylamide gel electrophoresis using a Bio-Rad MiniProtean cell (Bio-Rad Laboratories Inc., Hercules, USA). Two types of buffers were used to unfold the proteins (Nikiforidis and Kiosseoglou, 2009). Buffer 1 consisted of Tris–HCl (50.0 mmol/L), Urea (5.0 mol/L), 1 wt.% SDS and 4 wt.% 2-mercaptoethanol. Buffer 2 consisted of Tris–HCl (125.0 mmol/L), Urea (5.0 mol/L), 1 wt.% SDS, 20 wt.% Glycerol and 4 wt.% 2-mercaptoethanol. The creams were dispersed in ultrapure water (1:100 wt./v.) and were combined with Buffer 1 (1:1 v./v.) and agitated for 15 min at room temperature. Each sample was rested for 15 min before buffer 2 was added. The samples were vortexed once more for 15 min and rested for another 15 min. Afterward, the samples were heated at 90 °C for 5 min and kept at −20 °C overnight. Before the samples were loaded onto the gel, 3 freeze–thaw cycles were applied. An amount of 20 μL of each sample was then loaded on a 12% Tris–HCl SDS-ready gel, size range of 10−200 kDa; and 10 μL of Pre-Stained Protein Standard (Bio-Rad Laboratories Inc., Hercules, USA). The electrophoresis was carried out at 200 V for about 30 min. Subsequently, the gel was stained with Bio-safe Coomassie Stain (Bio-Rad Laboratories Inc., Hercules, USA), and analyzed with software from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, USA) to quantitatively determine the optical density (%) of the different proteins present in the samples.

2.9. Zeta-potential determination

Dynamic light scattering (DLS Zetasizer Nano ZS, Malvern Instruments Ltd, UK) was used to analyze the ζ-potential of the samples. The creams were diluted 1000 times (oil-basis) in ultra-pure water. After the dilution, the pH of the dispersions was adjusted manually in a range of 3–9 with either HCl (1.0 mol/L) or NaOH (1.0 mol/L) solution. The refractive indices used were 1.43 for the dispersed phase and 1.33 for the continuous phase.

2.10. Statistical analysis

A one-way analysis of variance (ANOVA) and an LSD post-hoc significance test were applied to establish differences among the extraction yields. The analyses were performed with the IBM SPSS statistics 23 software. Differences were significant when $p < 0.05$.

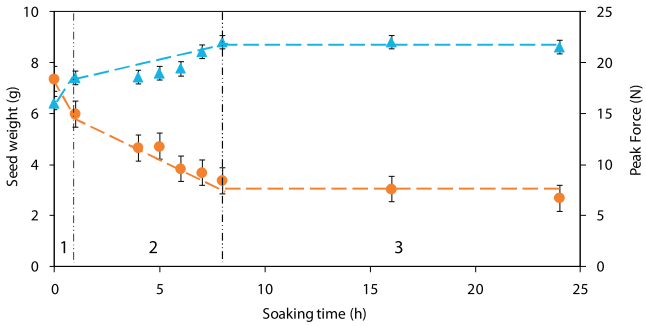

Fig. 1 – Effect of soaking time on the rapeseed weight (g) (▲) and peak force (N) (●) or necessary force to break a rapeseed. Three different stages of water absorption and modifications on the strength of the material are observed. (1) A steep increase on water content and loss of strength. (2) A less steep increase of water conent and loss of strength (3) A plateau region with no variation in seed weight or seed mechanical properties.

3. Results and discussion

3.1. Seed mechanical properties and seed changes during soaking

Fig. 1 shows the weight increase of the individual rapeseeds upon soaking and its effect on the mechanical properties, which were evaluated as the maximum force exerted during a compression test.

The weight of individual dry rapeseeds started at 6 g ± 1.3 g and increased to 7.4 g ± 1.7 g during the first hour. The slope of the weight increase was less pronounced between 1 h and 8 h. At 8 h, a plateau at 8.8 ± 1.4 g was reached. Similar patterns were previously reported for chickpeas and amaranth (Johnny et al., 2015; Resio et al., 2005). The slow diffusion of water is due to the complex mass transfer phenomena. The influx of water into the cells leads to the solubilization of cell components. This reduces the concentration gradients of solutes between the cytosol and the media, slowing down its diffusion of water into the cells (Johnny et al., 2015). In the reported cases, similar as in Fig. 1, three stages in the water absorption were also identified and related with the following phenomena: (1) A step increase in weight (from 0 to 1 h) was associated with the diffusion of water through larger capillaries between different parts of the seeds. (2) A less pronounced slope of weight increase (from 1 to 8 h), which was associated with the diffusion of water between the capillaries and inter-cellular spaces on the seed coat (i.e. hilum) (Johnny et al., 2015). Finally, (3) a plateau (from 8 h onwards), which starts when the water absorption matched the counter-diffusion of solutes from the seeds into the water (Sayar et al., 2001). In other words, when solutes from the cells diffused into the water at a similar rate than water diffuses inside the seeds.

Interestingly the results of the compression test perfectly followed the same trend of the seed weight increase (Fig. 1). As it has been reported, the hardness of the cellulose-rich materials is strongly related to their moisture content (Sun et al., 2010; Chami Khazraji and Robert, 2013). At low moisture content a significant part of the matrix components may be in glassy state, thus exhibiting high rigidity. The absorption of water, on the other hand, may bring the amorphous parts between the (cellulose) crystals in the rubbery state, and ensure higher flexibility of the matrix. At the same time,

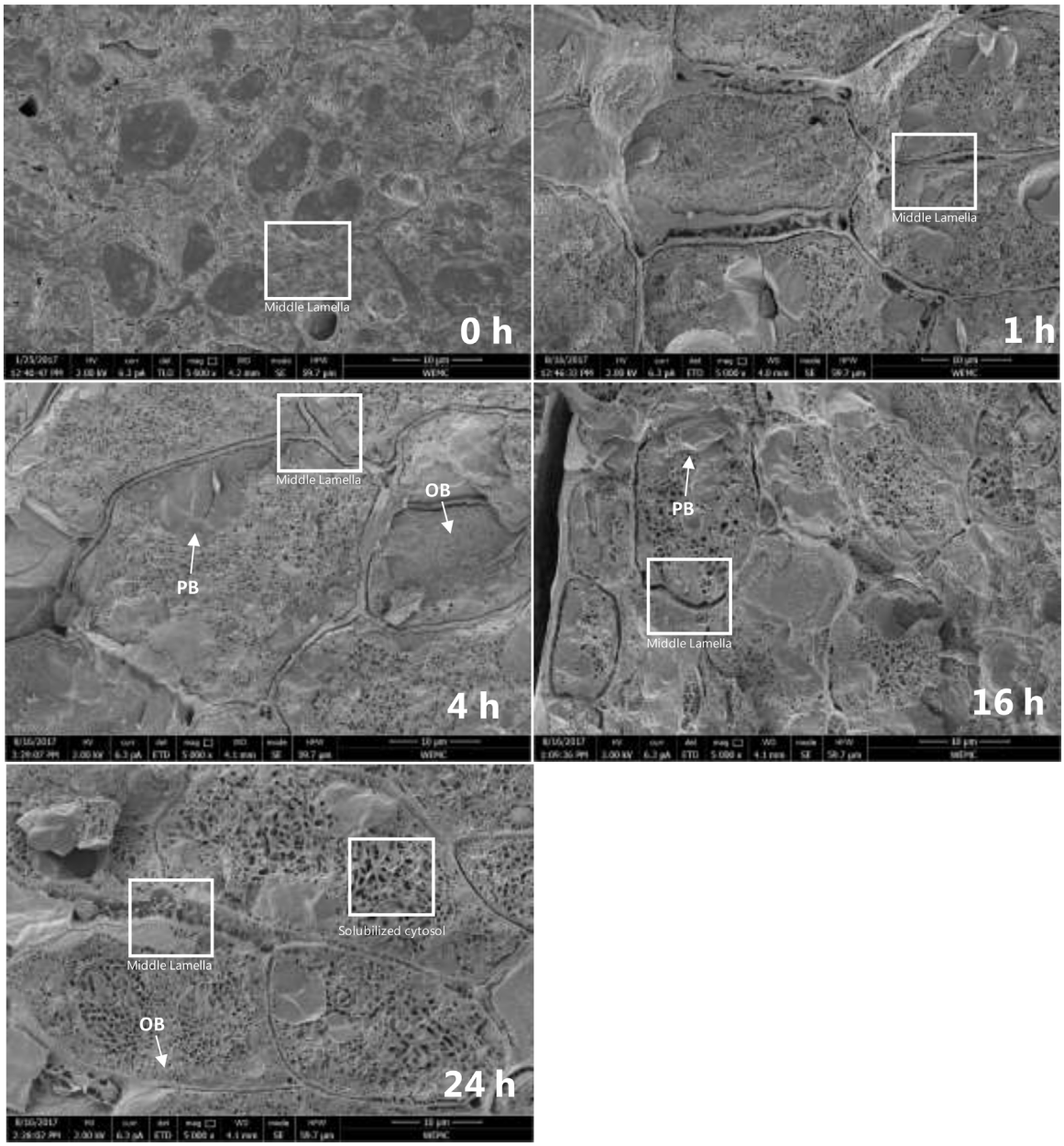

Fig. 2 – Cryo-SEM images of the rapeseeds soaked for different amount of time. The abbreviations OB and PB stand for oil bodies (or oleosomes) and proteins bodies. The hollow squares indicate the middle lamella.

cellulosic parts, still retain their coherence, due to the local crosslinking of the crystalline domains (O'Neill et al., 2017). The gradual solubilization and extraction of other components such as pectin, proteins, and ions may also lead to softening of the structure. Especially as most of these soluble components are constituents of the middle lamella (Carter and Manthey, 2019), which is identified as the cohesive material between cell-walls (Daher and Braybrook, 2015).

Recently it was reported that the softening of other plant material, such as beans during cooking, was related to the weakening of the middle lamella due to the solubilization of its polysaccharides (Chigwedere et al., 2018; Sila et al., 2005). Hence, to correlate the effect of water absorption and mechanical properties with the microstructure of the rapeseeds, the physiological changes of the rapeseed microstructure were monitored using cryo-SEM. The micrographs of the seeds are depicted in Fig. 2.

From the micrographs we can see that separation between cells occurred after the first hour of soaking time, which keeps increasing over time. Moreover, the threads of a viscous material, which we believe corresponds to the middle lamella, are more evident over time. Additionally, the density of the cellular structure also seems to increase as more gaps are observed in the cells. All these changes are more evident with the increase on soaking time. We believe that the observed gaps between cellular components corresponded to water crystals that were formed during the pre-preparation of the samples for cryo-SEM. These gaps are more abundant as the amount of water inside the cells increases, even when the weight of the seeds reaches a plateau. Hence we believe that these gaps are also related with the solubilization of cellular material (Budiaman and Fennema, 1987). Similar results, where the soaking time is strongly related with the solubilization of cellular material, have been reported for other materials such as chickpeas (Johnny et al., 2015).

Overall, the images are in line with the proposed mechanism for water diffusion and solubilization of components, previously described in Fig. 1. As the images confirm that the

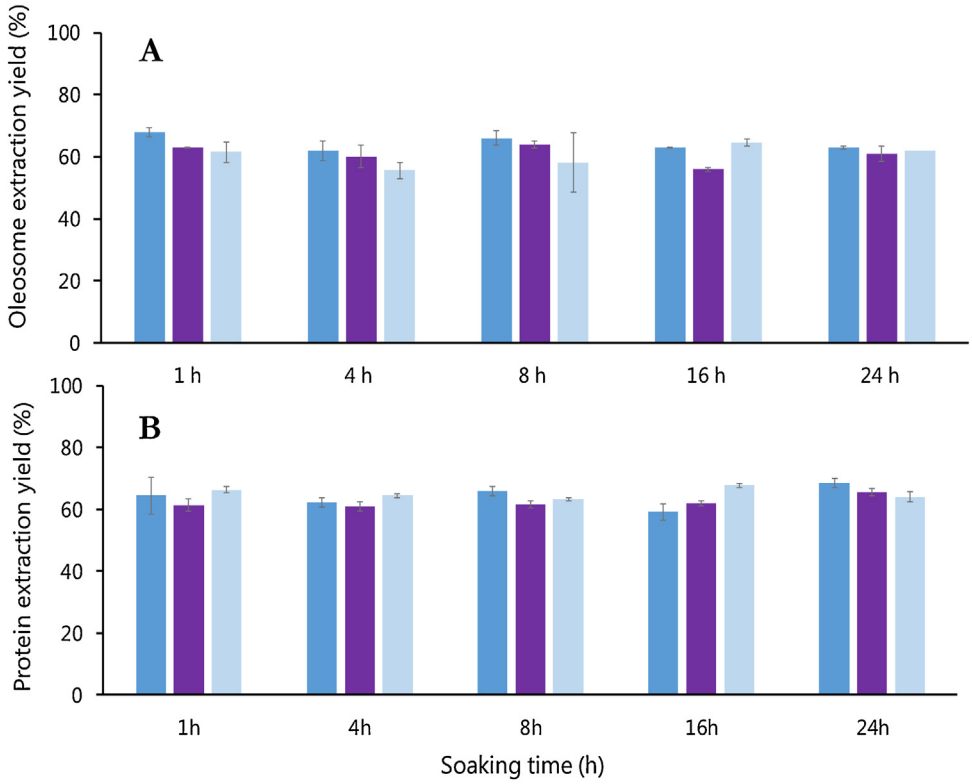

Fig. 3 – Oleosome extraction yield (A) and protein extraction yield (B). The extractions were performed with 0.1 M $NaHCO_3$ at pH 9.5 (■), with 0.2 M KCl at pH 7 (■) and with ultra-pure water at pH 7 (■).

migration of water inside the cells firstly affects the middle lamella and secondly it solubilizes the cellular material.

3.2. *Effect of soaking time on the extraction yields*

To determine the effect of soaking media and the seed softening on the release of intracellular oleosomes and proteins, the oleosome and protein extraction yields were determined for 3 different extraction media (0.1 mol/L $NaHCO_3$ at pH 9.5 and ultra-pure water at pH 7). Previously it was reported that when using twin-screw press, no significant difference was found on oleosome and protein yields between different extraction media (Romero-Guzmán et al., 2020a). However, in that work the effect of the soaking time was not depicted. Hence similar aqueous media in combination with different soaking times were used to perform the extraction employing a twin-screw press. The results are shown in Fig. 3.

Fig. 3 shows that there is no statistically significant difference between the oleosome and protein yields recovered after different soaking times for any of the soaking media. This indicates that, unlike the extraction using a blender, the moisture content of the seeds (and seed hardness) nor pH are relevant for the oleosome extraction, when using a twin-screw press. In our previous work, it was concluded that the shear forces inside the press mastered the extraction and that the solubilization effect of the different soaking media was superfluous. The same seems to hold for the different soaking times and states of the material. The similar yields obtained for all the treatments could be explained by the fact that friction inside the twin-screw press is favoured by the presence of dry material (Evon et al., 2007). The increase in friction is desired as it increases the shear forces created in the screws and as consequence, it enhances the extraction of material. Even though the solubilization of cellular material enhances oleosome and protein extraction, it is negatively related to mechanical seed strength (Matsakidou et al., 2015).

Therefore we hypothesized that the effect of solubilization of cellular material on yield is partly offset by this loss of shear forces associated with the mechanical strength of the seeds. The counterproductivity of both phenomena results in the extraction being unaffected past 8 h soaking time.

Due to the reduction of the friction inside the screws, resulting on the reduction of the dry mass, the overall extraction seems to be unaffected as both phenomena are counterproductive.

3.3. *Oleosome characterization*

To assess the quality of the extracted oleosomes at different soaking times, the oleosomes were recovered and their particle size distribution was characterized (Fig. 4).

The oleosomes recovered at alkaline conditions were extracted as individual droplets while those obtained with water at pH 7 were aggregated. This effect was reported before as an effect of the charge of the proteins that are associated with the surface of the oleosomes (De Chirico et al., 2018; Romero-Guzmán et al., 2020b; Tzen and Huang, 1992). The oleosomes extracted at alkaline conditions (Fig. 4A) after short soaking times (1 h and 4 h) showed evidence of significant aggregation and coalescence, while those extracted after longer soaking times (at 8 h, 16 h and 24 h) mostly consisted of individual oleosomes. In the case of oleosomes extracted with water at neutral pH, the different soaking times did not affect the aggregation behavior, as they remained aggregated, even when soaked for 24 h. To break the interactions holding these aggregated oleosomes extracted at neutral pH, 1 wt.% of SDS was added during the dilution of the primary extract. In Fig. 4C, we can see that the addition of SDS efficiently broke the aggre-

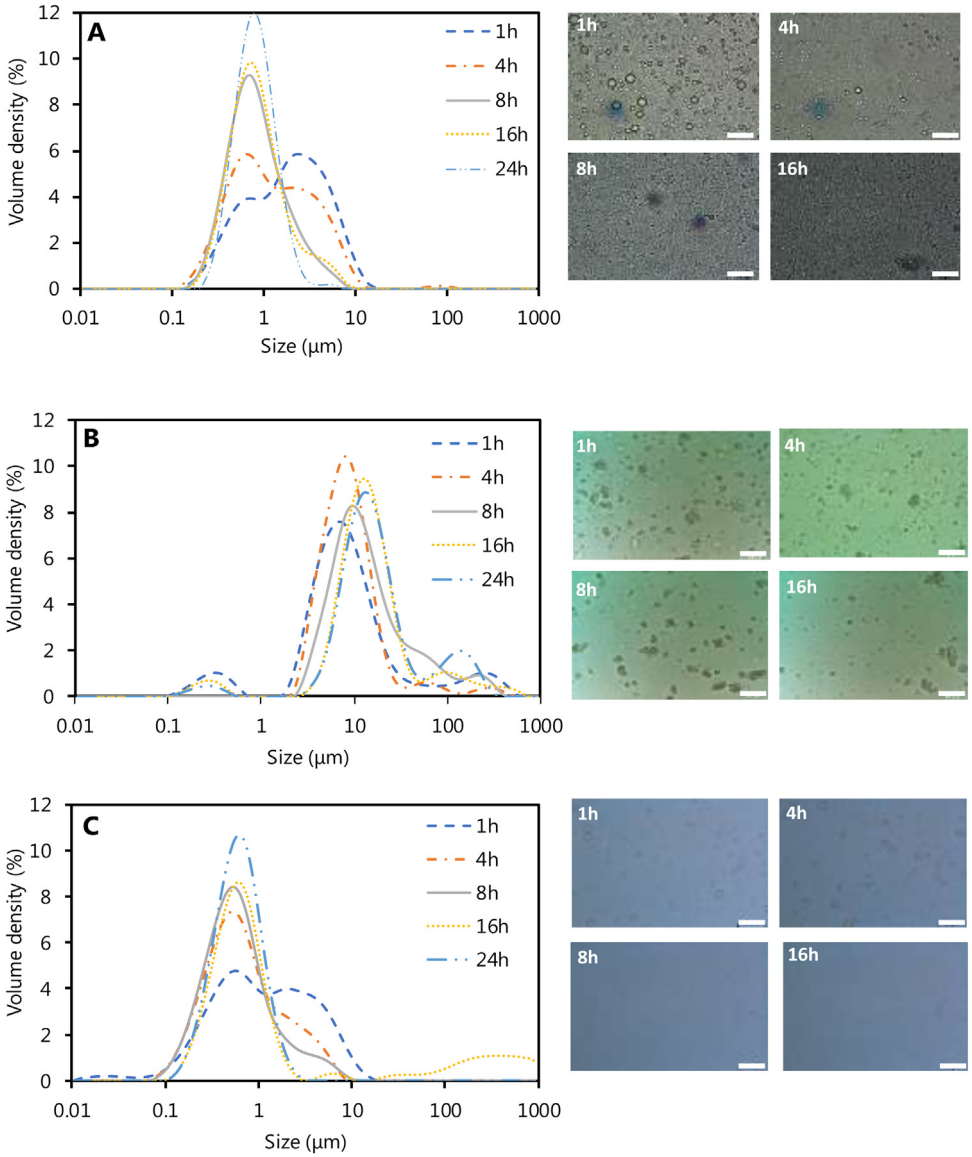

Fig. 4 – Particle size distribution and micrograph of oleosome diluted creams recovered at (A) pH 9.5 with $NaHCO_3$ 0.1 mol/L, (B) at pH 7 with H_2O and (C) at pH 7 with H_2O and 1 wt. % SDS. Scale bar 20 μm.

gates yielding individual oleosomes (Romero-Guzmán et al., 2020b). These oleosomes were like those obtained at alkaline conditions (0.1 mol/L $NaHCO_3$ at pH 9.5). Moreover, we see that the oleosomes recovered at neutral conditions after 1 and 4 h soaking was also enlarged in comparison with those recovered after 8 h or longer soaking times. Previously it was reported that the co-extracted material interacting with the oleosomes could act as a shield and protect oleosomes against coalescence during centrifugation (Nikiforidis et al., 2016; Nikiforidis and Kiosseoglou, 2009). Hence, we concluded that the enlarged oleosomes recovered at shorter soaking times at either alkaline or neutral conditions were enlarged during the extraction and not during the centrifugation step. We hypothesized that probably as the cellular material was still in glassy state during the extraction (making it more brittle), the shear forces produced during the extraction may damage the oleosomes.

Overall, while the yields of oleosomes and proteins were as high at all soaking times, the seeds soaked for less than 8 h, recovered damaged oleosomes. The incomplete hydration during short soaking times let the protein-oleosome cohesion in the cells intact, leading to brittle oleosomes, that may lose part of their native surrounding mono-layer during the extraction. At longer soaking times, all the cell material is hydrated and hence more flexible, leading to the release of intact oleosomes. To proof this hypothesis, we characterized the protein profile of pure oleosomes (those extracted at alkaline conditions) as it can be seen in Fig. 5.

Mostly the three oleosome associated proteins (Purkrtova et al., 2008): oleosin (18 kDa), caleosin (26 kDa) and steroleosin (42 kDa) are attached to the oleosome interface. However, at 1 h and 4 h there are more non-oleosome associated proteins present. These extraneous proteins are those very abundant rapeseed storage proteins, such as napin (10 kDa) (Wanasundara et al., 2016). Therefore, these results support the hypothesis that at short soaking times, oleosomes are damage and surface active extraneous material attaches to their interface, creating hybrid oleosomes (De Chirico et al., 2018).

4. Conclusion

We can conclude that the migration of the water molecules inside the seeds affects the mechanical properties of the seeds. This effect is due to the interactions of the cellulosic

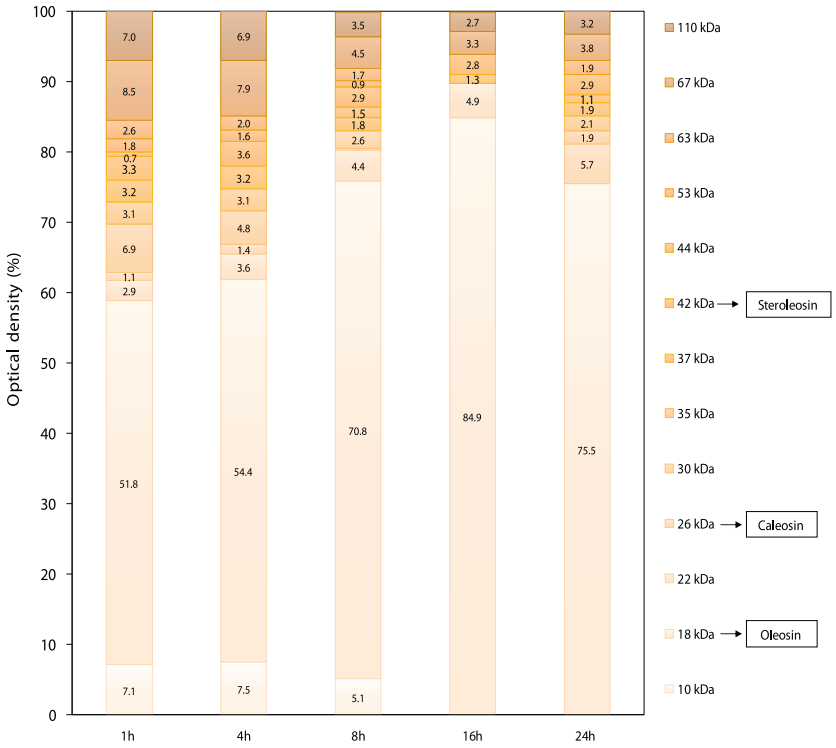

Fig. 5 – Interfacial composition of the oleosomes obtained after different soaking times, determined with SDS-PAGE densitometry.

materials with water, in combination with the solubilization and leaching of cellular material into the soaking fluid. Water diffusion, leads to solubilization of the middle lamella and extraction of solutes from the cytosol. These effects take place gradually and reach a plateau after about 8 h soaking time.

The changes in the mechanical properties and solubilization of the material are counter-effective when operating a twin-screw press. Dry material aids the friction generated inside the screws, and hence the extraction yields. Based on that statement, the short solubilization times, would favor the oleosome extraction; however, the solubilization of material happens at longer soaking times, which also favors the extraction of cellular material. Therefore, we concluded that there is a counteracting effect of these two phenomena, which results in similar oleosome and protein extraction yields at all soaking times.

The use of pH 9.5 aqueous media releases individual, non-aggregated oleosomes, while the use of water (pH 7) results on the extraction of aggregated oleosomes, even after 24 h soaking time. These aggregates are broken up when using a 1 wt.% SDS solution, showing that the aggregates are relatively easily dissociated as they are hold by electrostatic interactions. Regarding the effect of soaking time on the quality of oleosomes, it was concluded that shorter soaking times than 8 h lead to larger aggregates and damage oleosomes, with significantly more non-associated oleosome proteins bounded to their interface. These results are independent from the aqueous media used during the extraction.

Overall, we can conclude that when using twin-screw press short soaking times, such as 1 h, can be used without affecting the extraction yield. Nevertheless, at least 8 h soaking time are necessary to achieve the right mechanical properties of the seeds, which are vital to the recovery of intact oleosomes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A

Fig. A1

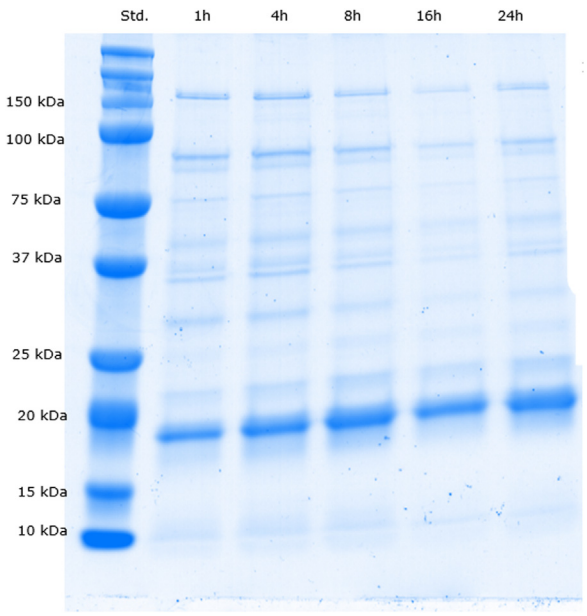

Fig. A1 – Original SDS-Page that depicts the effect of soaking time on the protein profile of the recovered creams.

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