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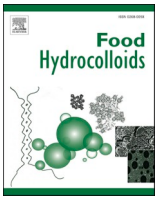

Extraction of oleosome and protein mixtures from sunflower seeds

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ABSTRACT

Oil seeds contain 10-20 wt% proteins and up to 50 wt% oil, organised in micron-sized oil droplets, named oleosomes. During oil extraction, which takes place using mechanical pressing or organic solvents or a combination of them, oleosomes are ruptured, and the oil is obtained. The oil extraction process might lead to the degradation of both the contained oil and proteins, therefore, as an effort to have a minimum impact on the quality of both, an aqueous extraction has been suggested, where oleosomes and proteins are simultaneously extracted. Oleosomes, being oil droplets themselves, can be used in emulsion-like food products, however, their extraction and purification from proteins is energy intensive. For a better understanding of the extraction and separation of oleosomes and proteins from sunflower seeds, we here explore the mass balance of oleosomes and proteins during each extraction step. Additionally, we investigate the effect of the process steps on oleosome physical stability. At the initial extract, oleosomes and proteins were at a 3:1 ratio, with an oleosome diameter of up to 10 μm. Three centrifugation steps were needed to separate proteins since the cream obtained had an oleosome/protein ratio close to 20:1. However, the removal of proteins had a significant effect on droplet coalescence, since oleosomes with a diameter up to 40–50 μm were observed. After a homogenisation step though, the oil droplets regained their initial size. Besides the effect on the physical stability of the sunflower oleosomes, the oleosome purification affected the obtaining yield, as from 87 wt% after the first extraction step, dropped to 66 wt%. By providing the mass balances during the oleosome/protein extraction from sunflower seeds, we highlight the effect of the sunflower oleosome purification steps on the obtaining yield and the role of the co-extracted storage proteins on their physical stability. Unlikely oleosomes from other sources, those derived from sunflower seeds, are prompt to coalescence when storage proteins are not present. With this insight, we provide tools for targeted sunflower oleosome and protein extraction depending on the potential applications and yield needed.

1. Introduction

A major trend in food science and industry is the creation of healthy and sustainable food products. Especially the sustainability aspect is driven by a growing global population and the high environmental stress of animal-based food products (Friel et al., 2009). As a result, alternative food ingredient sources are carefully investigated, and one such ingredient is lipids, and especially the oil droplets that comprise emulsion foods (Kim, Wang, & Selomulya, 2020). An interesting source of plant-derived oil droplets is the use of oleosomes, which are oil droplets that naturally exist in all oilseeds, like sunflower seeds (Nikiforidis, 2019).

Oleosomes have a triacylglycerol (TAG) core, which is surrounded by a monolayer of phospholipids with membrane proteins (Frandsen, Mundy, & Tzen, 2001; J. T.C. Tzen & Huang, 1992; Jason T. C. Tzen, 2012). In the conventional oil extraction process, oleosomes are disrupted to obtain the TAG core. However, the oil extraction process might lead to the degradation of both the extracted oil and remaining proteins, therefore, as an effort to have a minimum impat on the quality of both, an aqueous extraction has been suggested, where intact oleosomes and proteins are simultaneously extracted (Ntone, Bitter, & Nikiforidis, 2020).

Extracted oleosomes have diameters varying from 0.2 to 10.0 μm, depending on the source, extraction method, and environmental conditions during cultivation (Nikiforidis, 2019; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). A remarkable property of the oleosomes is

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their protective membrane due to strong interactions between the phospholipids and membrane proteins (Deleu et al., 2010), which provide high stability against coalescence (Ding, Wen, et al., 2020; Ding, Xu, et al., 2020; Ishii et al., 2017; Kapchie, Yao, Hauck, Wang, & Murphy, 2013). In addition, oleosomes can behave as an emulsifier by taking up free oil (Ishii et al., 2017), or even act as carriers by encapsulating therapeutic and flavour components (Fisk, Linforth, Taylor, & Gray, 2011; Zhang et al., 2022).

Aqueous extraction of oleosomes is possible due to the hydrophilic nature of the oleosome surface, as the polar phospholipid headgroups and hydrophilic domains of the membrane proteins are directed outwards (De Chirico et al., 2020; Iwanaga et al., 2007). The extraction takes place at an alkaline pH to increase the negative surface charge of oleosomes, as their isoelectric point (pI) is around 5.5–6.5 for various crops (i.e. sunflower seeds, rapeseed, and maize germ) (Nikiforidis et al., 2013). Under the same conditions, the storage proteins are also extracted, and as a result, oleosomes and proteins are obtained simultaneously. To separate oleosomes from the coextracted proteins, centrifugation steps are applied, and oleosomes are obtained from the upper cream layer, while proteins remain at the subnatant phase (Ishii et al., 2017; Karefyllakis, van der Goot, & Nikiforidis, 2019; Yang, Waardenburg, et al., 2021).

The extraction of oleosomes is a rather extensive process since copious amounts of water are required to remove the proteins. Such extensive oleosome extraction processes is commonly proposed, which lead to high losses of material and can also affect the integrity of oleosomes. A similar trend is seen for plant proteins, where extensive processing leads to protein isolates with high purity (>80%), but lower protein extraction yields. However, mild processing gives a higher yield with less pure protein concentrates (Loveday, 2019; Möller, Li, van der Goot, & van der Padt, 2021).

This work assesses the impact of each oleosome extraction step from sunflower seeds on oleosome purity, extraction yield and stability against coalescence. The integrity of oleosomes is investigated by performing laser diffraction measurements to measure the size of oleosomes and confocal laser scanning microscopy (CLSM) to visualise them. Understanding the various process steps contributes to the design of milder processes, which may then be targeted to obtain sunflower oleosome extracts with specific functional properties. These outcomes can further advance the industrial-scale utilisation of oleosomes, as high-end ingredients.

2. Material and methods

2.1. Materials

Organic dehulled sunflower seeds were obtained from the Notenstore (the Netherlands). Other chemicals were all used as received (Sigma-Aldrich, USA). All SDS-PAGE materials were provided by Thermo-Fisher, the Netherlands. All samples were prepared in demineralised (demi) water.

2.2. Sunflower oleosome extraction process

An overview of the sunflower oleosome extraction process is shown in Fig. 1. The extraction method was obtained from Karefyllakis et al. with several modifications (Karefyllakis, van der Goot, & Nikiforidis, 2019). Dehulled sunflower seeds (150 g) were soaked in demi-water in a 1:7 (w:w) seed-to-water ratio, and stirred with an overhead rotor for 4 h at room temperature. During stirring, the pH was adjusted to 7.8 every 30 min using 1 M NaOH. The pH was always ≤7.8, as a higher pH could induce phenol oxidation. The mixture was then blended (kitchen blender 400 W, Waring Commercial, USA) for 1 min at max speed. The obtained slurry was stirred again for 1 h, while adjusting the pH to 7.8 every 15 min. Afterwards, the slurry was filtered using a cheesecloth. This filtrate is labelled as filtered extract (FIL-extract).

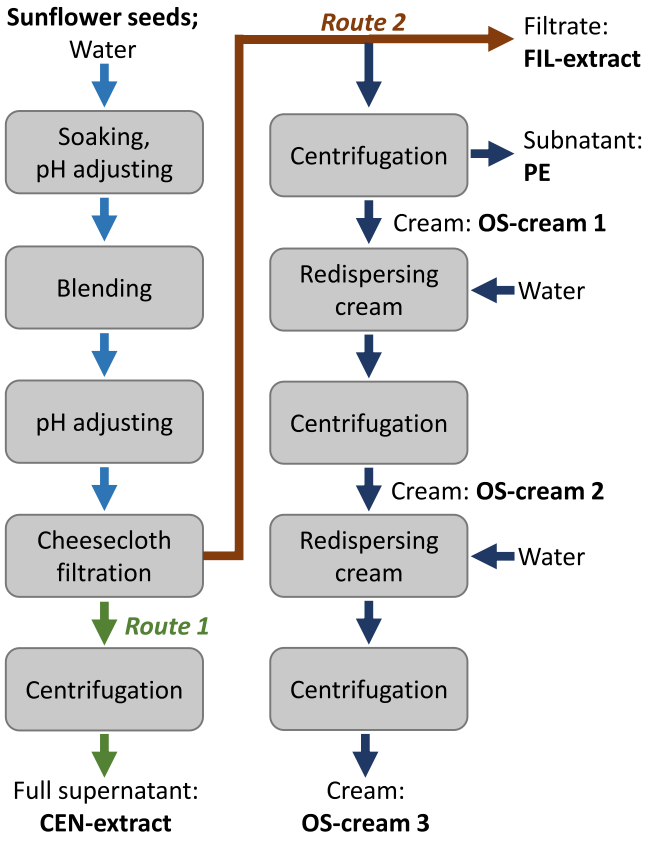

Fig. 1. Schematic overview of the oleosome extraction and washing process. CEN-extract: Centrifuged extraction; Fil-extract: Filtered extract; PE: Protein-rich extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

The FIL-extract was centrifuged at 10,000×*g* for 30 min at 4 °C. After centrifugation, three layers were obtained: a top cream layer, a protein-rich middle layer and a pellet with insoluble material. There are two possible process routes after centrifugation: 1. The collection of the full supernatant (cream layer + protein-rich subnatant) by pouring both layers out of the tube, which is called centrifuged extract (CEN-extract); 2. The separation of the cream layer and the protein-rich subnatant. The latter was performed by pouring both layers into a 170-mesh sieve. The protein-rich subnatant passes the sieve, while the cream is retained, allowing effective recovery of the cream. The protein-rich subnatant is labelled as protein-rich extract (PE), while the cream is called the first oleosome cream (OS-cream 1).

The OS-cream 1 was further purified by two additional washing steps. The OS-cream 1 was redispersed in a 1:4 cream-to-water ratio, and stirred for 30 min at room temperature. Afterwards, the mixture was again centrifuged (same conditions). The obtained cream is labelled as the second oleosome cream (OS-cream 2). The OS-cream 2 was washed once more by redispersing and centrifugation, which yielded the third oleosome cream (OS-cream 3).

2.3. Mass flow and compositional analysis

The process described above was repeated in triplicate to obtain accurate mass flow data. The dry matter content of all streams was determined by drying the sample at 105 °C for 4 h. The dry matter of each sample was determined in duplicate, and calculated using equation (1).

$$Dry\ matter\ content\ (\%) = \frac{Mass\ of\ dried\ sample\ (g)}{Mass\ of\ initial\ sample\ (g)}\ x\ 100 \quad (1)$$

The oil content of the (milled) seeds and all streams was analysed using Soxhlet extraction. All samples were dried overnight at 60 °C. About 2–5 g of material was used for oil extraction, which was performed for 16 h using petroleum ether as a solvent. The oil content of each sample was determined in duplicate. The oil content was calculated using equation 2

$$Oil\ content\ (\%) = \frac{Mass\ of\ extracted\ oil\ (g)}{Mass\ of\ dried\ sample\ (g)}\ x\ 100 \quad (2)$$

The protein content was measured in a Flash EA 1112 Series Dumas analyser (Interscience, The Netherlands). The nitrogen content of the samples was obtained and converted into protein content using a conversion factor of 5.7 (Fetzer et al., 2019). The protein content of each sample was determined in triplicate.

The dry matter, oil and protein extraction yields were determined using equation (3).

$$Yield\ (\%) = \frac{Dry\ matter, oil\ or\ protein\ extracted\ from\ 150g\ seeds\ (g)}{Dry\ matter, oil\ or\ protein\ content\ in\ 150\ g\ seeds\ (g)}\ x\ 100 \quad (3)$$

Mass flow charts were made using e!Sankey software v5 (iPoint Group, Germany).

2.4. Protein composition

The protein composition of the creams was studied using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were diluted in water to a protein concentration of 0.2% (w/w). Samples under non-reducing conditions were mixed with NuPAGE LDS sample buffer, followed by a heating step at 70 °C for 10 min. After the heating step, the samples were cooled to room temperature, and aliquots of 15 μL were loaded on a 4–12% (w/w) BisTris gel. A marker with a molecular weight range from 2.5 to 200 kDa was included. The electrophoresis step was performed at 200 V for 30 min, and the obtained gel was stained with SimplyBlue Safestain. The stained gel was scanned using a gel scanner.

2.5. Homogenisation of oleosome extracts

Before homogenisation, all creams were diluted to 10% (w/w) dry matter using demi water. The FIL-extract and CEN-extract fractions were homogenised as such. The pH of all samples was adjusted to the original extraction pH of 7.8 if necessary, using 1M NaOH (usually by 0.1–0.3 pH). All samples were pre-sheared with an Ultra-turrax (IKA, Germany) at 8,000 rpm for 1 min. Afterwards, the samples were homogenised with a high-pressure homogeniser (GEA, Niro Soavi NS 1001 L, Italy) for 5 passes at 500 bar on the first step, and 50 bar on the second step.

2.6. Droplet size determination

The droplet size was determined using static light scattering in a Bettersizer S3 Plus (3P Instruments, Germany). The refractive indices of the continuous and dispersed phases were set at 1.330 and 1.469, respectively. The droplet sizes of the full extract and (diluted) cream were measured before and after the homogenisation step. The unhomogenised creams were diluted to 10% (w/w) based on dry matter and stirred for 30 min before analysis. In addition, droplet flocculates were broken up by mixing the samples with a 1% (w/w) sodium dodecyl sulphate (SDS) solution in a 1:1 (v/v) ratio. The samples were carefully mixed by rotation and incubated for 5 min, followed by droplet size analysis. All measurements were performed in triplicate.

2.7. Confocal laser scanning microscopy

The samples were studied using confocal laser scanning microscopy to obtain a visual representation of the systems. All samples were diluted to 10% (w/w) dry matter and stirred for 30 min. Proteins and the oil phase were fluorescently labelled by adding 5 μL 0.1% (w/v) Nile Red and 10 μL 0.1% (w/w) Fast Green per mL sample. Aliquots of 150 μL sample were pipetted onto a glass bottom μ-Slide with 8 wells (Ibidi, Germany). The analysis was performed using a Leica TSC SP8x confocal laser microscope (Leica Microsystems Inc., Germany), equipped with a white laser and HyD detector). The slides were analysed with a 63x magnification and a water immersion lens (refractive index = 1.20). Nile Red was excited at 488 nm, and the emission was measured between 500 and 600 nm, and Fast green was excited at 633 nm, and the emission was measured between 650 and 750 nm.

2.8. Statistical analysis

The extraction process was performed at least three times to obtain three individual repliates of each sample, which were analysed separately. Analysis of variance was performed on the obtained data using a one-way analysis of variance (ANOVA) and Duncan's test at $p \leq 0.05$ to evaluate the statistical significance between samples. SPSS 25.0 software (SPSS, USA) was used to run the tests.

3. Results and discussion

3.1. Purity and yield during the extraction process

The moisture, oil and protein content and extraction yields of various oleosome extracts are shown in Table 1. The sunflower seeds used for oleosome extraction have an oil and protein content of 57.9 and 19.5% (w/w) based on dry matter (DM), respectively. An overview of the extraction process is shown in Fig. 1. First, the seeds were soaked and blended at pH 7.8. Normally, a higher pH would lead to higher negative surface charges on the oleosomes and of the storage proteins, giving a better separation between both components due to the higher electrostatic repulsion. However, a more alkaline pH accelerates the oxidation of phenol, mainly chlorogenic acid. At alkaline conditions, the phenol chlorogenic acid can turn into an oxidised form with quinone groups (Karefyllakis, Salakou, Bitter, van der Goot, & Nikiforidis, 2018). These quinones are highly reactive, and can form covalently bound complexes with proteins, thereby yielding a green colour (Pringent, Voragen, Visser, van Koningsveld, & Gruppen, 2007; Wildermuth, Young, & Were, 2016). After soaking, the seeds are blended to disrupt the cell walls, leading to the extraction of the oleosomes into the water phase due to their hydrophilic surface (J. T.C. Tzen & Huang, 1992).

After the extraction phase, the slurry is filtered using a cheesecloth and gives a filtered extract (FIL-extract). This FIL-extract has a DM content of 10.6% (w/w), and an oil and protein content of 66.0 and 20.4% (w/w) based on DM, respectively. The filtration step retains most of the insoluble material, as shown by a lower DM extraction yield of 76.6 than oil yield of 87.3%. The next step is a gravitational separation by centrifugation. This centrifugation step (10,000×g at 30 min) might not be suitable for large scale production of oleosomes due to high energy usage. Upscaling would be possible using continuous centrifuges (at lower speeds) and decanters, which would require more attention in future studies. The centrifugation step in this work resulted in three layers: a small pellet containing insoluble material, a protein-rich middle layer (known as subnatant), and an oleosome-rich cream layer. Here, two extraction routes were explored: 1) the joint recovery of the full supernatant (subnatant + cream layer) and 2) the separate recovery of both the subnatant and the cream layer.

The first route yields a centrifuged extract (CEN-extract) with comparable purity and extraction yields as FIL-extract. The oil content based on DM increased from 66.0 to 68.9% (w/w), while the oil yield remained

Table 1
The moisture, oil and protein content, and dry matter (DM), oil and protein extraction yield of sunflower seeds and the various extracts. The yield was calculated based on equation (3). Values are shown as mean ± standard deviation (n = 3). Means within a row with the same superscript are not significantly different ($p > 0.05$). Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; PE: Protein-rich extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

	Moisture content (% w/w)	Content based on DM (% w/w)		Extraction yield (%)		
		Oil	Protein	Total DM	Oil	Protein
Seeds	6.6 ± 0.2^{a}	57.9 ± 0.9^{a}	19.5 ± 0.3^{a}	100	100	100
FIL-extract	89.6 ± 2.1^{d}	66.0 ± 0.6^{b}	20.4 ± 0.7^{a}	76.6 ± 0.4^{a}	87.3 ± 0.5^{a}	77.1 ± 1.0^{a}
CEN-extract	89.3 ± 1.7^{d}	68.9 ± 1.3^{c}	18.1 ± 0.1^{b}	73.3 ± 0.5^{b}	87.2 ± 2.1^{a}	68.1 ± 1.8^{b}
OS-cream 1	$34.2.1 \pm 2.8^{b}$	87.6 ± 1.3^{d}	8.1 ± 0.4^{c}	55.2 ± 0.6^{c}	79.1 ± 1.0^{b}	20.7 ± 0.2^{c}
OS-cream 2	$41.4 \pm 3,5^{c}$	92.8 ± 0.9^{e}	5.8 ± 0.5^{d}	43.5 ± 1.2^{d}	70.7 ± 0.9^{c}	10.8 ± 0.2^{d}
OS-cream 3	$36.5 \pm 2,5^{b,c}$	94.6 ± 0.5^{f}	4.6 ± 0.4^{e}	40.2 ± 1.1^{e}	66.0 ± 1.7^{d}	9.6 ± 0.4^{e}
PE	$97.1 \pm 1,5^{e}$	9.4 ± 0.5^{g}	57.6 ± 0.4^{f}	18.0 ± 0.3^{f}	3.1 ± 0.2^{e}	50.2 ± 0.6^{f}

constant. A small amount of insoluble material and proteins is removed, leading to a lower DM and protein yield for CEN-extract compared to FIL-extract. In the second route, we obtained the cream, labelled as first oleosome cream (OS-cream 1), and a protein-rich subnatant, labelled as protein extract (PE). The OS-cream 1 has a substantially higher oleosome purity than CEN-extract, as the oil content increased to 87.6% (w/w), while the protein content decreased to 8.1%. The PE is high in protein with a protein content of 50.2% (w/w) based on DM, but still contains a minor amount of oleosomes (9.4%). These could be smaller oleosomes that remain in the subnatant after centrifugation or contamination from the OS-cream 1 upon separation. Comparable oil and protein contents were obtained, when using a comparable extraction method for rapeseeds (Ntone et al., 2020). In this study, separation of the OS-cream 1 and PE led to a slight loss of oleosomes, as the combined oil extraction yield of OS-cream 1 and PE is 82.2%, while that of CEN-extract is 87.2%.

In the following steps, the OS-cream 1 was washed to remove proteins and other solutes by redispersing the cream in water, followed by centrifugation. The cream layer was recovered and labelled as second oleosome cream (OS-cream 2). Finally, the OS-cream 2 was washed once more, yielding the third oleosome cream (OS-cream 3). Additional washing steps led to more pure oleosome extracts, as the oil increased up to 94.6% (w/w) based on DM for OS-cream 3. On the other hand, more extensive oleosome purification led to lower oil extraction yields of 70.7 and 66.0% for OS-cream 2 and OS-cream 3, respectively. Oleosomes might remain in the subnatant or in the centrifugation tubes during recovery.

Another method to assess the oleosome purity is by evaluating the protein composition using gel electrophoresis. A scan of the gel (under non-reducing conditions) containing all oleosome extracts is shown in Fig. 2. Here, three areas are highlighted: an area between 46 and 67 kDa, 16–19 kDa and 8–14 kDa. The group proteins with molecular weights (Mw) from 46 to 67 kDa are most likely the sunflower globulins, known as helianthins. This protein exists as a trimer around neutral and slightly alkaline pH(Gonzalez-Perez & Vereijken, 2007), and has a wide range of Mw for the subunits, varying from 46 to 66 kDa (Žilic et al., 2010). Another group of storage proteins has Mw varying from 8 to 14 kDa, which are likely the sunflower albumin proteins (González-Pérez, Vereijken, Van Koningsveld, Gruppen, & Voragen, 2005). The two aforementioned areas are prominent on the lane of the PE, which is expected for the storage proteins.

Additionally, the intensity of these bands slightly reduces for oleosome extracts with higher oil purities, suggesting the removal of storage proteins upon washing. However, the storage protein bands are present in all oleosome extracts, even in the most extensive purified OS-cream 3. A high alkaline pH is required to obtain a high negative net charge for proteins and oleosomes, and we could not perform the extraction at such an alkaline pH due to potential phenol oxidation. As a result, storage

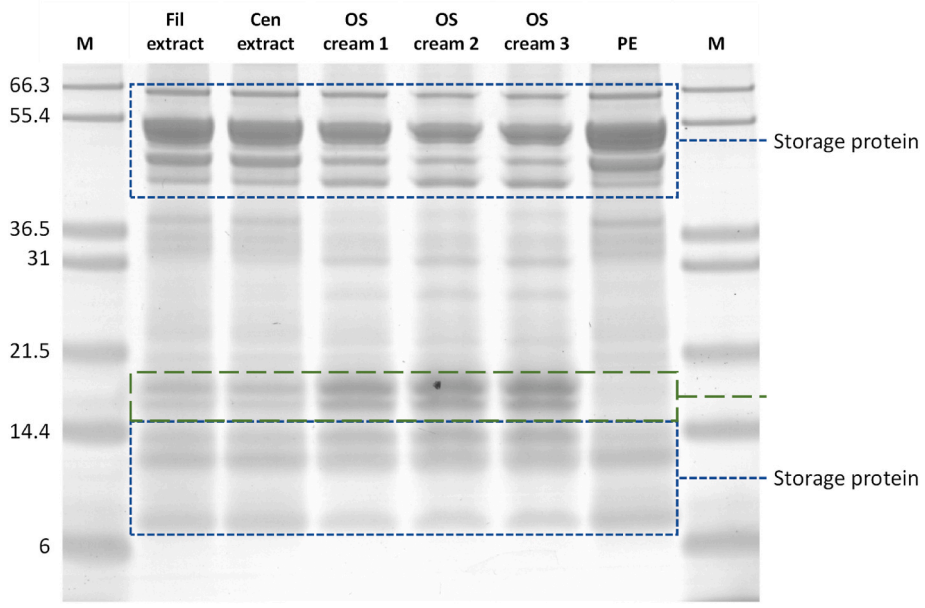

Fig. 2. SDS-PAGE profiles (non-reducing conditions) of the various oleosome-rich extracts. A molecular weight marker (M) is included, and the corresponding molecular weights (kDa) are indicated on the outer lanes. Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; PE: Protein-rich extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

protein may remain non-covalently bound to oleosomes during the extraction, leading to storage proteins in the creams, which will thus be visible on the SDS-PAGE gel scan.

The final group of proteins has bands at 16 and 19 kDa, which are likely oleosome membrane proteins. These bands become more prominent for more purified oleosome extracts and are also nearly absent in the PE. Sunflower oleosome membrane proteins were previously reported to have Mw varying from 15 to 26 kDa (Alexander et al., 2002). The band at 19 kDa is most likely oleosin, as an Mw of 19.2 was previously reported for this protein (UniProtKB/Swiss-Prot, 2022). In short, oleosome extracts with high purity can be obtained by multiple washing steps, but storage proteins seem to remain due to a limited extraction pH.

3.2. Oleosome extraction: purity versus yield

The extraction yields are graphically represented in Fig. 3, which illustrates the dry matter flows for oil, protein and other components for an extraction of 1,000 kg (dry matter) dehulled sunflower seeds. As mentioned in previous sections, the least processed FIL-extract gives the highest mass flows for oil (506 kg) and proteins (150 kg). In route 1, additional removal of insoluble material by centrifugation yielded a comparable oil mass flow of 505 kg. In route 2, the proteins and oleosomes are co-extracted, leading to two streams: the protein-rich extraction (PE) and the cream layer (OS-cream 1). About 104 kg of the protein is extracted in the side-stream PE. The OS-cream 1 already has a higher oil content compared to CEN-extract, which increases further by performing washing steps, yielding OS-cream 2 and OS-cream 3. More extensive processing also requires more water, as shown in the mass flow diagram in Figure S1 in the SI. The production of FIL-extract, CEN-extract, PE and OS-cream 1 requires 7,000 kg water, when starting with 1,000 kg of sunflower seeds. Addition washing steps further increase the water usage to 10,352 and 13,320 kg for OS-cream 2 and OS-cream 3, respectively.

The impact of processing on the oleosome purity and oil yield is shown in Fig. 4A by plotting the oil content (based in DM) of the various extracts over oil yield. Here, a clear relationship arises, where the production of oleosome extracts with higher oil contents leads to lower oil extraction yields, and vice versa. For proteins, we show lower protein contents and yields upon more extensive washing, which is expected as more proteins are removed with the washing steps. The most mildly processed FIL-extract and CEN-extract (route 1) have the highest oil yields of 87.2–87.3 wt%, but a relatively low oil content (purity) of 66.0–68.9 wt%. Such a mild process would be suitable if the presence of other components is acceptable. This includes presence of storage proteins, minerals, sugars, but also phenols. The FIL-extract and CEN-extract would only be stable at a more acidic pH or in the presence of antioxidants.

These non-oleosome components can be removed in route 2, yielding OS-cream 1 and PE. The PE is a side-stream with 50.2% protein, and about 9.4% oil, indicating the minor presence of oleosomes. PE can be further purified by, for instance, dialysis or diafiltration to remove small components, such as minerals, sugars and phenols. This method was previously proven effective for a PE from rapeseed, as the protein content increased from 39.5 to 65.1% after diafiltration over a 5 kDa cut-off, while the phenol content reduced from 6.3 to 2.7%. The diafiltrated rapeseed PE also possessed good functional properties in foaming, emulsifying and gelation (Ntone, Kornet, et al., 2022; Ntone, Qu, et al., 2022; Yang, Berton-Carabin, Nikiforidis, van der Linden, & Sagis, 2021). Therefore, our sunflower PE could have a high potential as a functional protein extract, especially after additional processing. Sunflower proteins have shown promising functionality in previous studies (González-Pérez et al., 2005; Gonzalez-Perez & Vereijken, 2007; Rodríguez Patino et al., 2007).

Another advantage of OS-cream 1 over CEN-extract and FIL-extract is its water content, as the majority of the water is separated into the PE. The OS-cream 1 could be utilised as a dense oil content system (58% w/w oil). The oil purity can be improved from 87.6 to 94.6% (w/w) by two additional washing steps, leading to an OS-cream 3 with high oil purity. A drawback is the use of more water (Fig. 4B), as 34.9 kg of water is now required to extract 1 kg oil in OS-cream 3, while extracting 1 kg oil of a more mildly purified FIL-extract, CEN-extract or OS-cream 1 only require 13.8–15.3 kg of water, which is less than half. Nonetheless, oleosome extracts OS-cream 2 and OS-cream 3 could be suitable as systems with high oil purity.

A final stream from oleosome extraction is the fibre-rich fraction, which is present as the cake after the filtration step and the pellet after the centrifugation step. If we would take the production of CEN-extract

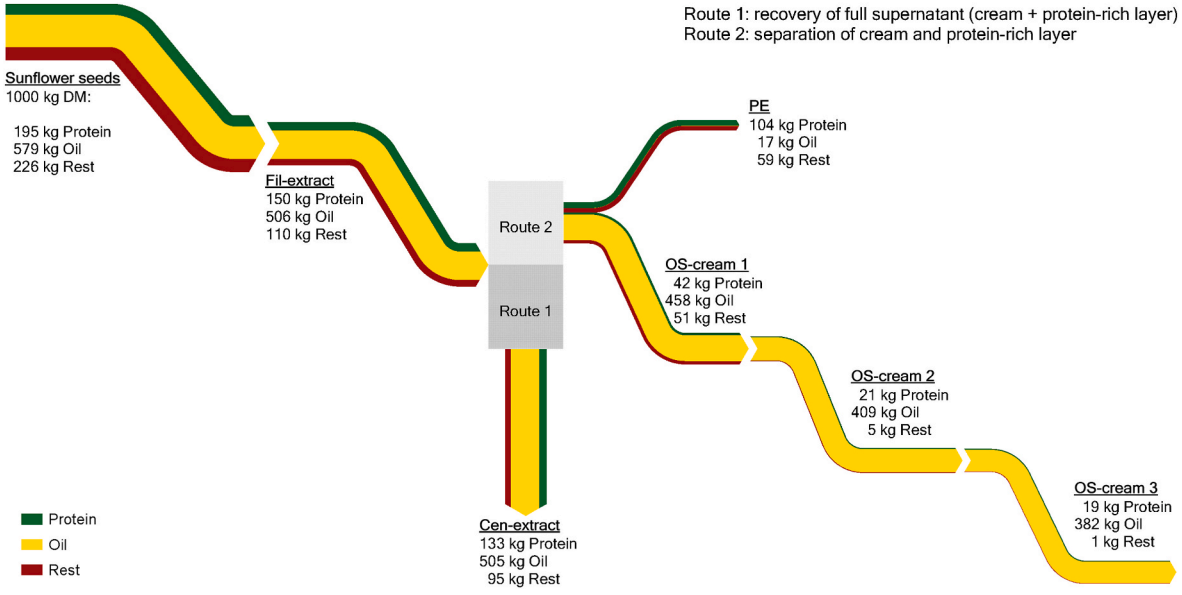

Fig. 3. Mass flow diagram of the protein, oil and rest streams during oleosome extraction and washing steps. A total of 1,000 kg of sunflower seeds (based on DM) was chosen as the starting material. The green, yellow and red stream show the amount of protein, oil and rest, respectively, in the dry matter. The weight of each component is shown for each stream. The water streams are not shown in this diagram, but can be found in the SI. Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; PE: Protein-rich extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

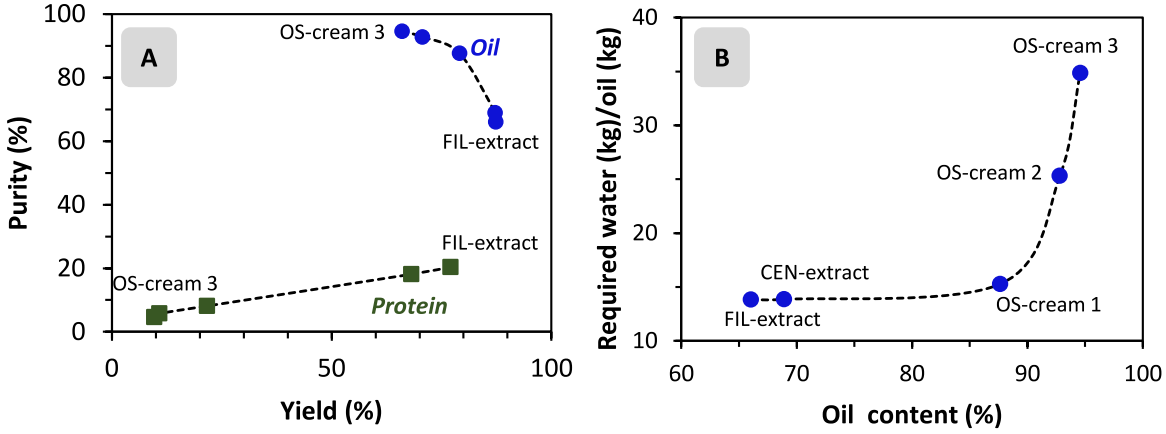

Fig. 4. (A) The oil (blue spheres) and protein (green squares) content (wt%) based on dry matter over yield of the various oleosome-rich extracts. (B) The oil content (% w/w) based on DM over the required water during extraction (kg) to extract one kg of oil. Each of the marker is labelled with the corresponding extract. The line is added as a guide to the eye. Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; PE: Protein-rich extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

from 1,000 kg sunflower seeds as an example; roughly 62 kg of protein, 74 kg of oil and 131 kg of other components (mostly fibres) enters the side-streams, as a fibre-rich cake. Such a cake can be of use, as shown by Karefyllakis et al. (Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019), where a similar sunflower seed fibre-rich cake was shown to be able to stabilize oil droplets that are stable against coalescence. In addition, such fibre-rich side-stream could be valuable in bakery product application (Bhise, Kaur, Ahluwali, & Thind, 2014; Gültekin Subaşı, Vahapoğlu, Capanoglu, & Mohammadifar, 2021; Martins, Pinho, & Ferreira, 2017).

In short, more extensive processing leads to more pure oleosomes, but lower oil extraction yields. The extraction intensity can be tuned based on the desired application of the extracts. The extracts (FIL- and CEN-extract) can be utilised if the non-oleosome components are acceptable. A purer and denser cream can be obtained as OS-cream 1, which yield a protein-rich side-stream (PE) that can be processed into a functional protein isolate. Finally, oleosome extracts with high purity (MP- and OS-cream 3) can be obtained by additional washing steps.

3.3. Oleosome droplet functionality

The droplet size distribution is shown in Fig. 5. The FIL-extract had a size distribution ranging from 1.0 to 21.2 μm, with a peak size of 3.8 μm and a $d_{3,2}$ of 4.2 μm. CEN-extract showed a lower peak height in the similar range with a small shoulder peak ranging up to 40.1 μm, leading to a $d_{3,2}$ of 4.8 μm. The shoulder peak increased with more extensive processing, up to 111.4 μm for OS-cream 3. The $d_{3,2}$ increased to 4.9, 5.0 and 5.4 μm for OS-cream 1, OS-cream 2 and OS-cream 3, respectively.

More extensive oleosome extraction led to larger droplet sizes, which was visualised using confocal laser scanning microscopy (CLSM). In Fig. 6, the proteins and lipids are stained in green and red, respectively. In the images of the CEN-extract, mainly single droplets are present, but some small flocculates occur. The continuous phase around the droplets was high in protein, which is expected, as both oleosomes and proteins are abundantly present in the CEN-extract. Also, a bright green halo is present around the oleosomes, suggesting the accumulation of proteins around the oleosomes. Such behaviour is expected, as the storage proteins may interact with the oleosomes due to the (relatively) low extraction pH. The halo is also present in more purified oleosome extracts OS-cream 1 and OS-cream 3. In addition, two major observations can be made when extracting OS-cream 1 and OS-cream 3. The single droplet size seems to slightly increase, which is most prominently visible

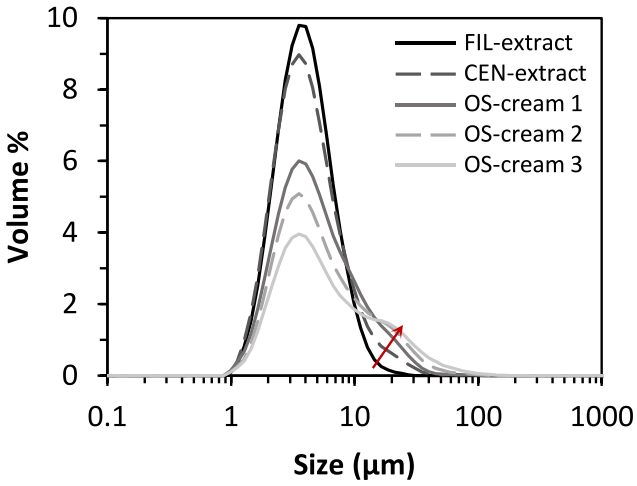

Fig. 5. The droplet size distributions of the oleosome-rich extracts at their extraction pH (7.8). The CEN-extract were analysed as such, while the creams were diluted to 10% (w/w) based on DM prior and stirred for 30 min to analysis. Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

for OS-cream 3. Also, OS-cream 3 showed significant droplet flocculation, and this flocculation is the likely explanation for the increasing shoulder peak in Fig. 5. Centrifugation helps to obtain pure sunflower oleosomes, but it might induce oleosome flocculation due to extensive oleosome interactions. The present storage proteins can prevent extensive oleosome interactions and their coalescence. After each centrifugation step, storage proteins are removed and the coalescence rate increases. (CEN-extract < OS-cream 1 < OS-cream 2 < OS-cream 3), as shown in Fig. 5.

Flocculates of oleosomes can be broken up using sodium dodecyl sulphate (SDS). The sizes of the resulting de-flocculated emulsions are shown in Fig. 7A, with five overlapping size distributions for all oleosome extracts with sizes between 0.7 and 6.7 μm, and a $d_{3,2}$ around 1.9 μm. We did see evidence of the larger single droplet sizes that were observed in the CLSM images for OS-cream 3. This observation suggests that these larger droplets be a very small part of the droplet population.

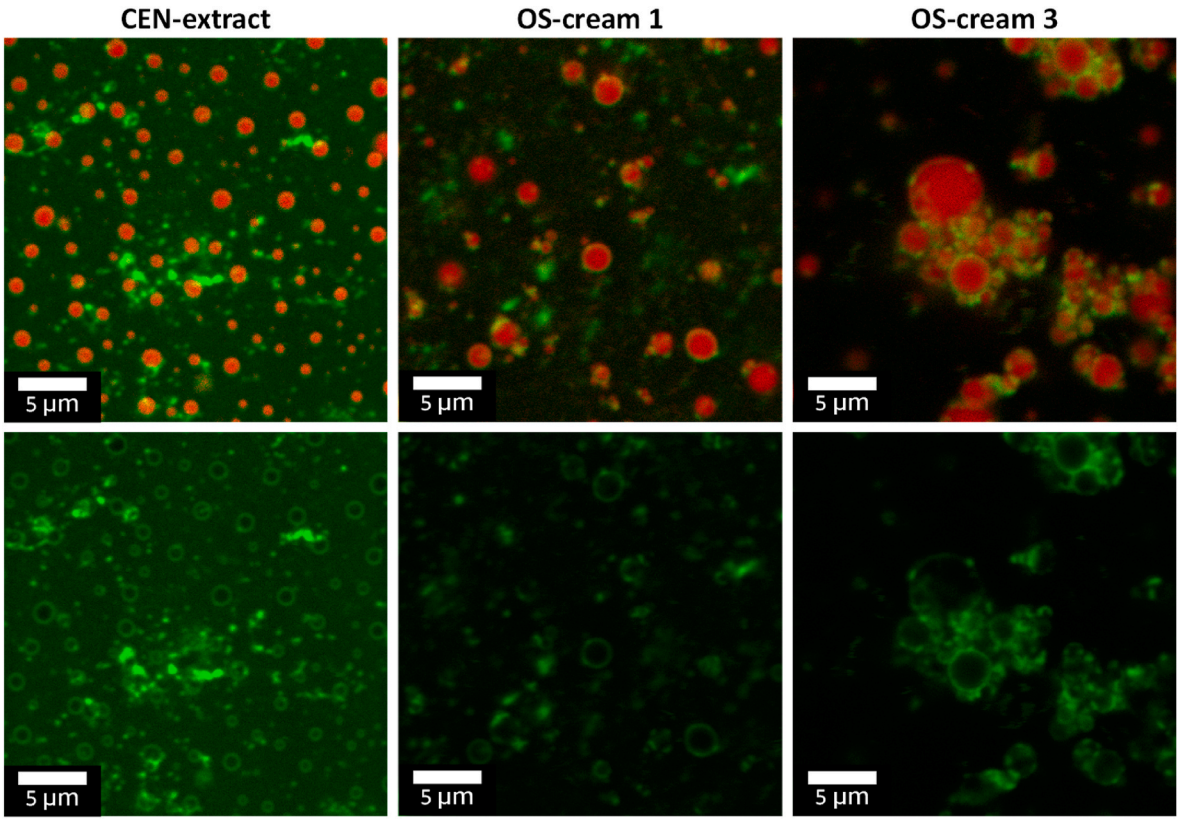

Fig. 6. CLSM images of CEN-extract, OS-cream 1 and OS-cream 2 at their extraction pH (7.8). Proteins are stained with Fast Green dye and are shown as green in the images. Lipids are stained with Nile Red dye and are shown as red in the images.

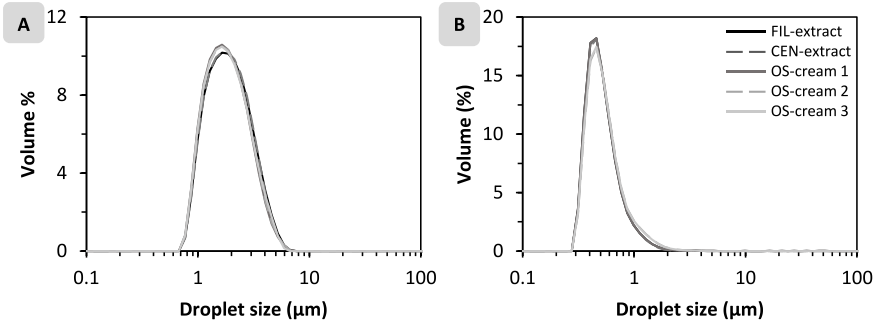

Fig. 7. (A) The droplet size distributions of the oleosome-rich extracts at their extraction pH (7.8). Single droplet sizes were shown after the break-up of flocculates using SDS, and (B) droplet sizes of homogenised oleosome-rich extracts were shown. Abbreviations: CEN-extract: Centrifuged extract; Fil-extract: Filtered extract; OS-cream 1: first oleosome cream; OS-cream 2: second oleosome cream; OS-cream 3: third oleosome cream.

In this case, these large droplets could be the result of free oil after the oleosome break-up and coalescence, as a small amount of oil was observed on top of the cream layer for OS-cream 2 and OS-cream 3 after centrifugation.

The flocculated and could be broken up using high-pressure homogenisation. In addition, coalesced droplets in the MP and LP cream can be reduced in size to increase their stability against creaming. Therefore, all oleosome extracts were homogenised, and the droplet size distribution is shown in Fig. 7B. Also here, the five curves overlap with sizes ranging from 0.3 to 2.1 μm and a $d_{3,2}$ around 0.5 μm. These are single droplets, as the addition of SDS led to similar size distribution curves (data not shown). Homogenisation of oleosomes was previously shown to lead to smaller droplets (Bernat, Cháfer, Rodríguez-García, Chiralt, & González-Martínez, 2015). The smaller homogenised single droplets than non-homogenised droplets would lead to a major increase in oil-water surface area that need to be stabilised by emulsifiers. For FIL-extract and CEN-extract, we could expect the interface stabilisation of the free proteins in the bulk. On the other hand, HP-, MP- and OS-cream 3 is substantially lower in free protein. In this case, the oleosome membrane might be diluted upon homogenisation, which allows the stabilisation of the newly formed surface area.

In short, extensive processing led to increased droplet flocculation and minor droplet breakage and coalescence. On the other hand, similar droplet sizes were obtained after homogenisation. The functionality of the oleosomes does not seem to depend on the extent of purification.

4. Conclusion

Sunflower oleosomes were extracted at an increasing degree of oil purity. More extensive purification led to higher oil contents in the form of olesomes, but lower oil extraction yields, due to losses in the process. Also, extensive purification requires more than twice the amount of water. The degree of processing only had a minor impact on droplet size, as droplet flocculation occurred for more extensively processed oleosome extracts. On the other hand, no differences in droplet size were found after homogenisation. Therefore, the extraction intensity only has a minor impact on oleosome functionality.

Here, we show the advantages and disadvantages of oleosome extracts after various purification steps. A diluted oleosome extract can be obtained with oleosomes, proteins and other solutes. The cream and protein-rich liquid layer can be separated, yielding a dense oleosome cream and a mildly extracted sunflower protein concentrate. Additional washing steps can be performed when oleosomes with high purity are required, but one should be aware of the requirement for more resources, such as water. Additionally, in contract to rapeseeds or other oleosome sources, purified sunflower oleosomes was not stable against coalescence. However, after applying a high pressure homogenisation step oleosomes regained their original size without further coalescence. This brings us to our key message on oleosome extraction; oleosome extracts with different oil content, purity and yield can be produced by varying the extraction method. These findings allow us to effectively design extraction processes for oleosomes to obtain high-end lipid ingredients, which are present as stabilised oil droplets in the seeds.

CRediT author statement

Jack Yang: Conceptualisation, Methodology, Investigation, Validation, Visualisation, Writing – Original Draft; Umay S. Vardar: Methodology, Investigation; Remko M. Boom: Visualisation, Writing – Review & Editing; Johannes H. Bitter: Writing - Review & Editing; Constantinos V. Nikiforidis: Conceptualisation, Methodology, Supervision, Writing – Review & Editing.

Declaration of competing interest

The authors have declared that no competing interest exist. This manuscript has not been published and is not under consideration for publication in any other journal. All authors approve this manuscript and its submission to Food Hydrocolloids.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.109078.

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