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The emulsifying ability of oleosomes and their interfacial molecules

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ABSTRACT

Oleosomes are natural oil droplets, present in all organisms and abundant in oilseeds. After their aqueous extraction from oilseeds, they can be directly utilized as oil droplets in food, cosmetics and all types of oil-in-water emulsion systems. However, to expand the potential uses of oleosomes as green ingredients and to valorize oilseeds as efficient as possible, we explored their emulsifying ability. Oleosomes were extracted from rapeseeds, and 10.0 wt% oil-in-water emulsions were created after homogenization with 0.5–6.0 wt% oleosomes, and the droplet size of the emulsions and their structure was measured by laser diffraction and confocal laser scanning microscopy (CLSM), respectively. The emulsion with an oleosome concentration lower than 1.0 wt% gave unstable emulsions with visible free oil. At oleosome concentrations at 1.5 wt% or higher, we obtained stable emulsions with droplet sizes between 2.0 and 12.0 μm. To investigate the role of the oleosome interfacial molecules in stabilizing emulsions we also studied their emulsifying and interfacial properties (using drop tensiometry) after isolating them from the oleosome structure. Both oleosomes and their isolated interfacial molecules exhibited a similar behavior on the oil-water interfaces, forming predominantly elastic interfacial films, and also showed a similar emulsifying ability. Our results show that oleosomes are not stabilizing the oil-in-water emulsions as intact particles, but they provide their interfacial molecules, which are enough to stabilize an oil-water surface up to about 2 times bigger than the initial oleosome surface. The understanding of the behavior of oleosomes as emulsifiers, opens many possibilities to use oleosomes as alternative to synthetic emulsifiers in food and pharma applications.

1. Introduction

A significant number of materials, commonly used in daily activities by humankind, like the creams and pastes used in foods and cosmetics are dispersions of oil droplets in water. To stabilize the oil droplets and prevent their coalescence, interfacial active molecules are used, which are adsorbed on the oil droplet interface. Due to the efforts to develop more sustainable ecosystems, there are efforts to substitute the synthetic and animal surface active ingredients currently used with biobased and sustainable alternatives [11]. Plant-derived proteins and phospholipids are at first site great alternatives as sustainable emulsifiers, as they are abundant in oilseeds, like soy and rapeseeds, however their extraction from the complex oilseed matrix is not trivial, and it is questionable whether their use provides a significant gain in terms of resources used [3]. The extraction of proteins and also phospholipids from oilseeds involves the use of solvents, like hexane, derived from petrochemicals, but besides that many process steps are involved to obtain the proteins and phospholipids that perform well in terms of emulsification properties [7].

However, after a closer look at the colloidal structures available in oilseeds, one could find that there are oil droplets (oleosomes) present, which have sizes similar to those used in foods and cosmetics. The core of the oil droplets is comprised by triacylglycerols (vegetable oil) and are stabilized by phospholipids in combination with highly interfacially active interfacial proteins, called oleosins [10,12,18]. The oleosome interface is approximately 2.0–4.0 wt% of the oleosome total mass, and

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the ratio between phospholipids and oleosins at the interface is about 1:1 [13,15,24]. The oleosome interfacial molecules are selected by nature and create an interface around the oleosome lipid core that prevents their the coalescence [14].

Another function of oleosomes as reported in literature is to accumulate free fatty acids present in the cellular environment and the ability to expand in size when enzymes locate at the oleosome interface and synthesize further TAGs in the oleosome core. Both processes result into the growth of oleosomes in size, indicating that their interfacial molecules can emulsify additional oil and rearrange around the newly created larger oleosome interface [17].

The inherent physical stability of oleosomes as provided by their interfacial molecules has received quite some attention [1,5,6,16,20], concluding that this property, make them good candidates as biobased alternative for oil-in-water emulsions. However, in the current conventional oil extraction from seeds, the oleosome structure is disrupted, where the oil core is extracted into a bulk of plant oil and the oleosome interfacial molecules mostly remain in the side stream.

Given the above, to expand the potential use of oleosomes as alternative ingredients to stabilize oil-in-water emulsions, and to exploit the advanced properties of their interface, there is a need to understand further the ability of the oleosomes and their interfacial molecules to emulsify oil. To our knowledge, there is little research addressing the ability of oleosomes as emulsifiers and the role of the oleosome interfacial molecules in this function.

The oleosomes, as a whole droplet, is not expected to be surface active, as several works show the mechanism of interface stabilization is the result of oleosomes disruption at the interface. As a result, the surface-active membrane proteins and phospholipids will spread over the interface, thus decreasing interfacial tension and forming an interfacial layer [25,29]. Finally, the surface activity of the oleosome membrane proteins was proven by Plankensteiner et al. [18], where a pure oleosin (87 wt% protein) was highly surface active and could form stiff oil-water interfacial layers. It is crucial to understand the role of the interfacial material at the oleosome interface.

Therefore, in this research we aim to explore the ability of oleosomes as a whole and of only their interfacial molecules to stabilize oil and create new emulsified oil droplets. We did that by homogenizing oleosomes with 10 wt% bulk oil at different oleosome concentrations. The same experiment was repeated where we used only the oleosome interfacial molecules, after separating them from the oleosome structure. The microstructure of the formed emulsions was studied using light scattering in combination with confocal laser scanning microscopy (CLSM), while drop tensiometry was used to get an indication of the potentially formed interfacial structure. With this work we provide insights into the emulsifying properties of oleosomes and their interface, which can be extracted from oilseeds or oilseed cakes and used to stabilize oil droplets with advanced functionality in terms of physicochemical stability.

2. Materials and methods

2.1. Materials

Untreated Alize rapeseeds (stored at − 18 °C; 18.7 wt% protein and 41.9 wt% oil based on dry matter) were used for the extraction of the oleosomes [16]. Rapeseed oil was kindly provided by Nutricia Research B.V. All other chemicals were of analytical grade and obtained from Sigma Aldrich (Steinheim, Germany). All samples were prepared with deionized water.

2.2. Methods

2.2.1. Oleosome extraction

Purified oleosomes were obtained using the method reported by de Chirico et al. [4] with some adaptations; dehulled rapeseeds were soaked at room temperature in 0.1 M sodium bicarbonate at pH 9.5 (pH was adjusted using 0.5 M NaOH) in a ratio of seeds to buffer of 1:7 w/w for 4 h under continuous stirring (RW 20 digital stirrer, IKA ®, Staufen, Germany) to ensure proper mixing. The high alkaline pH is necessary to obtain high interface charges for oleosomes and proteins, resulting in a more effective removal of the proteins from the oleosomes. Also, the buffer is necessary to retain the pH at 9.5 during the processes. After soaking, the seeds were blended at maximum speed for 90 s (Philips Avance HR2093 kitchen blender, Eindhoven, the Netherlands). To remove the solids, the mixture was passed through a cheesecloth. The filtrate was centrifuged (30 min; 10,000 *g*; 4 °C; SORVALL Legend XFR centrifuge by Thermo Fischer SCIENTIFIC, Waltham, USA) in 250 mL centrifuge tubes to remove extraneous proteins and fibers. After centrifugation, the top layer (cream) was collected. The cream layer was spread over a filter paper (Whatman ®, grade 4) to absorb most of the remaining liquid. The cream was resuspended in fresh extraction medium (1:4 w/w) and centrifuged under the same conditions. After the second centrifugation step, the cream layer was collected in the same manner and resuspended in deionized water (1:4 w/w). A third and final centrifugation step followed, and the cream layer containing purified oleosomes was again collected and stored at 4 °C until further use.

To separate the oleosome interfacial molecules, oleosomes were freshly extracted with the method described above and dispersed in a 1:10 (w/v) ratio with water. The dispersion was then frozen using liquid nitrogen, and freeze-dried.The freeze-dried oleosomes were defatted by adding hexane in a 1:10 (w/v) ratio and stirred for 2 h at room temperature. The mixture was filtered over filtration paper, and the defatting steps were repeated another three times. The final defatted powder which consisted of the oleosome interfacial molecules was dried for 16 h in a desiccator and then stored frozen until further use.

2.2.2. Preparation of emulsions

The purified oleosomes were used as emulsifiers to stabilize 10.0 wt % (rapeseed) oil emulsions. The oleosomes were dispersed in deionized water to a final concentration of 0.5–6.0 wt% (standardized at the oleosome concentration in the cream, based on dry matter) at pH 7.0 (pH adjusted with 0.1 M HCl). The oleosome dispersion was first sheared using a disperser (Ultra-Turrax, IKA®, Staufen, Germany) at 8000 rpm for 30 s. Next, rapeseed oil was slowly added to the dispersion and sheared for 1 min at 10,000 rpm. The formed coarse emulsion was further processed with high-pressure homogenizer (GEA®, Niro Soavi NS 1001 L, Parma, Italy) for 5 cycles at 300 bars. The experiments and additional analyses were performed in duplicates.

Emulsions stabilized using the oleosome interfacial molecules were prepared by dispersing the extracted molecules in water at a concentration of 0.053 % (w/w). The sample was stirred for 4 h at room temperature, and hydrated overnight at 4 °C. On the following day, the sample was treated in an ultrasonic water bath (Ultrasonic Cleaner, VWR International, Radnor, PA, USA) for two cycles of 5 min with a 5 min pause, and the pH was adjusted to 7.0. Afterwards, we followed the same steps we used to add rapeseed oil to oleosomes, as mentioned in the previous paragraphs. The final oil and oleosome interfacial molecule concentration were 6.95 % (w/w) and 0.05 % (w/w), respectively.

2.2.3. Characterization of emulsions

2.2.3.1. Particle size distribution analysis. The particle size distribution of the emulsions stabilized by oleosomes or by oleosome interfacial molecules, was determined by laser diffraction using a Bettersizer S3 Plus (3 P Instruments GmbH & Co. KG, Odelzhausen, Germany). The measurement settings were adjusted to a refractive index of 1.46–1.47 and a density of 0.91 g/cc for rapeseed oleosomes. The stirring speed of the small volume sample dispersion unit was set to 1600 rpm.

To determine the individual droplet size, 1.0 wt% sodium dodecyl sulfate (SDS) was added to the samples in a ratio of 1:1 (v/v) to disrupt

possible aggregates. SDS is a low molecular weight surfactant that can break protein hydrophobic interactions [19], and it was added to our system to prevent bridging between the lipid droplets.

The measurements were reported as volume-weighted diameter ($d_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$) where n_i is the number of droplets with a diameter of d_i. The average values are a result of measurements of two individual samples (n = 2), and are reported as average value ± the standard deviation.

2.2.4. Characterization of emulsion microstructure

The structure of the emulsions was studied using a Confocal Laser Scanning Microscope (Leica SP8-SMD microscope, Leica Microsystems, Wetzlar, Germany) with a 63 × magnification water immersion lens. Nile Red (0.01 wt% in ethanol) was used to stain the lipid phase in a ratio dye solution to sample of 1:200 (v/v) (i.e. 5 μl dye solution to 1 mL emulsion) and DiO (0.01 wt% in ethanol) was used to stain the phospholipid phase in a ratio dye solution to sample of 1:200 (v/v). The samples were excited at λ = 488 nm, and the emission was captured between 500 and 600 nm for both Nile Red and DiO, using a white light laser source. The images were analyzed using the Leica Application Suite X software.

2.2.5. Characterization of interfacial properties

The interfacial properties of oleosomes and of their interfacial molecules were studied using an automated drop tensiometer (ADT Tracker, Teclis, Longessaigne, France). Solutions with 0.1 % (w/w) oleosome or 0.002 % (w/w) of oleosome interfacial molecules were prepared at pH 7.0 using a 10 mM phosphate buffer. The pendant drop method was used, where a hanging (water) droplet of the solutions was created at the tip of a needle in a continuous oil phase to create an oil-water interface. Stripped rapeseed oil was used, and we refer to our previous work for the method of purification [27]. A water droplet with a surface area of 30 mm^2 was created, and analyzed for 2 h at 20 °C. The contour of the droplet was fitted with the Young-Laplace equation, which gave the interfacial tension.

Here, γ (water) is the interfacial tension of the pure oil-water interface, equal to 29.0 mN/m.

After 2 h the droplets were subjected to oscillatory dilatational deformations in an amplitude sweep, where the deformation amplitude was increased at a fixed frequency. The range of deformations varied from 5.0 % to 30 % at a frequency of 0.02 Hz. For each deformation amplitude, five consecutive cycles were performed, followed by a pause of 50 s.

The obtained sinusoidal interfacial tension signal was analyzed using a Fast Fourier transform, as described elsewhere [22]. The outputs are the elastic and viscous surface dilatational moduli (E_d' and E_d") . An alternative analysis was performed by constructing Lissajous plots of the interfacial pressure ($\Pi = \gamma - \gamma_0$) versus deformation, as previously shown by Kempen et al. [22]. Here, γ_0 is the interfacial tension of the non-deformed interface. All measurements were performed at least in triplicate.

3. Results and discussion

3.1. Emulsions stabilized with oleosomes

Oleosomes were initially purified from dehulled rapeseeds. The resulting oleosome cream contained about 25.0–30.0 wt% moisture, and the remaining 70.0–75.0 wt% consisted mainly of oleosomes, while about 2.6 % protein is left [20]. The proteins present in the oleosome cream are most likely oleosins, as shown by a SDS-PAGE scan oleosomes, with a major band around 18 kDa [20]. This SDS-PAGE shows the near absence of rapeseed storage proteins. At pH 9.5, the oleosomes and storage proteins have a high negative surface charge, resulting in strong repulsion of the components, finally, leading to an effective removal of the storage proteins. The obtained oleosomes were then dispersed in deionized water at a wide concentration range of 0.5–6.0 wt% at pH 7.0. This concentration range was chosen based on preliminary experiments to define the minimum amount of oleosomes needed to form emulsion droplets.

The obtained oleosomes had an initial size distribution ranging from 0.5 to 10.0 μm ($d_{4,3}$ = 1.6 μm). We then added oil (10.0 wt%) to the dispersion and applied high-speed shearing, followed by high-pressure homogenization. To investigate the ability of oleosomes to stabilize free oil, we first determined the droplet size of the emulsions. Fig. 1**a** shows the particle size distribution of the extracted oleosomes and 10.0 wt% oil-in-water emulsions as a function of oleosome concentration. To show whether the size distribution is related to individual droplet size or to aggregates, we provide at Fig. 1b the size distribution directly after adding SDS, which has shown to break the aggregated droplets. The size distribution of the oleosomes found in this work was similar to what has been previously reported [4,16,20,28]. At oleosome concentrations ≤ 1.0 wt%, free oil was present in the emulsions; therefore, we were unable to include the size distribution graphs at these concentrations.

At 1.5 wt% oleosome concentration, the size of emulsion droplets ranged from 10.0 to 500.0 μm, with a $d_{4,3}$ of 94.4 ± 9.4 μm (Fig. 1**a**). After adding SDS (Fig. 1**b**) to break possible aggregates present, the size distribution range was smaller, corresponding to an individual droplet size of 11.8 ± 0.8 μm. This result indicates that emulsion droplets were already formed at 1.5 wt% oleosome concentration, but were extensively aggregated. In a 1.5 wt% oleosome emulsion, we estimate that about 0.03 wt% of oleosome interfacial molecules are present, as the phospholipid and structural proteins at the interface represent about 2.0 wt% of the total oleosome mass. This would suggest that a concentration of 0.03 wt% oleosome interfacial molecules is sufficient to stabilize 10.0 wt% of added free oil into oil droplets, especially as when adding 1.0 % oleosomes to 10.0 wt% gave free oil in the beaker glass. In addition, we calculated the surface area increase from an original oleosome to the oil droplets.

By increasing the oleosome concentration to 2.0 wt% and 3.0 wt%, the aggregate size was reduced to 63.7 ± 8.5 μm and 55.1 ± 1.8 μm, respectively (Fig. 1**a**), while the individual droplet size also decreased to 7.2 ± 0.3 μm and 4.5 ± 0.1 μm respectively (Fig. 1**b**). Further increase of the oleosome concentration to 6.0 wt% led to even further reduction of the droplet aggregate size to 31.3 ± 3.4 μm, with an individual droplet size of 2.2 ± 0.0 μm, approaching the natural oleosome size. These results indicate that as the oleosome concentration increased, and thus increased the number of available interfacial active molecules from the oleosome interface, better coverage of the emulsion droplets was achieved. As more interfacial active molecules are present, smaller droplets are formed with a denser interface, resulting in a reduction in the strength of the hydrophobic interactions between the droplets and increasing electrostatic repulsion, leading to a decrease in droplet aggregation.

3.1.1. Emulsion microstructure – studied by CLSM

To confirm the measurements given by laser diffraction analysis and further visualize the microstructure of the emulsions, we used confocal laser scanning microscopy (CLSM). To do so, the emulsion droplet lipid core was dyed using Nile Red (Fig. 1c, top row). The CLSM images of the emulsions stabilized with 1.0 wt% oil (Fig. 1**c**) confirmed the presence of free oil. At oleosome concentrations of 3.0 and 6.0 wt%, it was observed that droplets with a diameter below 10 μm were formed, while they tend to cluster into aggregates. The droplet aggregation shown upon static light scattering (Fig. 1**a & b**) of samples before and after addition of SDS is confirmed by the CLSM images. To understand whether the molecules from the oleosome interface were equally distributed around the formed oil droplets, we stained only the areas rich in phospholipids using DiO (Fig. 1c, bottom row). With an oleosome concentration of 3.0 and 6.0 wt%, the phospholipid interface appeared to be more homogeneous around the droplet interface, with the fluorescence intensity of the interface increasing as the oleosome

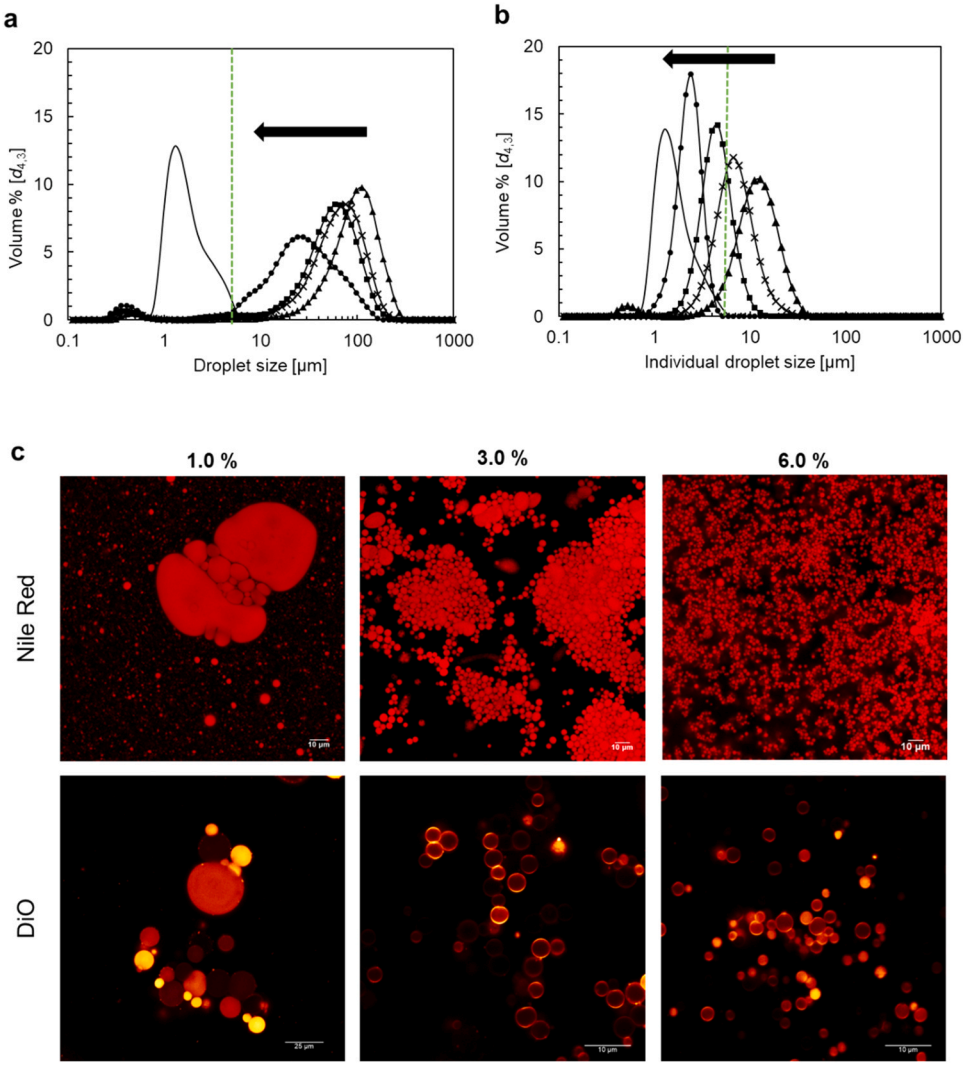

Fig. 1. a) Droplet size distribution of 10.0 wt% oil-in-water emulsions at 1.5 wt% (triangle symbol), 2.0 wt% (x symbol), 3.0 wt% (squared symbol) and 6.0 wt% (circle symbol) oleosome concentrations showing a) the emulsion droplet aggregate size and b) the emulsion individual droplet size (after the addition of SDS). Purified oleosomes are shown as continuous line. c) CLSM images of emulsions at different oleosome concentrations stained with Nile Red (top row) and DiO (bottom row). The oil droplets shown in panel 'c' correspond to the droplet sizes shown in panel 'a'.

concentration increased.

3.1.2. *Emulsions stabilized by isolated oleosome interfacial molecules*

To further confirm the stabilization mechanism and the role of the oleosome interface on emulsion droplet formation, we isolated the molecules (proteins and phospholipids) of the oleosome interface and used them to stabilize 10.0 wt% oil-in-water emulsions. Fig. 2 shows the individual droplet size of the emulsion stabilized with isolated oleosome interfacial molecules at the same concentration as in 6.0 wt% oleosome (0.12 wt%). For comparison, the size distribution of the emulsions stabilized with 6.0 wt% oleosomes was included in the figure. The individual droplet size of the emulsion stabilized by the oleosome interfacial molecules has a similar size distribution as the 6.0 wt% oleosome-stabilized emulsion, ranging from 0.5 to 10.0 μm, with an individual droplet size ($d_{4,3}$) of 2.8 ± 0.0 μm. This result clearly shows that the ability of oleosomes to stabilize free oil and act as an emulsifier is attributed to the interface-active molecules present on the oleosome interface (i.e. phospholipids and structural proteins).

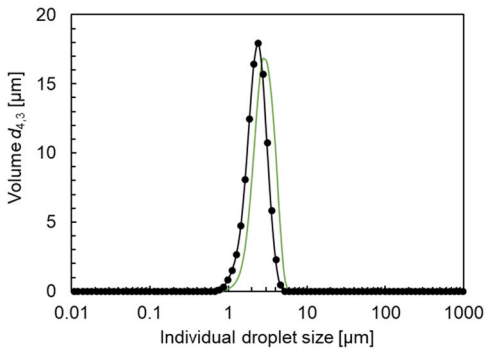

Fig. 2. Individual droplet size of emulsions stabilized with 6.0 wt% oleosomes (circle symbol) and with oleosome interfacial molecules (continuous green line) at the same oleosome interfacial molecules to oil concentration.

3.2. *Interfacial properties of oleosomes*

3.2.1. *Interfacial activity of oleosomes*

Effective stabilization of the oil-water interface is crucial in emulsion stabilization. Therefore, we have determined the oil-water interface stabilizing properties of oleosomes and the extracted interfacial molecules by measuring the interfacial activity and mechanical properties using surface dilatational rheology. Fig. 3**a** shows the interfacial tension increase over time of oleosomes and their isolated interfacial molecules. Also here, we assume that 0.02 wt% of the oleosomes is the interface, which was used to correct for the interfacial molecule concentration, so, 0.1 wt% olesomes and 0.002 wt% isolated interfacial molecules were used for analysis. Oleosomes had a lag phase of 190 s, where the interfacial tension remained zero. After this point, the interfacial tension

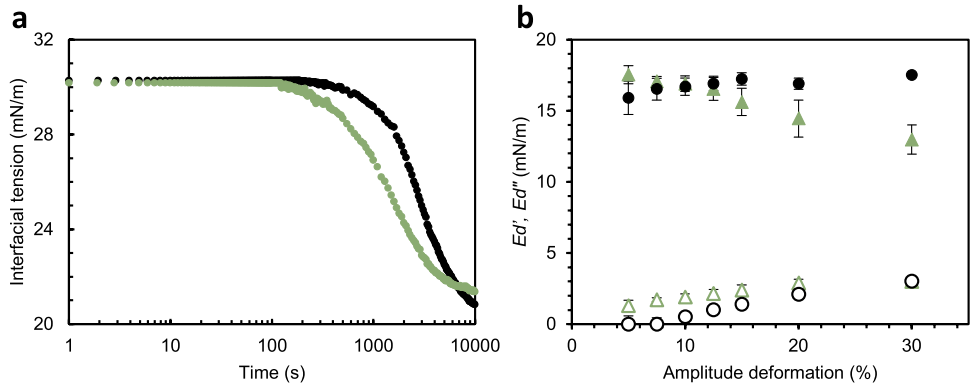

Fig. 3. a) Interfacial tension as a function of time of oleosomes (black circle) and oleosome interfacial molecules (green triangle) at 0.1 and 0.002 wt%, respectively. b) Dilatational elastic modulus (E_d': filled symbol) and viscous modulus (E_d'': hollow symbol) of oleosome (black circle) and oleosome interfacial molecules (green triangle) as a function of deformation amplitude at a constant oscillatory frequency of 0.02 Hz.

rapidly increased to 9.5 mN/m after 10,800 s of adsorption. The oleosome interfacial molecules exhibited a slightly lower lag time of 122 s, followed by a fast increase up to 8.9 mN/m. Here, we demonstrate a comparable adsorption behavior of oleosome and their isolated interface, indicating that the interfacial molecules of the oleosomes are responsible for the surface activity.

The slow initial phase of oleosome adsorption was also shown in previous work for air-water interfaces [25,26,29]. Waschatko et al. used Brewster angle microscopy on a Langmuir trough to demonstrate the diffusion of soybean oleosomes to the interface [25]. Upon reaching the interface, oleosomes may rupture, leading to the spreading of the oleosome interfacial molecules (i.e. phospholipids and structural proteins) and the TAG core. Here, the interfacial tension did not increase in the diffusion phase, but the interfacial tension increased rapidly upon oleosome rupture. Oleosomes may not rupture directly upon reaching the air-water interface, as intact ones were previously found for oleosomes from rapeseed [29]. The result is an interfacial film with TAG- and interfacial molecule-rich regions, and a low amount of intact oleosomes. We expect a similar adsorption behavior and droplet rupture of oleosomes at the oil-water interface. The interfacial composition might differ, as the TAG-rich regions are not expected to remain at the interface, as the oils are expected to merge with the oil phase. The type of interfacial films formed by oleosome and oleosome interfacial molecules were further explored by performing surface dilatational deformations.

3.2.2. Interfacial dilatational rheology

Fig. 3**b** shows the interfacial dilatational moduli versus amplitude of deformation of an amplitude sweep, where the deformation was gradually increased at a fixed oscillatory frequency. The oleosome-stabilized interface showed elastic moduli (E_d') values ranging from 16.0 to 17.6 mN/m. These E_d' values are (nearly) independent of amplitude of deformation, which is often typical for weaker and stretchable interfacial films, and which was also previously shown for oleosome-stabilized oil-water interfaces [29]. Such behavior was expected based on the explanation in the previous paragraph, where we assume an interface dominated by the oleosome interfacial molecules.

The interfacial film stabilized by the oleosome interfacial molecules showed markedly different E_d', with a value of 18.7 ± 1.4 at 5 % deformation, followed by a gradual decrease at higher deformations, ending with an E_d' of 12.6 ± 1.0 mN/m. These amplitude-dependent E_d' values indicate disruption of the interfacial microstructure upon larger deformations. Such behavior is expected for viscoelastic solid-like interfacial layers, where strong in-plane interactions are present between the adsorbed oleosome interfacial molecules.

3.2.3. Lissajous plots

In order to analyze the interfacial structure and interaction upon oscillatory deformations, we constructed Lissajous plots. In previous work, Lissajous plots were proven as a valuable supplementary tool to evaluate the mechanical properties of interfacial films [2,21,30], as the surface dilatational moduli do not capture the non-linearities of the surface stress response. These non-linearities can be analyzed when plotting the surface pressure against deformation. We refer to previously published work for a more extensive discussion on Lissajous plots [22].

Fig. 4 shows the Lissajous plots of interfacial films stabilized by oleosomes or isolated oleosome interfacial molecules at various deformations. The Lissajous plot of oleosomes at 5 % and 10 % deformation (Fig. 4**a–b**) are narrow and symmetric plots, suggesting a predominantly elastic response. At higher deformations (Fig. 4**c–d**), slightly wider plots can be observed, indicating an increase in viscous dissipation. In general, the Lissajous plots of the oleosome-stabilized interfaces remain fairly narrow, while the plots of the interfaces stabilized by oleosome interfacial molecules show wider plots, especially at deformations above 5 % (Fig. 4**f–h**). The more open plots of the interfaces stabilized by oleosome interfacial molecules suggest higher energy dissipation at larger deformations, resulting from the earlier mentioned microstructural disruption.

Another major difference is the plots at 30 % deformation, where the Lissajous plot of the interface stabilized by the oleosome interfacial molecules (Fig. 4**h**) reached a less negative surface pressure (− 4.7 mN/m) at the end of the compression cycle compared to oleosomes (− 6.9 mN/m, Fig. 4**d**). The same plot shows a slight degree of strain softening at the end of the compression cycle, as evident from the sudden change in slope (indicated by the arrow in Fig. 4h). This type of strain softening behavior in compression was also observed in other systems, and previously attributed to three phenomena: 1) expulsion of interface stabilizer from the interface into the bulk phase, 2) buckling of the interface, or 3) interfacial structures starting to slide over each other, i.e. multilayer formation [23]. A slight strain softening in compression was also observed for air-water interfacial films stabilized by the oleosome interfacial molecules [29], where the behavior was mainly attributed to the expulsion of phospholipids into the bulk upon (extensive) compression. On the other hand, we should not exclude structural breakdown and sliding of the microstructure, as the oleosome interfacial molecules seem to exhibit in-plane interactions at the oil-water interface, as shown by the strong amplitude-dependent moduli (Fig. 3**b**). Buckling is not expected, as this generally occurs for extremely stiff or glass-like structures, such as escin-stabilized interfaces [8,9].

By using Lissajous plots, we have demonstrated to formation of a weaker and more stretchable interfacial layer for an oleosome-stabilized oil-water interface, compared to an interface stabilized by oleosome interfacial molecules. The latter, formed a stiffer and solid-like layer, which showed microstructural breakdown at higher deformations. For both interfaces, we expect interfacial films that are dominated by the

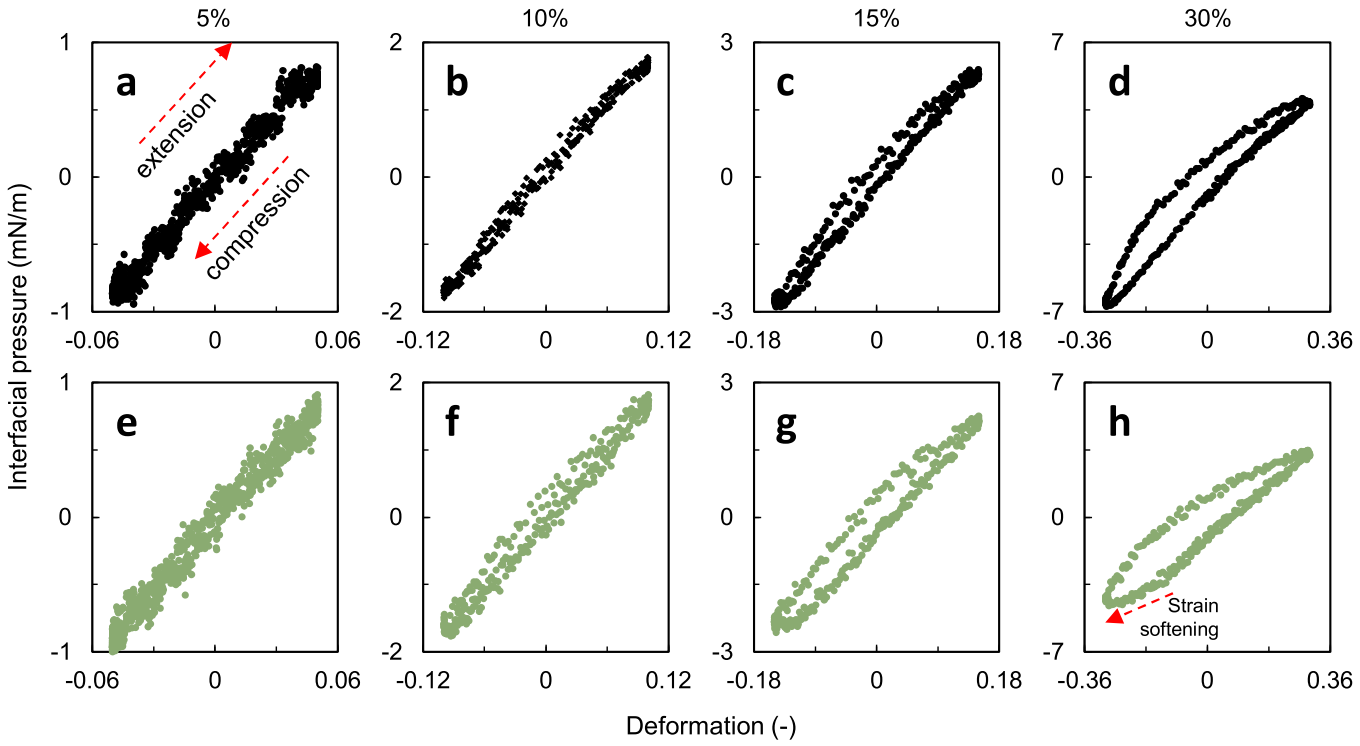

Fig. 4. Lissajous plots of interfacial films stabilized by oleosome (black, panel a–d) and oleosome interfacial molecules (green, panel e–h) at 5 %, 10 %, 15 % and 30 % deformation amplitude.

oleosome interfacial molecules (i.e. phospholipids and structural proteins). In summary, oleosomes are able to provide surface-active material that can stabilize the oil-water interface.

4. Conclusions

In this study, we showed the emulsifying ability of oleosomes, which are able to take up free oil and form stable emulsions. Emulsions with 10.0 wt% free oil could be effectively stabilized by $>$ 1.0 wt% oleosomes, leading to the smallest average droplet sizes of 2.2 μm. At 1.0 wt % or lower concentrations, we observed ineffective oil incorporation in the oleosomes, with large free oil droplets, as shown by CLSM. In addition, we proved that the interfacial molecules around the oleosomes are mainly responsible for the oil droplet stabilization in the oleosome-based emulsions. The oil-water interfacial properties were also evaluated, where the oleosomes found to be surface-active and able to stabilize an oil-water interface. Using about 0.03 wt% of isolated oleosome interfacial molecules (amount of phospholipids and proteins on oleosome interface) was enough to stabilize the oil droplets. These concentrations are much lower (about 10 times) compared to the ones commonly used for protein-stabilized emulsions and highlight how nature has efficiently designed the oleosome interface to stabilize free oil. In summary, oleosomes are natural oil droplets from plant seeds, which have the potential to act as natural emulsifiers. These natural emulsifiers can potentially be implemented in food or pharmaceutical applications, alleviating the sustainability impact of synthetic emulsifiers.

CRediT authorship contribution statement

E. Ntone: Conceptualization, Methodology, Investigation, Validation, Visualization, Writing – original draft. **J. Yang:** Methodology, Investigation, Validation, Visualization, Writing – original draft. **J.H. Bitter:** Writing – review & editing. **L.M.C. Sagis:** Writing – review & editing. **C.V. Nikiforidis:** Conceptualization, 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 the Journal of Colloids and Surfaces B: Biointerfaces.

Data Availability

Data will be made available on request.

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