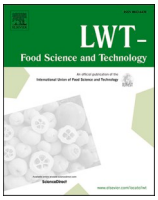
LWT - Food Science and Technology 189 (2023) 115551

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt



Thermal treatment improves the physical stability of hemp seed oleosomes during storage

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ARTICLE INFO

Keywords:
Hemp seeds
Oleosomes
Extraction
Thermal treatment
Stability

ABSTRACT

Oleosomes are the intracellular vesicles that store oil in oil-bearing seeds. By extracting them intact from the seed cells, an emulsion is obtained that does not require the addition of stabilizers and could be used in foods, cosmetics and pharmaceutical products. We investigated the physicochemical properties of hemp seed oleosomes. Oleosomes were extracted with an alkaline aqueous solvent at an extraction yield of 27.3 ± 2.2 wt%. The fresh oleosomes were physically stable at pH values between 2.0 and 12.0, at ionic strengths up to 500 mM and at temperatures up to 100 °C. However, after 14 days of storage, the extracts showed extensive microbial growth. To prevent the observable microbial growth a treatment of the oleosomes at 80 °C was applied. This treatment did not change the physicochemical properties of the oleosomes. Therefore, this treatment opens the possibility of storing oleosomes extracts for longer times before using them in a range of potential applications in food, cosmetics and pharmaceuticals.

1. Introduction

Hemp (*Cannabis sativa* L.) is the main species in the Cannabaceae family. Its tenacious vitality makes it suitable for different growth environments and widely growing all over the world (Rupasinghe et al., 2020). Due to its high fiber content, hemp has been extensively used by humankind to make clothes, paper, and other products, and in recent decades has emerged as a source of nutraceuticals (Di Mola et al., 2021). In recent years, many researchers have found that hemp is not only a fast-growing short-rotation plant but also could remediate contaminated soils and efficiently convert atmospheric CO_2 to biomass (Adesina et al., 2020; Finnan & Styles, 2013). As hemp cultivation expands, hemp seeds are a significant waste of hemp fiber production (Callaway, 2004; Farinon et al., 2020; Leonard et al., 2020). Hemp seeds contain lipids (25–35 wt%) and proteins (20–25 wt%) that could be extracted and used in food, cosmetics and pharmaceutics (Farinon et al., 2020). To achieve a circular economy, we must make the best use of all potential sources of functional materials.

The triacylglycerols (TAGs) in hemp seeds contain over 90 wt% polyunsaturated fatty acids (PUFAs), with a good balance between ω-6 and ω-3 unsaturated fatty acids (3:1) (Garcia et al., 2021; Lopez et al., 2021). The TAGs are located in the seeds in natural micron-sized oil vesicles, called oleosomes, which are separated by a phospholipid monolayer and proteins. The monolayer of oleosomes provides them with extraordinary stability against coalescence and also oxidation of the unsaturated fatty acids (Abdullah, Weiss, & Zhang, 2020; Ding et al, 2018; Nikiforidis, 2019). Oleosomes can be extracted from various seeds and remain stable against coalescence using an aqueous extraction method (Lan et al., 2020; Ntone et al., 2023; Yang et al., 2023; Zhao, Xu, & Liu, 2022). Due to they are naturally formed lipid droplets, oleosomes are used in emulsion-type formulations (Tan & McClements, 2021). It is however not clear how stable hemp seed oleosomes are outside their natural seed cell tissue, especially during processing, formulation and storage into products that are based on liquid emulsions.

We here therefore investigate the properties and stability of hemp seed oleosomes extracted by the aqueous extraction procedure, as functions of facing different environmental stresses. Hemp seed oleosomes were characterized for their proximate compositions (moisture, lipids and proteins contents), particle size and surface charge. This study presented a successful approach for extracting purified oleosomes from hemp seeds and also provided crucial insights into the stability of hemp seed oleosomes, which are essential to their potential use in food, cosmetic, or pharmaceutical products.

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https://doi.org/10.1016/j.lwt.2023.115551
Received 19 June 2023; Received in revised form 11 November 2023; Accepted 14 November 2023
Available online 16 November 2023

2. Materials and methods

2.1. Materials

Hemp seeds were provided by Botaneco Inc. (Calgary, Canada) and stored in a dark container at −20 °C until further use. Milli-Q water was used for preparing all solutions. Petroleum ether (40–60 °C PEC grade) was purchased from Actu-All Chemicals B.V. (Oss, The Netherlands). All other chemicals used in this work were of analytical grade and were purchased from Sigma Aldrich (St Louis, MO, USA).

2.2. Oleosome extraction

Oleosomes were extracted from hemp seeds using an existing aqueous extraction procedure with modifications (Yang et al., 2022). Hemp seeds (100 g) were mixed with Milli-Q water at ratios of 1:7 (w/w), adjusted to pH 8.0 with 1.0 M NaOH, and steeped overnight at 4 °C. After steeping, the pH of the seed dispersion was readjusted to pH 8.0 with 1.0 M NaOH, transferred to a laboratory blender (8010 ES, Waring Product Division, New Hartford, CT, USA), and rigorously blended for 1 min. The obtained slurry was filtered with a cheesecloth to remove solids. The initial extract was readjusted to pH 8.0 with 1.0 M NaOH and transferred to 250 mL centrifuge bottles to centrifuge at 4, 000 *g* at 4 °C for 30 min (Sorvall Legend XFR, ThermoFisher Scientific, Waltham, MA, USA). Following centrifugation, the oleosome-rich cream layer mixed with extraneous proteins was collected. The excess water was removed by placing the cream on a filter paper (Diameter 150 mm, Whatman 1, UK) for 2 min. The cream then was removed from the filter, analyzed for its protein content and then redispersed in water at pH 8.0 at the ratio of 1:9 (w/w). A second centrifugation step was applied at 10, 000 *g* for 30 min at 4 °C to remove extraneous proteins and the cream (purified oleosomes) was again collected and placed again on the filter paper for 2 min to remove excess water and stored for further analysis. The oleosome dispersions were prepared using purified oleosomes diluted with Milli-Q water to reach the lipid content at 7.5 wt% and pH was adjusted to 9.0 with 1.0 M NaOH.

2.3. Characterization of oleosomes

2.3.1. Moisture content

To determine the moisture content of purified oleosomes, approximately 1.0 g of purified oleosomes were placed in a laboratory oven (UFB 400, Memmert, Germany) at 60 °C and dried to constant weight. The moisture content was calculated from the weight difference between the initial and dried oleosomes. Intact hemp seeds, as a control, were treated with the same procedure.

2.3.2. Lipid content

Lipids were extracted from 1 g of hemp seeds and 1 g of dried oleosomes by using Soxhlet extraction with 150 mL petroleum ether for 8 h according to the AOAC method (Chemat et al., 2020). The lipid content was calculated by weighing the lipids in the distillation flask and dividing by the weight of initial hemp seeds and purified oleosomes as following equations:

$$\text{Lipid content in hemp seeds}(\text{wt}\%) = \frac{m_1}{m_2} \times 100 \quad (1)$$

$$\text{Lipid content in purified oleosomes}(\text{wt}\%) = \frac{m_3}{m_4} \times 100 \quad (2)$$

in which m_1 and m_3 were the mass (g) of lipids extracted from hemp seeds and purified oleosomes in the distillation flask, respectively. m_2 and m_4 were the mass (g) of initial hemp seeds and purified oleosomes, respectively.

2.3.3. Protein content

The protein content of the hemp seeds and purified oleosomes were analyzed by using the Dumas method (Ntone, Bitter, & Nikiforidis, 2020). A nitrogen-protein conversion factor of 5.7 was used to calculate the protein content.

2.3.4. Droplet size distribution

The droplet size distribution of oleosome dispersions (7.5 wt% lipid content) was measured using laser diffraction with a Bettersizer S3 Plus instrument (Bettersizer Instruments Ltd., Dandong, China), using a refractive index of 1.47 for the oleosomes, and 1.33 for the continuous phase. Next to the droplet size distributions, the average droplet size was represented as the volume mean diameter ($d_{4,3}$). To assess possible droplet aggregation, 10 mL of the oleosome dispersion was mixed with an equal volume of 1.0 wt% SDS solution. SDS breaks droplet aggregation driven by hydrophobic interactions, allowing us to measure the droplet size distributions of the individual oleosomes.

2.3.5. ζ-potential measurements

The ζ-potential of the oleosome dispersions (7.5 wt% lipid content) was analyzed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C. The refractive indexes for the dispersed and continuous phases were set at 1.47 and 1.33, respectively. The dispersion was diluted 100 times (0.075 wt% lipid content) and then adjusted pH to 9.0 with 1.0 M NaOH. The ζ-potential was measured automatically with 10–100 runs per analysis. All measurements were conducted in triplicates.

2.4. Protein profile characterization

The protein profiles of the initial extract, oleosomes mixed with storage proteins, and the purified oleosomes were analyzed using SDS-PAGE. Every oleosome dispersion sample (100 μL) was mixed with 250 μL of sample buffer (NuPAGE® LDS sample buffer: 4X, ThermoFisher, Landsmeer, the Netherlands), and 650 μL of Milli-Q water. The mixtures (1 mL) were transferred to Eppendorf tubes and centrifuged for 1 min at 2000 rpm. Then, the centrifuged mixtures were heated in a heating block for 10 min at 70 °C and centrifuged again. The 10 μL of protein marker (PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa, ThermoFisher, Landsmeer, the Netherlands) and 20 μL of the obtained samples were loaded on the gel (NuPAGE® Novex® 4–12 % Bis-Tris Gel, ThermoFisher, Landsmeer, the Netherlands). The electrophoresis was executed for 30 min at 200 V. The gel was rinsed with Milli-Q water and stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories B.V., Lunteren, the Netherlands).

2.5. Confocal Laser Scanning Microscopy (CLSM)

The oleosomes were inspected using a Confocal Laser Scanning Microscope (CLSM) (Leica TSC SP8x, Leica Microsystems Inc., Germany). One mL of oleosome dispersion was stained with 5 μL of Rhodamine DHPE (1 mg/mL in ethanol), 5 μL of Fluorescein isothiocyanate (FITC) (20 mg/mL in ethanol), and 10 μL of Nile Red (1 mg/mL in ethanol) for labelling the phospholipids, proteins and lipids, respectively.

2.6. The stability of oleosome dispersions

2.6.1. Effect of pH

Oleosome dispersion samples (8 mL, 7.5 wt% lipid content) were adjusted to a pH value ranging from 2.0 to 12.0 by using 1.0 M HCl or 1.0 M NaOH and transferred into glass test tubes (12 mL) with screw caps. Every sample was stored at 4 °C overnight. Next the sample was brought to room temperature before analysis.

2.6.2. Effect of ionic strength

Oleosome dispersion samples (4 mL, 7.5 wt% lipid content) were diluted with the same volume of different concentrations of NaCl

solution to obtain a mixture containing the final salt concentrations in the range of 0–500 mM and transferred into glass test tubes (12 mL) with screw caps. Every sample was stored at 4 °C overnight and restored to room temperature before analysis.

2.6.3. Effect of thermal treatment

Oleosome dispersion samples (8 mL, 7.5 wt% lipid content) were transferred into glass test tubes (12 mL) with screw caps and placed in a water bath. Treatments at different temperatures (20–100 °C) were applied to all samples for 30 min after the core of the sample reached the targeted temperature. Every sample was cooled in an ice bath to reach room temperature immediately and then their droplet size distribution was analyzed.

2.6.4. The long-term storage stability of oleosomes

Oleosome dispersions (7.5 wt% lipid content) were treated as mentioned in section *2.6.3* and incubated at 4 °C for 14 days in the dark condition for observation. Samples were added with antibacterial agents (Sodium Azide, 0.02 wt%) as the control group. The droplet size of oleosome dispersion was determined during long-term storage.

2.7. Statistical analysis

All experiments were conducted in triplicate. The results were presented as mean ± standard deviation. IBM SPSS Statistics software (Version 28.0.1.1, IBM SPSS, USA) was used for statistical analysis. Statistical analysis was performed using the analysis of variance (ANOVA) to compare different samples at a significant level of 95 % ($P < 0.05$).

3. Results and discussions

3.1. Characterization of hemp seeds and oleosome extracts

First, the hemp seeds were analyzed for their moisture, lipid, and protein contents and listed in Table 1. The hemp seeds contained 5.1 ± 1.3 wt% moisture, 28.5 ± 5.6 wt% lipids, and 25.6 ± 2.9 wt% proteins similar to what is reported in the literature (Farinon et al., 2020). The composition of the purified oleosomes was 23.2 ± 2.2 wt% moisture, 74.9 ± 3.5 wt% lipids, and 5.9 ± 0.4 wt% proteins. Different from recently reported results by another group, protein content in our oleosomes is higher, possibly due to the different material sources and extraction methods (Garcia et al., 2021).

To assess the type of proteins present in the oleosome extracts electrophoresis was used. Fig. 1a shows the protein profiles of the crude extract of oleosomes (lane 1), and the oleosomes after their purification (lane 2). A marker was included to indicate the molecular weight of the proteins present in the samples. Oleosome proteins, i.e. oleosins, are expected to be around 15 KDa (Garcia et al., 2021). Lane 1 shows that besides the 15 KDa proteins, also proteins with higher molecular masses were present, which were probably co-extracted proteins. Therefore an additional basic redispersing and centrifugation step of oleosomes was applied to remove these proteins. The SDS-page of the washed i.e., purified, oleosomes are shown in Fig. 1a, lane 2. Clearly, after the washing only the proteins at 15 KDa remained, which mostly like are the proteins that are absorbed on the oleosome membrane and cannot be removed with the washing step (Aluko, 2017; Garcia et al., 2021; Wang, 2008).

Table 1
Proximate composition of hemp seeds and purified oleosomes.

Parameter	Composition (wt %)	
	Hemp seeds	Purified oleosomes
Lipids	**28.5 ± 5.6**	**74.9 ± 3.5**
Proteins	**25.6 ± 2.9**	**5.9 ± 0.4**
Moisture	**5.1 ± 1.3**	**23.2 ± 2.2**

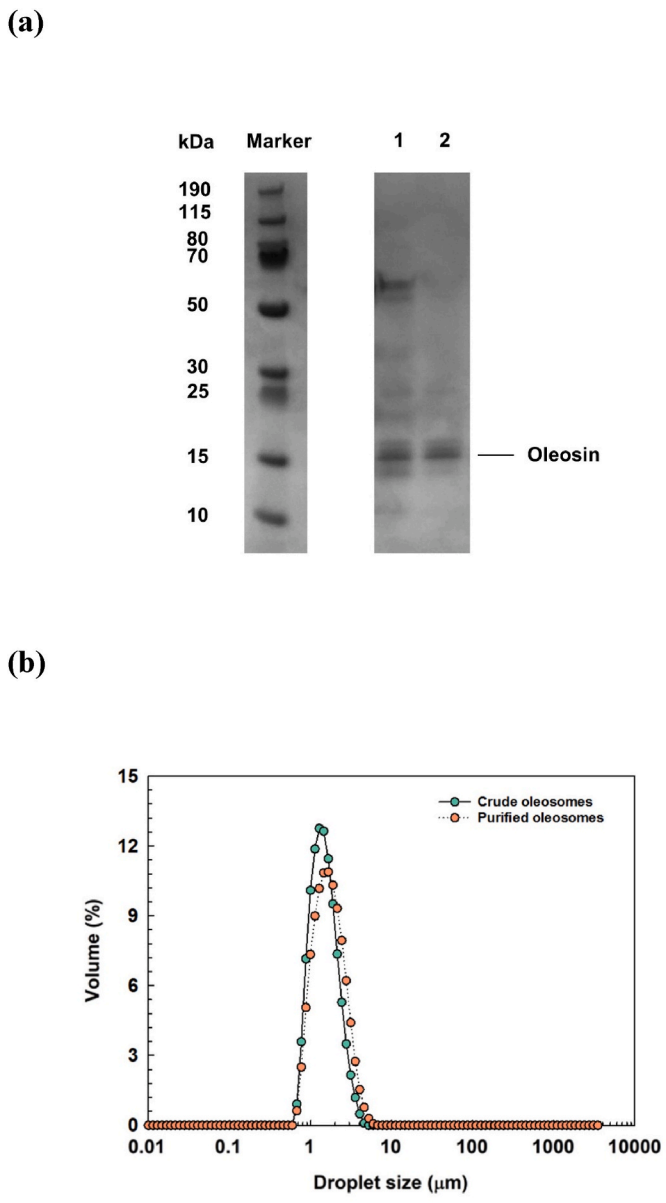

Fig. 1. Characterization of oleosomes. (**a**) SDS-PAGE of proteins present in the crude oleosomes mixed with storage proteins (lane 1) and the purified oleosomes (lane 2), respectively. (**b**) Droplet size distributions of 10 wt% the crude oleosome and 10 wt% purified oleosome in dispersions at pH 8.0, respectively.

In Fig. 1b size distributions of the extracted crude oleosomes and those after their purification are shown. The droplet size (about 2 μm) and size distribution of oleosomes are similar for both the crude oleosomes and the purified oleosomes. Thus no coalescence occurred during the purification which is in line with the fact that the ζ-potential of the oleosomes both before and after their purification was the same and at the range of −42 ± 6 to −36 ± 4 mV at pH 8.0. These values are similar to those reported in the literature for oleosomes (Bibat, Ang, & Eun, 2022; Pereira et al., 2022).

To investigate the macroscopic structure of purified oleosomes confocal microscopy was used (Fig. 2). Three different staining procedures were used i.e. Rhodamine DHPE to visualize phospholipids, FITC to visualize proteins and Nile Red to visualize lipids (Fig. 2). Rhodamine DHPE-labeled phospholipids are visible as bright orange structures (Yamaguchi et al., 2017) (Fig. 2a). The even distribution of the orange areas in circular shapes indicates that the phospholipids are uniformly distributed on the oleosome surface. Additionally, proteins

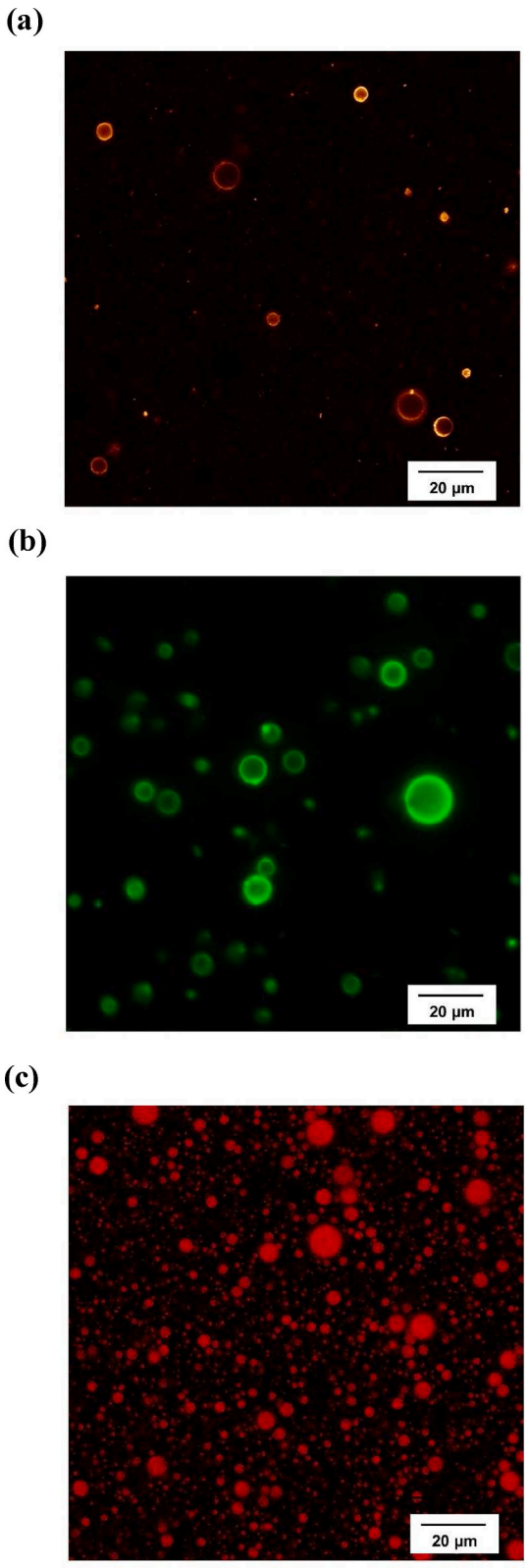

Fig. 2. Confocal Laser Scanning Microscopy images of purified oleosomes showing the interfacial distribution of phospholipids stained by Rhodamine DHPE (**a**), proteins stained by FITC (**b**), and neutral lipids stained by Nile Red (**c**), respectively.

were labeled using FITC (green) (Jin et al., 2023) (Fig. 2b). The green areas are visible as spherical structures which are the proteins present on the surface of oleosomes. No free proteins are visible. This result confirms the results obtained from the SDS-PAGE, which showed for the purified sample that mostly oleosome-related proteins were present (at 15 kDa). Finally, Nile Red was used to confirm that the core of the droplets were indeed TAGs, which was indeed the case as shown in Fig. 2c. (Nikiforidis, Kiosseoglou, & Scholten, 2013).

3.2. *Effect of environmental stress on the stability of oleosome dispersions*

3.2.1. *Effect of pH on the stability of oleosome dispersions*

The surface charge of oil droplets plays a crucial role in the electrostatic interactions and potential droplet aggregation or coalescence of oleosomes (Abdullah et al., 2020; McClements, 2016; Wang et al., 2019). Therefore the surface charge, expressed as ζ-potential, and volume mean diameters as a function of pH were investigated for purified

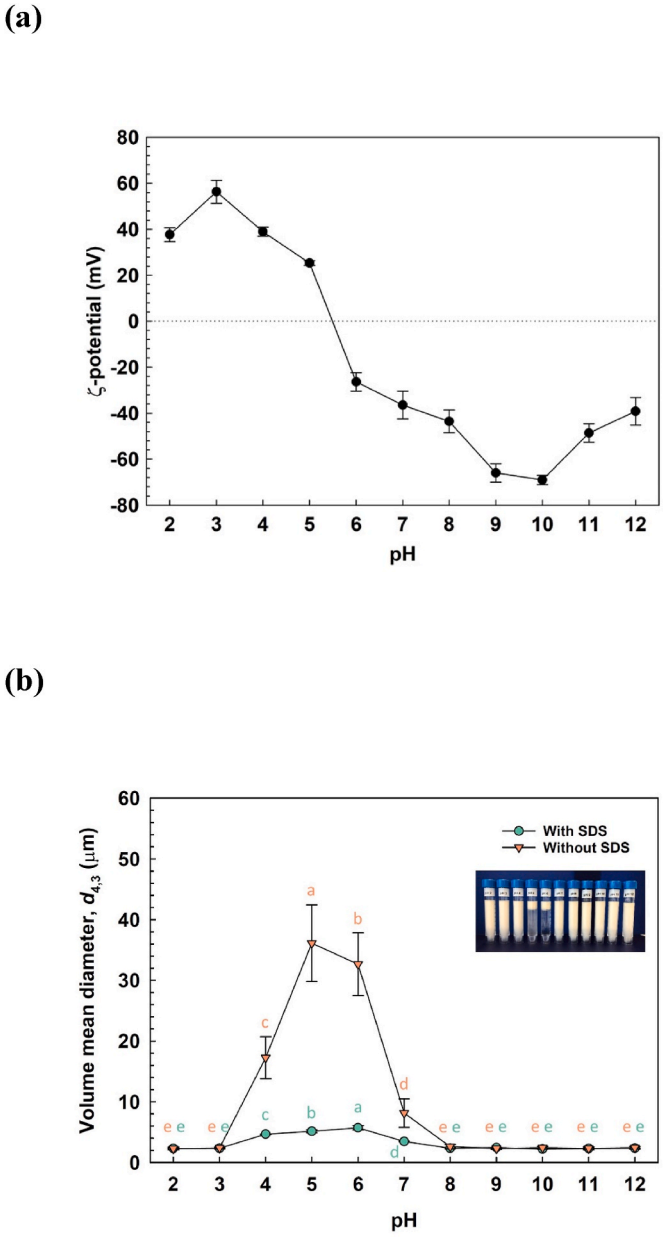

Fig. 3. Effect of pH on the ζ-potential (**a**) and $d_{4,3}$ (**b**) of purified oleosomes. The a-e values with a different letter are significantly different ($p < 0.05$).

oleosomes (Fig. 3). Fig. 3a, shows that the ζ-potential of purified oleosomes depended on the pH, with the ζ-potential ranging between +56 ± 5 mV at pH 3.0 to −69 ± 2 mV at pH 10.0 (Fig. 3a). The curve that describes the change of the surface charge of the oleosomes when pH is changing, crosses axis X at pH values around 5.5, which is the zero charge point of the system (Fig. 3a). The stronger electrostatic repulsion of oleosomes away from (below and above) the zero charge point than that close to the zero charge point, resulting in a better stability of oleosomes with less aggregation. Interestingly, a decrease of the absolute ζ-potential was observed below pH 3.0 (from −56 mV to −38 mV) and above pH 10.0 (from −69 mV to −31 mV), but it did not affect the stability of oleosomes due to the high absolute surface charge (>30 mV). As suggested, the drop in pH values below 3.0 can be due to the increased ionic strength at these extreme conditions, or possibly due to partial hydrolysis and/or desorption of some of the charged components from the oleosome surface (Garcia et al., 2021; Iwanaga et al., 2007; Lopez et al., 2021).

In Fig. 3b, the average droplet sizes of purified oleosomes as a function of the pH are presented. To understand whether the droplet sizes refer to droplet aggregates or individual droplets, we treated the dispersions with the SDS solution, which may break attractive hydrophobic forces between droplets. To avoid the adsorption of SDS on the oleosome interface and competitive displacement of molecules from the oleosome interface, we added SDS just a few minutes before the size analysis (Romero-Guzmán et al., 2020). The $d_{4,3}$ of purified oleosomes at pH 4.0–7.0 was higher compared to the values outside this pH range. The large particles were found at the condition without SDS, especially at pH 5.0 (36.1 ± 6.3 μm) and pH 6.0 (32.7 ± 5.2 μm). When SDS was added to the purified oleosome, a slight change in particle size as a function of pH was observed, but still within a reasonable range of oleosome sizes. This suggests that the enormously increased droplet size (without SDS) was mainly caused by the aggregation of purified oleosomes and not by coalescence. Thus purified oleosomes themselves are stable as a function of pH.

After this analysis, the pH of the dispersed oleosomes was kept at 9.0, to guarantee their stability against aggregation.

3.2.2. *Effect of ionic strength on the stability of oleosome dispersions*

The addition of ions to oleosome dispersions can result in a change in their surface charge (Lopez et al., 2021). In Fig. 4a, we provide the surface charge, represented by the ζ-potential, as a function of the ionic strength in the continuous phase. The charge of the purified oleosomes was affected by the ionic strength, with the ζ-potential slowly increasing from −66 ± 11 mV to −33 ± 10 mV as the ionic strength increased (Fig. 4a). This can be caused by an increased charge screening caused by the added ions which leads to less charge on the surface of the droplets (Ma et al., 2023). Generally, droplets with lower electric charge are prone to aggregation or coalescence due to weak electrostatic repulsion that cannot overcome the interaction between droplets (Abdullah et al., 2020; Lopez et al., 2021; Wang et al., 2019). In the case of the purified oleosomes, with the reducing ζ-potential, purified oleosomes still have good stability against flocculation due to the high absolute surface charge (>30 mV). At all NaCl concentrations, the van der Waals forces and hydrophobic force between purified oleosomes were overcome by electrostatic repulsion and prevented flocculation (Li et al., 2022). With the addition of NaCl, the system was affected by the electrostatic screening effect decreasing the electrostatic repulsion between purified oleosomes. Meanwhile, with the increase of NaCl concentration, the van der Waals forces and the attractive interaction between purified oleosomes also probably decreased. Moreover, the positively-charged protein residues in oleosins at the surface of purified oleosomes under higher NaCl concentrations might electrostatically attract with anionic chloride ions which could form a "layer" on purified oleosomes and prevent aggregation (Ishii, Matsumiya, & Matsumura, 2021).

Furthermore, Fig. 4b shows the droplet size distribution of the purified oleosomes under different ionic strengths from 0 to 500 Mm NaCl

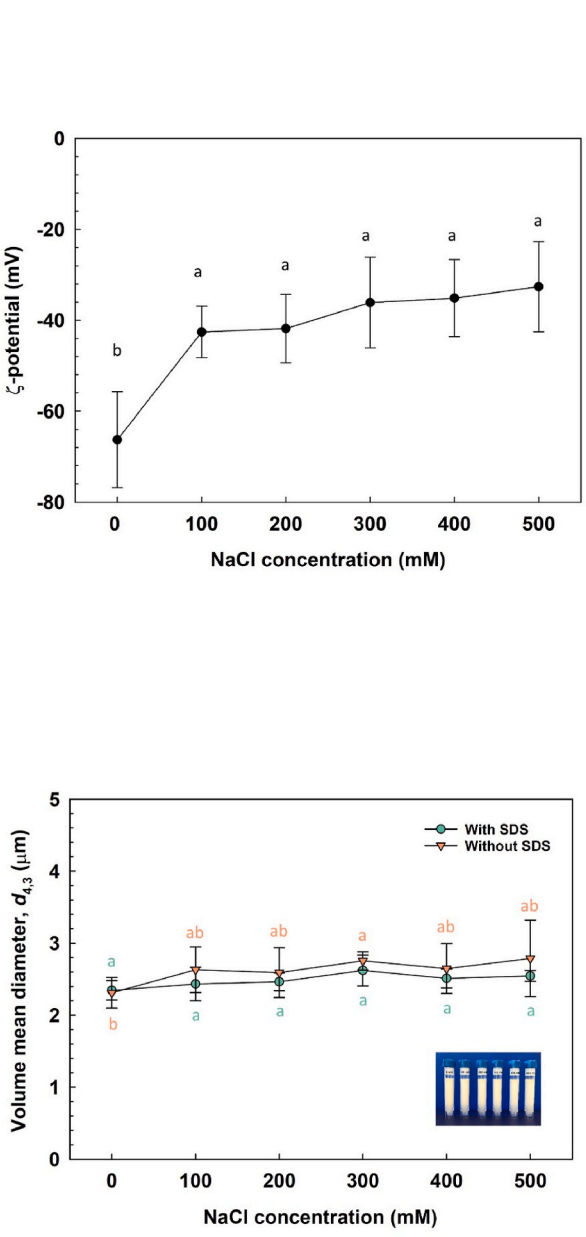

Fig. 4. Effect of ionic strength on the ζ-potential (**a**) and $d_{4,3}$ (**b**) of purified oleosomes. The a and b values with a different letter are significantly different ($p < 0.05$).

with and without treatment with SDS. All samples have a similar droplet size. There is no significant effect on the droplet size of the ionic strength on the emulsion stability with SDS. Similar results were reported earlier, where when the ionic strength was increased, no significant change in droplet size was observed. Oleosins provide not only electrostatic repulsion but also a steric hindrance that is resistant to the flocculation of oleosomes. Compared with the electrostatic interactions, the steric effect of oleosins is a more important key to the stability of oleosomes even if the charge density is reduced. (Garcia et al., 2021).

3.2.3. *Effect of thermal treatment on the stability of oleosome dispersions*

Temperature is a crucial factor that can influence the interfacial properties of oleosomes, as the proteins at the interface could denature and change their conformation on the interface (Ding et al., 2020). Additionally, heating might affect the mobility of the phospholipids on the interface (Jash, Ubeyitogullari, & Rizvi, 2020), leading to oleosome size changes. To investigate whether the protein profile on the oleosome

interface changed after the heating step we used SDS-PAGE (Fig. 5a). After the heating steps only one band at around 15–17 kDa was observed, indicating that the oleosins remained on the oleosome interface, and no big changes occurred. Oleosins have a long central hydrophobic hairpin that is inserted into the oil core, while they have two short arms that are relatively hydrophilic and are horizontally oriented on or extend from the oleosome surface. The hydrophilic arms of oleosins could hold the hydrophobic hairpin with 5–6 nm long to specific secondary structures in the oil core (Huang, 2018). This specialized structure might inhibit interactions of the hairpins of two adjacent oleosins so that oleosin will not aggregate and denature due to thermal treatment and remain available for SDS-PAGE analysis. This is probably one of the reasons that oleosins are not affected by heating at these temperatures.

An indication of the changes that might occur on the oleosome interface is the change of the ζ-potential. Therefore, in Fig. 5b the changes of the ζ-potential of the oleosome dispersions under thermal treatment at 20–100 °C for 30 min are reported. The ζ-potential of oleosomes was changed only slightly after thermal treatment, therefore, the surface of oleosomes was not expected to change during the heating steps at these conditions.

Fig. 5c shows the changes in the droplet size distribution of oleosomes after the heating steps. There was no obvious change in the $d_{4,3}$ values showing that no droplet-droplet interactions took place over the full range of the applied temperatures. Additionally, as it is shown in the insert picture of Fig. 5c, the lack of aggregation could also be noticed by the lack of creaming during storage.

The stability of oleosomes overheating has also been observed earlier, where it was found that pasteurization could improve the stability against coalescence and aggregation of oleosomes (from soybean, rapeseeds, hazelnuts, sesames and peanuts) (Chirico et al., 2020; Nikiforidis, Donsouzi, & Kiosseoglou, 2016; Wu et al., 2022; Zhou et al., 2022).

3.3. Physical storage stability of purified oleosome dispersions

The physical stability of oleosomes is crucial when oleosomes are used in real products such as foods and beverages. In the case of rapeseed oleosomes, it has been reported that there might be lipolytic enzymes (i.e. lipase) present that have lipolytic activity and affect the integrity of oleosomes during storage (Chirico et al., 2020). Therefore, we stored oleosomes for up to 14 days after the thermal treatment steps and measured their physical stability presented as $d_{4,3}$. Fig. 6a shows the droplet size of oleosomes after being thermally treated at different temperatures and stored at 4 °C for up to 14 days. When samples were heated above 80 °C, their droplet size remained stable during 14 days of storage (Fig. 6a). Meanwhile, samples treated at a temperature lower than 50 °C were not stable and showed oiling-off and microbial spoilage after 14 days of storage. Samples treated at 60 and 70 °C did not have obvious phase separation, but the average droplet size became larger from 2.0 ± 0.2 μm to 5.0 ± 3.3 μm and 3.2 ± 2.0 μm, respectively. The delayed droplet size increase (samples treated at 20–40 °C after 7 days storage, samples treated at 50–70 °C after 14 days storage) might be due to samples being stored at dark and low temperature conditions, resulting in the bacteria not growing rapidly but being delayed.

During the long-term storage, the enzymes and bacteria in purified oleosome dispersions might undergo some reactions with purified oleosomes, such as proteolysis, lipid oxidation and microbial fermentation, resulting in droplet instability similar to previous studies (Chen et al., 2012; Chirico et al., 2020; Nionelli et al., 2018; Zhou et al., 2022). Over 80 °C treated samples could be regarded as pasteurized samples, in which most of the enzymes and bacteria were inactivated and inhabited (Smelt & Brul, 2014; Wu et al., 2022). Pasteurization is widely used in the production of food and beverages to make products safe, increase shelf life, and reduce spoilage (Jay, Loessner, & Golden, 2005). Therefore, samples treated at over 80 °C were more physically stable and

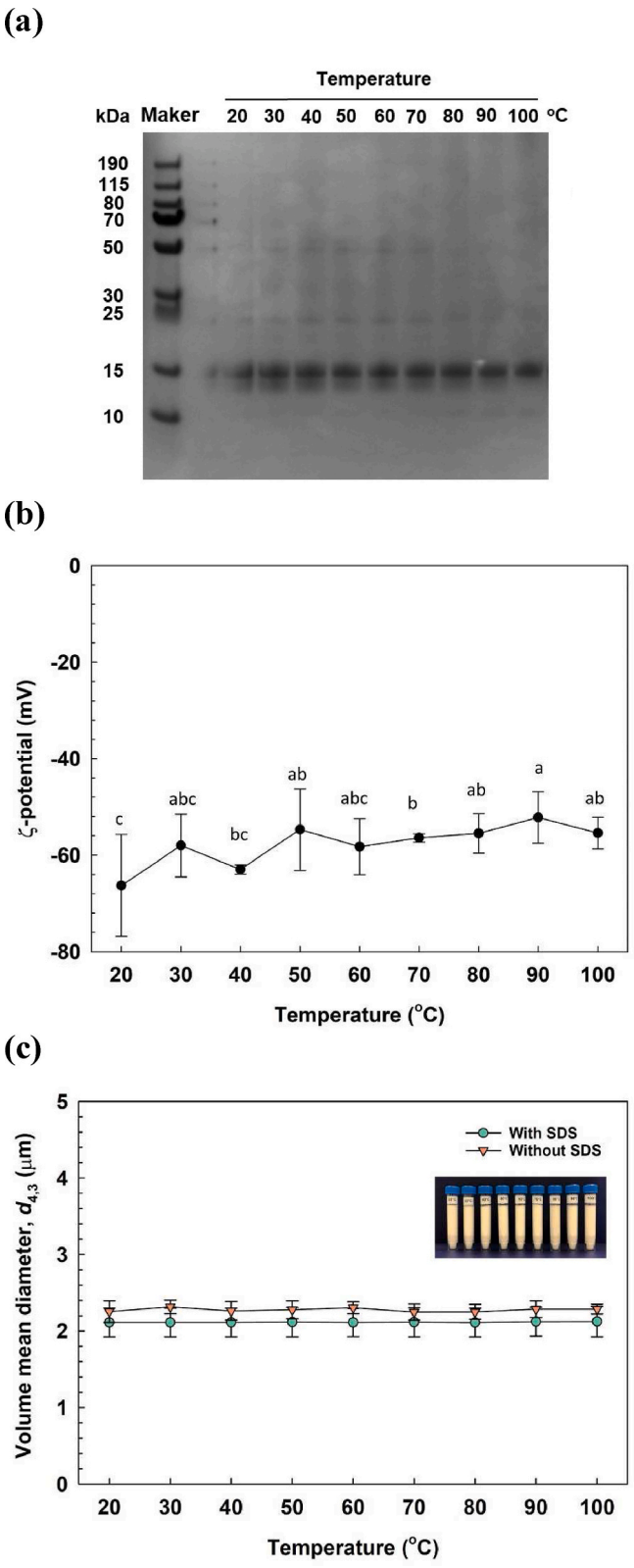

Fig. 5. SDS-PAGE of proteins profiles (**a**), Effect of thermal treatment on the ζ-potential (**b**), and $d_{4,3}$ (**c**) of purified oleosomes. The a-c values with a different letter are significantly different ($p < 0.05$).

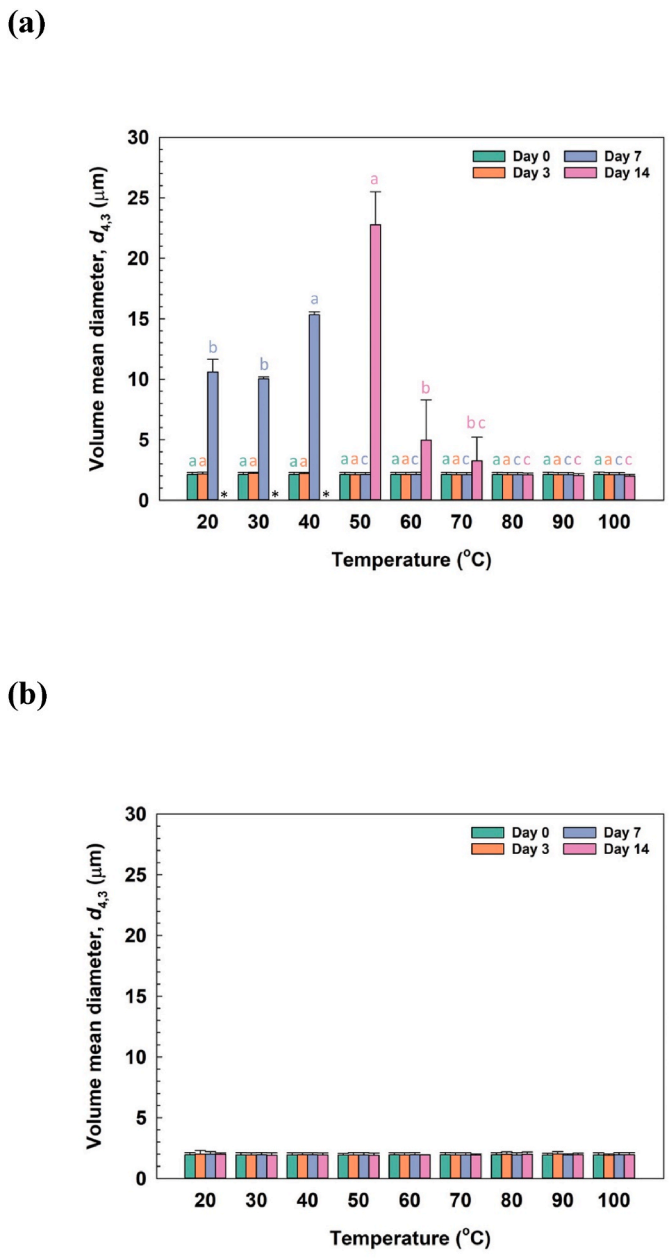

Fig. 6. Effect of thermal treatment on the particle size of purified oleosome dispersions during 14 days storage at 4 °C. The $d_{4,3}$ derived size of purified oleosomes without antibacterial agents (**a**) or with antibacterial agents (**b**) during storage, respectively. (*) The particle size of the samples could not be analyzed due to complete oiling off or microbial spoilage. The a-c values with a different letter are significantly different ($p < 0.05$).

advantageous than those with temperatures lower than 80 °C.

To investigate whether there were any bacteria present, which might affect the stability of oleosomes, we used control samples, but with the addition of an antibacterial agent. When an antibacterial agent was used, no changes in the droplet size distributions were observed regardless if a heating step was applied or not (Fig. 6b). These findings indicate that the coalescence observed when no antibacterial agent was present, could be attributed to the presence of bacteria, which are affecting the stability of oleosomes. By heating the oleosome dispersions above 80 °C, we prevented the growth of bacteria in the 14 days measured and no spoilage or oleosomes coalescence was observed.

These data are in contradiction with other reports where the need for heating of rapeseed-derived oleosomes was mentioned due to the presence of lipolytic enzymes that had to be deactivated (Chirico et al., 2020). In that case, the enzymatic activity was attributed to exogenous storage proteins that had contaminated the oleosomes (De Chirico, 2018; Zhou et al., 2022). In our work, we used alkaline conditions to purify oleosomes to reduce the exogenous storage protein content. Apparently, in the case of hemp seeds exogenous proteins and thus enzymes were most washed out and not present in hemp seed purified oleosome dispersions (Fig. 1a) and their destabilization during storage could be attributed to the presence of bacteria. A thermal treatment above 80 °C for 30 min was enough to act as a pasteurization step and de-activate the bacteria.

4. Conclusions

Hemp seed oleosomes were successfully extracted from hemp seeds and the composition of the extracted hemp seed oleosome extracts was 23.2 ± 2.2 wt% moisture, 74.9 ± 3.5 wt% lipids, and 5.9 ± 0.4 wt% proteins. The purified oleosomes had a lipid core surrounded by a phospholipids/proteins membrane as observed with CLSM. The oleosomes did not coalesce during the extraction and purification and retained their characteristic structure.

Thermal treatment did not affect oleosome physical stability, even at temperatures of 100 °C for 30 min. When the oleosome dispersions were stored for 14 days without the use of an antibacterial agent, the oleosome dispersions had to be heated at 80 °C or above to remain stable. However, in the presence of an antibacterial agent, the dispersions maintained good physical stability even after storage for 14 days irrespective of their thermal treatment. Therefore, we conclude in the case of the extracted and purified hemp seed oleosomes there are no enzymes present that could cause lipolysis and need to be activated, but a heating step is mostly needed to prevent the growth of bacteria. The results of this work will assist the holistic use of hemp plants that are currently underutilized and can lead toward the use of oleosomes in commercial products.

CRediT authorship contribution statement

Zhaoxiang Ma: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Visualization, Data curation, Writing – original draft. **Johannes H. Bitter:** Supervision, Writing – review & editing. **Remko M. Boom:** Supervision, Writing – review & editing. **Constantinos V. Nikiforidis:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to express their gratitude to Botaneco Inc. for providing the financial supportand Umay Sevgi Vardar for capturing the CLSM images.

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