



# The effect of monovalent ( $\text{Na}^+$ , $\text{K}^+$ ) and divalent ( $\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ ) cations on rapeseed oleosome (oil body) extraction and stability at pH 7

Maria Juliana Romero-Guzmán<sup>a</sup>, Vasileios Petris<sup>a</sup>, Simone De Chirico<sup>b</sup>, Vincenzo di Bari<sup>b</sup>, David Gray<sup>b</sup>, Remko M. Boom<sup>a</sup>, Constantinos V. Nikiforidis<sup>c,\*</sup>

<sup>a</sup> Food Process Engineering, Wageningen University and Research, Bornse Weilanden 9, Wageningen 6708 WG, The Netherlands

<sup>b</sup> Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

<sup>c</sup> Biobased Chemistry and Technology, Wageningen University and Research, Bornse Weilanden 9, Wageningen 6708 WG, The Netherlands

## ARTICLE INFO

### Keywords:

Oil bodies  
Extraction  
Natural emulsion  
Rapeseed  
Oleosomes

## ABSTRACT

Oleosomes are storage vehicles of TAGs in plant seeds. They are protected with a phospholipid-protein monolayer and extracted with alkaline aqueous media; however, pH adjustment intensifies the extraction process. Therefore, the aim of this work was to investigate the extraction mechanism of rapeseed oleosomes at pH 7 and at the presence of monovalent and divalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{+2}$ ). The oleosome yield at pH 9.5 was 64 wt%, while the yield at pH 7 with  $\text{H}_2\text{O}$  was just 43 wt%. The presence of cations at pH 7, significantly enhanced the yield, with  $\text{K}^+$  giving the highest yield (64 wt.%). The cations affected the oleosome interface and their interactions. The presence of monovalent cations resulted in aggregation and minor coalescence, while divalent cations resulted in extensive coalescence. These results help to understand the interactions of oleosomes in their native matrix and design simple extraction processes at neutral conditions.

## 1. Introduction

Oleosomes or oil bodies, as they are widely known, are the triacylglycerols (TAGs) storage organelles in plants, serving as the main energy source during seed germination. To retain the chemical quality of the TAGs against extreme environmental stresses, plant cells are building an amphipathic phospholipid-protein membrane around them (Tzen & Huang, 1992). Besides the *in situ* functionality of oleosomes, plant oils (i.e. soybean oil, rapeseed oil, sunflower oil) are generally extracted and used for numerous applications in food, pharmaceutical products, and as biofuels (Hammond, Johnson, Su, Wang, & White, 2005). However, plant oil extraction requires the disruption of the oleosome membrane by a pressing step, followed by toxic organic solvent extraction (Thiyam-Hollaender, Eskin, & Michael, 2012). When plant oils are extracted, they are used as bulk oils or as dispersed phases in oil-in-water emulsions, which requires an emulsification step and the use of an emulsifier (McClements, 2004). Nevertheless, looking back to the oleosome physiology, all these process steps seem unnecessary, as oleosomes, are naturally emulsified oil droplets that could readily serve as the dispersed phase of oil-in-water emulsions. Therefore, instead of focusing only on oil extraction, efforts should be made towards the optimization of the oleosome extraction. For this reason, we have to

deeply understand the properties of oleosome membrane and the interactions at the molecular level.

The most abundant proteins on the oleosome membrane are oleosins, which represent up to 75–80% of the oleosome membrane protein content (Jolivet et al., 2011; Tzen, 2012). Oleosins are a group of proteins with a low molecular weight (14–17 kDa) and are composed by a hydrophobic tail that is anchored in the oil core and two short fairly hydrophilic terminals that are on the oleosome surface (Lin, Liao, Yang, & Tzen, 2005). The other group of proteins present on the oleosome membrane are caleosins (24–28 kDa) and steroleosins (35–60 kDa) (Lin et al., 2005). Similar to oleosins, these proteins have also a hydrophobic tail, which is smaller than the one of oleosins and a longer domain exposed to the bulk phase (Shimada & Hara-Nishimura, 2010). Even though the exact biological functions of the membrane proteins are still to be defined (Purkrtova, Jolivet, Miquel, & Chardot, 2008; Song et al., 2014), it is known that caleosins have a unique  $\text{Ca}^{2+}$  binding site on the N-terminal of the protein that can also bind  $\text{Mg}^{2+}$  (Allouche, Parello, & Sanejouand, 1999; Chen, Tsai, & Tzen, 1999), while steroleosins have a hydrophilic sterol-binding dehydrogenase domain (Purkrtova et al., 2008). Regarding the phospholipids at the oleosome interface, the main type present is phosphatidylcholine representing 65% (wt%) of the total phospholipids, followed by phosphatidylserine, phosphatidylinositol

\* Corresponding author.

E-mail address: [costas.nikiforidis@wur.nl](mailto:costas.nikiforidis@wur.nl) (C.V. Nikiforidis).

<https://doi.org/10.1016/j.foodchem.2019.125578>

Received 9 July 2019; Received in revised form 11 September 2019; Accepted 22 September 2019

Available online 04 October 2019

0308-8146/ © 2019 Elsevier Ltd. All rights reserved.

and phosphatidylethanolamine (Deleu et al., 2010; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993).

The understanding of the architecture of the oleosome interface, the molecular combination and the forces that might occur, will help towards optimizing their extraction. Both proteins and phospholipids are charged molecules and electrostatic forces can occur between neighbouring oleosomes and also between oleosomes and surrounding charged material (Nikiforidis & Kiosseoglou, 2011). Besides electrostatic forces, hydrophobic attractive forces might take place as well. The domains of the oleosome proteins that are exposed to the bulk phase are fairly hydrophilic, however, they also contain hydrophobic patches that can attract each other and lead to aggregation of neighbouring oleosomes (Jolivet et al., 2017; Nikiforidis, Donsouzi, & Kiosseoglou, 2016; Nikiforidis & Kiosseoglou, 2011). Furthermore, the hydrophobic domains of extrinsic proteins might interact with the oleosome proteins leading to bridging flocculation (Eren, Narsimhan, & Campanella, 2016). Hydrophobic attractive forces can be prevented by using surfactants, like Tween or SDS (Nikiforidis et al., 2016; Nikiforidis & Kiosseoglou, 2011). Nevertheless, the addition of surfactants may affect the oleosome membrane, therefore this research was mostly focused on affect the hydrophobic interactions by electrostatic interactions.

Oleosomes have a zero charge point between pH values of 4 and 6, therefore, to increase electrostatic repulsion and to enhance the extraction yield it has been proposed to perform the extraction at pH values above 9.0, where the electrokinetic potential is below  $-40$  mV (De Chirico, di Bari, Foster, & Gray, 2018; Matsakidou, Mantzouridou, & Kiosseoglou, 2015). However, in order to reduce the number of steps and chemicals used during the oleosome extraction, efforts should be made towards understanding the oleosome extraction mechanism at neutral pH values. An alternative to pH adjustment for altering the electrostatic interactions between proteins is the addition of cations (Collins, 2004; Dumetz, Snellinger-O'Brien, Kaler, & Lenhoff, 2007; Levy & Onuchic, 2004; Zhang & Cremer, 2006). Ionic environments weaken or strengthen the protein-protein electrostatic interactions, which can cause protein unfolding and affects its solubility. Therefore, the aim of this work was to investigate the effect of monovalent ( $\text{Na}^+$ ,  $\text{K}^+$ ) and divalent ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) cations on oleosome extraction at pH 7. The effect of the cations was evaluated by comparing the oleosome extraction yields and the effect on the physical stability of the obtained oleosomes.

## 2. Materials and methods

### 2.1. Materials

Untreated rapeseeds (*Brassica napus*), type Allize were kindly purchased by the Division of Food Sciences, University of Nottingham, Sutton Bonington, UK. Magnesium Chloride ( $\text{MgCl}_2$ ) was obtained from Merck (Darmstadt, Germany). All other chemicals including the sodium chloride, potassium chloride and calcium chloride ( $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{CaCl}_2$ ) were obtained in analytical grade from Sigma-Aldrich (St. Louis, MO, USA). Solutions and dispersions were made with ultrapure water (MilliQ) obtained with a Merck Millipore device (Darmstadt, Germany).

### 2.2. Oleosome aqueous extraction

Rapeseed oleosomes were isolated using the extraction method proposed by De Chirico et al. (2018), with some modifications based on the method proposed by Nikiforidis and Kiosseoglou (2009). The different aqueous media were prepared by dissolving the different salts ( $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ ,  $0.2$  mol/L) in ultra-pure water (MilliQ) and adjusting their pH to 7.0 with a solution of  $\text{NaOH}$  ( $0.1$  mol/L) or  $\text{HCl}$  ( $0.1$  mol/L). The additional aqueous solution made by  $\text{NaCl}$  ( $0.3$  mol/L) was elaborated in a similar way than the other salted-aqueous media. The alkaline aqueous media was prepared similarly, by dissolving

$\text{NaHCO}_3$   $0.1$  mol/L and adjusting the pH to pH 9.5 with  $\text{NaOH}$  ( $1.0$  mol/L). A SevenMulti™ dual meter pH/conductivity (Mettler Toledo, Greifensee, Switzerland) was used to monitor the pH. The seeds were soaked ( $1:1$  w/v) in the different aqueous media for 16 h at  $4^\circ\text{C}$ . After soaking, the solid/solvent ratio was adjusted to  $1:7$  w/v and the dispersion was blended for 60 s at 7200 rpm (Thermomix TM31, Utrecht, The Netherlands). The mixture was then filtered through two layers of cheesecloth (GEFU®, Eslohe, Germany). The first extract (filtrate) was centrifuged at 3000g for 15 min at  $4^\circ\text{C}$ . After the centrifugation step, three different layers were observed: the cream, the serum and the precipitate. The oleosome cream was manually collected, dispersed in ultra-pure water (MilliQ) ( $1:4$  w/v) and centrifuged at 10000g for 30 min at  $4^\circ\text{C}$ . This washing step was repeated twice. The oleosome extraction yield was calculated based on the difference between lipid content remaining in the cake and the initial lipid content in the seeds.

### 2.3. Compositional analysis of all streams

The moisture content of the retentate and oleosome cream was determined using a Moisture Analyzer (MA35M, Sartorius Gottingen, Germany). Oil quantification was performed on dry samples that were placed in a Soxhlet device (Buchi extractor, Büchi, Flawil, Switzerland) for 9 h, while the oil was extracted using petroleum ether. The oleosome extraction yield was calculated based on the difference between the oil left in the solid residue after the extraction (cake) and the initial amount of oil in the seeds ( $36.6 \pm 0.5\%$ ). The protein content of the defatted samples was calculated by determining the amount of Nitrogen in the samples using the Dumas method and using a conversion factor of 5.5 as suggested in literature (Lindeboom & Wanasundara, 2007) (Nitrogen analyzer, FlashEA 112 series, Thermo Scientific, Interscience, The Netherlands).

### 2.4. Determination of oleosome particle size distribution

The droplet size distribution of oleosome emulsions was determined by laser light scattering (MalvernMastersizer 3000, Malvern Instruments Ltd, UK). The refractive index used was 1.47 for the dispersed phase (oleosomes) and 1.33 for the continuous phase (water). Average droplet sizes are reported using the surface weighted ( $d_{3,2}$ ) mean diameter. All measurements were conducted on fresh oleosome creams diluted in ultrapure water ( $1:100$  w/v).

### 2.5. Determination of oleosome zeta potential

A dynamic light scattering apparatus (DLS ZetasizerNanoZS, Malvern Instruments Ltd, UK) was used to analyze the  $\zeta$ -potential of the emulsions. The creams were diluted  $1000$  w/v with ultra-pure water. After the dilution, the pH of the dispersions was adjusted manually to pH 7. The refractive indexes used were 1.47 for the dispersed phase and 1.33 for the continuous phase.

### 2.6. Optical microscopy analysis of oleosome emulsions

Images of the oleosome emulsions were taken with the microscope AxioVision V 4.8.3.0 (Carl Zeiss MicroImaging, GmbH) equipped with a digital camera (Axiocam MRc 5). The oleosome cream for each treatment was diluted with ultrapure water ( $1:100$  w/v) and one drop of the emulsion was added on a glass slide and placed onto the microscope. The magnification used was  $100\times$ .

### 2.7. Statistical analysis

All the measurements and extractions were performed at least in triplicates. One-way analysis of variance (ANOVA) test was applied to detect differences among the extraction yields as function of the

**Table 1**  
Extraction yield of oleosomes recovered with different aqueous solvents.

Aqueous solvent	Oleosome extraction yield (wt%)	Standard Deviation
H <sub>2</sub> O (pH 7.0)	42.7 <sup>a</sup>	± 1.9
Na <sup>+</sup> (0.2 mol/L, pH 7.0)	50.2 <sup>b</sup>	± 2.0
Na <sup>+</sup> (0.3 mol/L, pH 7.0)	55.3 <sup>c</sup>	± 1.8
K <sup>+</sup> (0.2 mol/L, pH 7.0)	64.2 <sup>d</sup>	± 0.6
Mg <sup>2+</sup> (0.2 mol/L, pH 7.0)	52.5 <sup>e</sup>	± 4.9
Ca <sup>2+</sup> (0.2 mol/L, pH 7.0)	55.0 <sup>e</sup>	± 2.3
NaHCO <sub>3</sub> (0.1 mol/L, pH 9.5)	63.6 <sup>d</sup>	± 0.5

Values with different letters are significantly different with  $p < 0.05$ .

aqueous extraction media. Analyses were performed with the IBM SPSS statistics 23 software. Differences were considered to be significant at  $p < 0.05$ .

### 3. Results and discussion

#### 3.1. Effect of cations on oleosome extraction yield and stability

To achieve high oleosome extraction yields, pH values above 9.0 are necessary, where proteins and oleosomes are soluble due to the high electrokinetic potential (De Chirico et al., 2018; Nikiforidis & Kiosseoglou, 2009). For example, maize oleosomes have a zero charge point at around pH 4.5. Their extraction at pH 6.0 has a yield about 15 wt.% while at pH 9.0 it reaches a yield of up to 90 wt.%, (Nikiforidis & Kiosseoglou, 2009). As an effort towards an alternative path to increase oleosome solubility without adjusting pH, we decided to investigate oleosome extraction and stability at neutral pH (7.0) and in the presence of monovalent or divalent cations (Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup>).

The extraction yields of rapeseed oleosomes in the presence of cations are shown in Table 1. When only ultra-pure water was used the lowest extraction yield was achieved, which was 42.7 wt.%. At the presence of K<sup>+</sup> (0.2 mol/L), the extraction yield was significantly enhanced and reached the highest value, of 64.2 wt.%. In contrast, the extraction performed with Na<sup>+</sup> (0.2 mol/L) reached a yield of 50.2 wt.%. When divalent cations were present, the yield was 52.5 wt.% after the extraction with Mg<sup>2+</sup> (0.2 mol/L) and 55.0 wt.% with Ca<sup>2+</sup> (0.2 mol/L). The minimum amount of extracted rapeseed oleosomes was achieved when only ultra-pure water was used (42.7 wt.%), indicating that the cations interacted with the oleosome membrane, enhancing oleosome solubility and subsequently their extraction.

According to Hofmeister series (Roberts et al., 2015), a small difference between the effect of the two monovalent cations (Na<sup>+</sup> and K<sup>+</sup>) was expected. More precisely a slightly stronger solubilization effect from Na<sup>+</sup> than K<sup>+</sup> was expected, due to the order of these cations in the series, being K<sup>+</sup> exactly to the left of Na<sup>+</sup> on the series; however, the expected difference was not of this significant extent as extraction yield at the presence of K<sup>+</sup> was higher than at the presence of Na<sup>+</sup>. Besides the interaction with the membrane proteins, this phenomenon could be attributed to the interaction of the cations with the other membrane component, like the phospholipids and more specifically, phosphatidylcholine (Gurtovenko & Vattulainen, 2008; Mao et al., 2013). It has been reported that in comparison to K<sup>+</sup> the binding capacity of Na<sup>+</sup> to phosphatidylcholine is 2.2 folds higher, most likely due to its larger surface charge (Gurtovenko & Vattulainen, 2008). This would mean that maybe a significant amount of Na<sup>+</sup> binds to phosphatidylcholine and is not available for the oleosome extraction, but interacting with the phospholipid oleosome membrane. To understand whether the available concentration of Na<sup>+</sup> had an effect to oleosome extraction yield, a solution with higher Na<sup>+</sup> concentration (0.3 mol/L) was also used. The oleosome extraction yield with higher concentration of Na<sup>+</sup> (0.3 mol/L) slightly increased and resulted significantly different from

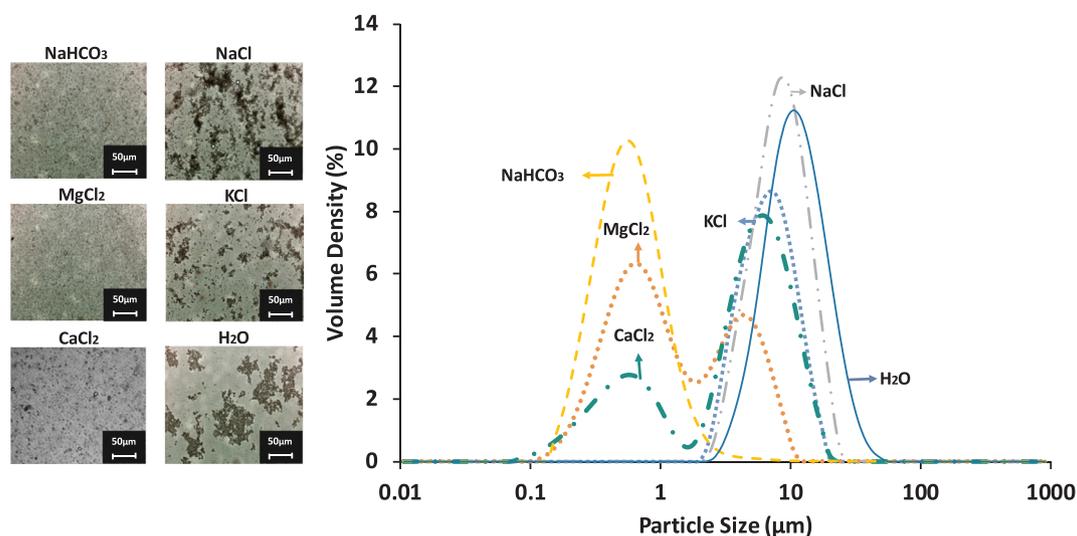
the obtained with Na<sup>+</sup> at 0.2 mol/L, reaching 55.3 wt.%, these difference could mean that when increasing the excess of cations not interacting with the phospholipid membrane could aid the extraction; however, still this higher concentration of Na<sup>+</sup> did not reach the extraction yield obtained when K<sup>+</sup> (0.2 mol/L) was present. Therefore, besides the interactions with other components of the interface and the affect on concentration, K<sup>+</sup> led to higher extraction yields. Furthermore, it is important to state that the yield in the presence of K<sup>+</sup> (0.2 mol/L) at pH 7 did not significantly differ from the yield obtained when NaHCO<sub>3</sub> buffer (0.1 mol/L) at pH 9.5 was used.

With regards to the divalent cations, they interacted as expected with oleosome interfacial proteins and significantly enhanced their extraction yield in comparison to pure water at the same pH. Divalent cations can affect salt bridges in proteins causing hydration and subsequent extraction (Arakawa & Timasheff, 1984). This mechanism explains the fact that divalent cations had a positive effect on oleosome extraction in comparison to pure water, however, the formation of new bridges resulted in a lower extraction yield in comparison to K<sup>+</sup>. Between the effect of the two divalent cations, no significantly differences were measured. According to Hofmeister series, this should be expected, since their effect on protein unfolding and solubility is similar (Roberts et al., 2015). The increase of the oleosome extraction yield with the aid of cations at neutral pH values is an important finding proving that high extraction yields of oleosomes cannot only be achieved in strongly alkaline environments.

Besides the effect of the cations on extraction yield, their effect on the stability against aggregation of the extracted oleosomes was also investigated. Fig. 1, shows the particle size distribution and the optical micrographs of the initially obtained oleosome extracts. Two types of peaks are observed, the first one observed from 0.1 to 2.0 μm, corresponding to individual oleosomes and the second one from 5 to 50 μm, corresponding to aggregates of oleosomes. The emulsions extracted at pH 9.5 (NaHCO<sub>3</sub>, 0.1 mol/L) yielded oleosomes of around 1 μm, evident of native individual oleosomes (De Chirico et al., 2018). The extracts with H<sub>2</sub>O or the monovalent cations at pH 7 exhibited extensive aggregation, showing a broad peak between 10 and 50 μm. The oleosome aggregation when Na<sup>+</sup> and K<sup>+</sup> were present at pH 7 has been previously reported (Iwanaga et al., 2007; Tzen, Lie, & Huang, 1992). This behaviour was expected due to the low electrokinetic potential (< 21.5 mV) (Table 2) and resulting from low electrostatic repulsion. The aggregates were probably formed due to hydrophobic forces between oleosomes and also between oleosomes and co-extracted extraneous proteins that can bridge neighboring oleosomes (Nikiforidis & Kiosseoglou, 2009). On the other hand, the emulsions extracted with divalent cations showed bimodal distributions as some of the oleosomes extracted with these cations were recovered as individual droplets with a similar distribution to those extracted at pH 9.5; however, aggregation was also observed. According to Table 2, the electrokinetic potentials of the divalent cations were in the same range (between -9.7 and -21.5 mV) as when the monovalent cations were present and copious protein-protein hydrophobic interactions should be expected. However, the presence of individual oleosomes indicates interactions of the divalent cations with the membrane proteins and also with the extraneous proteins inhibiting hydrophobic attractive forces. As caleosins' N-terminal containing the calcium binding site (Chen et al., 1999), is exposed to the bulk phase, it has been reported that both Ca<sup>2+</sup> and Mg<sup>2+</sup> interact with this site affecting the protein configuration and overall hydrophobicity (Allouche et al., 1999), however, more research is necessary to support this hypothesis.

#### 3.2. Effect of cations on the physical stability of dense oleosome creams

To investigate further the effect of the cations on oleosome stability, high-speed centrifugation (10,000g for 30 min) was applied to obtain densely packed oleosome creams. The ratio of oil and proteins obtained relates to the interactions of oleosomes with extraneous proteins



**Fig. 1.** Particle size distribution and microscopy images of the initial extracts obtained with (—) H<sub>2</sub>O (pH 7) (---) Na<sup>+</sup> (0.2 mol/L, pH 7), (●) K<sup>+</sup> (0.2 mol/L, pH 7), (●) Mg<sup>2+</sup> (0.2 mol/L, pH 7), (-.-.-) Ca<sup>2+</sup> (0.2 mol/L, pH 7) and (-.-.-) NaHCO<sub>3</sub> (0.1 mol/L, pH 9.5). The scale bar is 50 μm.

**Table 2**

Zeta potential of oleosomes final recovered creams.

Treatment	Zeta potential (mV)	Standard Deviation
Na <sup>+</sup> (0.2 mol/L, pH 7.0)	-21.5 <sup>a</sup>	± 0.4
K <sup>+</sup> (0.2 mol/L, pH 7.0)	-9.8 <sup>b</sup>	± 0.5
Mg <sup>2+</sup> (0.2 mol/L, pH 7.0)	-9.7 <sup>b</sup>	± 0.4
Ca <sup>2+</sup> (0.2 mol/L, pH 7.0)	-21.8 <sup>a</sup>	± 0.4
H <sub>2</sub> O (pH 7.0)	-20.24 <sup>c</sup>	± 0.4
NaHCO <sub>3</sub> (0.1 mol/L, pH 9.5)	-56.7 <sup>d</sup>	± 0.3

Values with different letters are significantly different with  $p < 0.05$ .

(Nikiforidis, Kiosseoglou, & Scholten, 2013) while possible physical destabilization indicates conformational changes on the membrane (Nikiforidis & Kiosseoglou, 2010). As it is presented in Table 3, the oleosome creams with K<sup>+</sup>, Na<sup>+</sup> or Mg<sup>2+</sup> had a lower oil to protein ratio compared to those that were extracted in the presence of Ca<sup>2+</sup>. On one hand the higher protein content with K<sup>+</sup> and Na<sup>+</sup> could explain the observed aggregates (Fig. 1), where extraneous proteins bridge oleosomes through hydrophobic forces and hence they are difficult to remove (Qi et al., 2017). On the other hand, the lower protein content observed when Ca<sup>2+</sup> was present indicates that there is less extraneous protein entrapped in the cream (Nikiforidis, Matsakidou, & Kiosseoglou, 2014).

As it is shown in Fig. 2 and as has been previously reported, extraneous proteins had a significant impact on oleosome stability against coalescence (Nikiforidis & Kiosseoglou, 2011; Zhao, Chen, Chen, Kong, & Hua, 2016). The oleosome creams obtained with H<sub>2</sub>O were the most stable against coalescence. Their size distribution showed a bimodal distribution with a peak corresponding to small individual oleosomes

**Table 3**

Protein and lipid content of the recovered oleosome creams extracted with different aqueous solvents.

		H <sub>2</sub> O		Na <sup>+</sup>		K <sup>+</sup>		Mg <sup>2+</sup>		Ca <sup>2+</sup>		NaHCO <sub>3</sub>	
		wt. %	STDv	wt. %	STDv	wt. %	STDv	wt. %	STDv	wt. %	STDv	wt. %	STDv
Wet basis	Lipids	42.8 <sup>a</sup>	± 2.8	56.3 <sup>b</sup>	± 3.5	52.2 <sup>c</sup>	± 0.4	69.2 <sup>d</sup>	± 0.4	66.6 <sup>e</sup>	± 0.5	70.9 <sup>d</sup>	± 1.2
	Protein	7.5 <sup>i</sup>	± 1.2	7.1 <sup>i</sup>	± 0.5	8.2 <sup>iii</sup>	± 1.3	9.4 <sup>ii</sup>	± 0.5	5.1 <sup>iii</sup>	± 0.5	3.9 <sup>iii</sup>	± 0.1
	Ratio lipids:proteins	5.7	-	7.8	-	6.3	-	7.3	-	12.8	-	17.5	-
Dry basis	Lipids	60.6 <sup>a</sup>	± 2.8	81.1 <sup>bc</sup>	± 3.5	73.6 <sup>d</sup>	± 0.4	79.1 <sup>b</sup>	± 0.4	85.1 <sup>e</sup>	± 0.5	84.2 <sup>c-e</sup>	± 1.2
	Protein	10.6	± 1.2	10.3 <sup>i</sup>	± 0.5	12.6 <sup>i</sup>	± 1.3	11.6 <sup>i</sup>	± 0.5	6.6 <sup>ii</sup>	± 0.5	4.7 <sup>iii</sup>	± 0.1
	Ratio lipids:proteins	5.7	-	7.6	-	6.4	-	7.4	-	12.4	-	17.0	-

Values with different letters are significantly different with  $p < 0.05$ .

from 0.05 to 0.7 μm and another peak corresponding to aggregates with a size between 0.3 and 20 μm, but no coalesced droplets were observed. The oleosome creams obtained with K<sup>+</sup> or Na<sup>+</sup>, show similar distributions, where slight coalescence was observed. The case of Ca<sup>2+</sup> and Mg<sup>2+</sup> was different since there was minor aggregation after the oleosome extraction in comparison with the extracts recovered with monovalent cations, however, the applied centrifugal forces lead to extensive coalescence and subsequent oil separation.

Besides the effect of extraneous proteins that can form an additional film around oleosomes and prevent coalescence, interactions of the cations with the membrane molecules might also lead to reconfiguration and destabilization. When pure water was used to extract oleosomes, large aggregates were formed, while the droplets were very stable against coalescence, as the smallest individual droplets were recovered with this medium (Fig. 2). However, at the presence of Na<sup>+</sup> and K<sup>+</sup>, the oleosomes were less stable against coalescence, indicating an effect of the monovalent cations on the membrane molecular interactions. The extensive coalescence when divalent cations were present (Fig. 2) shows that divalent cations had a stronger effect on the membrane inter- and intra-molecular interactions. The specific Ca<sup>2+</sup> binding site on caleosins is an indication of potential interactions of caleosins with the excess of Ca<sup>2+</sup> or in general with divalent cations. It has been reported that the exposure of caleosins to divalent cations (Ca<sup>2+</sup> or Mg<sup>2+</sup>) affects the tertiary and quaternary structure (Allouche et al., 1999; Purkrtova et al., 2008) however, the type of the interactions and the effect on oleosome membrane stability have to be further investigated.

Finally, regarding the presence of NaHCO<sub>3</sub> (pH 9.5) the mechanism is completely different. The electrokinetic potential of the oleosomes at

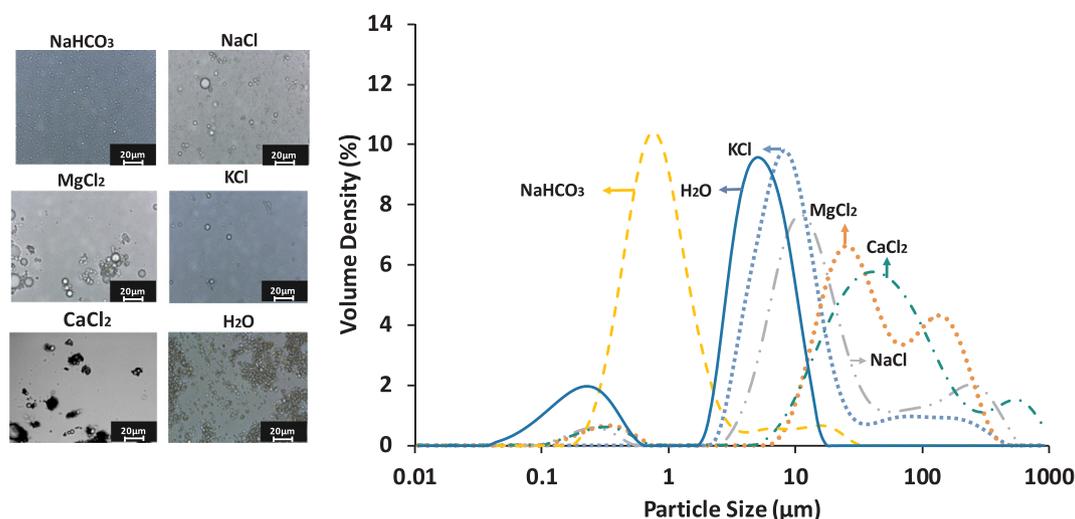


Fig. 2. Particle size distribution and microscopy images of the final oleosome creams after high speed centrifugation obtained with (—) H<sub>2</sub>O (pH 7) (---) Na<sup>+</sup> (0.2 mol/L, pH 7), (---) K<sup>+</sup> (0.2 mol/L, pH 7), (---) Mg<sup>2+</sup> (0.2 mol/L, pH 7), (---) Ca<sup>2+</sup> (0.2 mol/L, pH 7) and (---) NaHCO<sub>3</sub> (0.1 mol/L, pH 9.5). The scale bar is 20 μm.

this pH is very high,  $-57$  mV (Table 2), which creates strong repulsive electrostatic forces and prevents both aggregation and coalescence. This performance has reported for most cases where pH values between 9.0 and 9.5 were used (De Chirico et al., 2018; Wang et al., 2019).

#### 4. Conclusion

The presence of monovalent (K<sup>+</sup> or Na<sup>+</sup>) and divalent (Ca<sup>2+</sup> or Mg<sup>2+</sup>) cations significantly enhanced the extraction of oleosomes at pH 7. All extraction yields achieved in the presence of cations were significantly different than the one with H<sub>2</sub>O at pH 7, which was about 43 wt.%. More specifically, the presence of K<sup>+</sup> at pH 7, reached a yield of 64 wt.% that was no significantly different that the one obtained when pH 9.5 was used. Cations at specific concentrations can break the salt bridges in proteins, interrupt their interactions and lead to an increase of their extraction yield. These results show that the interactions between oleosomes and between oleosomes and co-extracted proteins can be inhibited either by pH adjustment to strong alkaline environments or at the presence of cations. Moreover, the interactions of the cations with the oleosome membrane had an effect on the stability of oleosome extracts. In the absence of cations at pH 7, extensive aggregation was observed, which can be attributed to hydrophobic forces and the low electrokinetic potential of the system. The addition of monovalent cations caused extensive aggregation as well, while the divalent cations partly reduced the formation of aggregates. Divalent cations probably interacted with the oleosome membrane proteins, altering their re-configuration and inhibited the protein-protein hydrophobic interactions. However, when a dense oleosome cream was created, the oleosomes obtained with H<sub>2</sub>O retained their integrity, while those obtained with monovalent cations showed slightly coalescence and those obtained with divalent cations where extensively coalesced. These results suggest that, membrane protein re-configuration due to the presence of divalent cations has a significant negative impact on oleosome stability.

#### 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.

#### Acknowledgments

The authors would like to thank the National Council on Science and Technology of Mexico (CONACYT) for sponsoring this project. Fellow No. 218820.

#### References

- Allouche, D., Parello, J., & Sanejouand, Y. H. (1999). Ca<sup>2+</sup>/Mg<sup>2+</sup> exchange in parvalbumin and other EF-hand proteins. A theoretical study. *Journal of Molecular Biology*, 285(2), 857–873.
- Arakawa, T., & Timasheff, S. N. (1984). Mechanism of protein salting in and salting out by divalent cation salts: Balance between hydration and salt binding. *Biochemistry*, 25, 5912–5923.
- Chen, J. C., Tsai, C. C., & Tzen, J. T. (1999). Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. *Plant Cell Physiology*, 40(10), 1079–1086.
- Collins, K. D. (2004). Ions from the Hofmeister series and osmolytes: Effects on proteins in solution and in the crystallization process. *Methods*, 34(3), 300–311.
- De Chirico, S., di Bari, V., Foster, T., & Gray, D. (2018). Enhancing the recovery of oilseed rape seed oil bodies (oleosomes) using bicarbonate-based soaking and grinding media. *Food Chemistry*, 241, 419–426.
- Deleu, M., Vaca-Medina, G., Fabre, J.-F., Roiz, J., Valentin, R., & Mouloungui, Z. (2010). Interfacial properties of oleosins and phospholipids from rapeseed for the stability of oil bodies in aqueous medium. *Colloids and Surfaces B, Biointerfaces*, 80(2), 125–132.
- Dumetz, A. C., Snellinger-O'Brien, A. M., Kaler, E. W., & Lenhoff, A. M. (2007). Patterns of protein-protein interactions in salt solutions and implications for protein crystallization. *Protein Science*, 16, 1867–1877.
- Eren, N. M., Narsimhan, G., & Campanella, O. H. (2016). Protein adsorption induced bridging flocculation: The dominant entropic pathway for nano-bio complexation. *Nanoscale*, 8, 3326–3336.
- Gurtovenko, A. A., & Vattulainen, I. (2008). Effect of NaCl and KCl on phosphatidylcholine and phosphatidylethanolamine lipid Membranes: Insight from atomic-scale simulations for understanding salt-induced effects in the plasma membrane. *Journal of Physical Chemistry B*, 112(7), 1953–1962.
- Hammond, E. G., Johnson, L. A., Su, C., Wang, T., & White, P. J. (2005). Soybean Oil. In F. Shahidi (Ed.), *Bailey's Industrial Oil and Fat Products* (6th ed.). New York: John Wiley & Sons in press.
- Iwanaga, D., Gray, D. A., Fisk, I. D., Decker, E. A., Weiss, J., & McClements, D. J. (2007). Extraction and characterization of oil bodies from soy beans: A natural source of pre-emulsified soybean oil. *Journal of Agricultural and Food Chemistry*, 55(21), 8711–8716.
- Jolivet, P., Aymé, L., Giuliani, A., Wien, F., Chardot, T., & Gohon, Y. (2017). Structural proteomics: Topology and relative accessibility of plant lipid droplet associated proteins. *Journal of Proteomics*, 3(169), 87–98.
- Jolivet, P., Boulard, C., Bellamy, A., Valot, B., D'Andréa, S., Zivy, M., ... Chardot, T. (2011). Oil body proteins sequentially accumulate throughout seed development in *Brassica napus*. *Journal of Plant Physiology*, 168(17), 2015–2020.
- Levy, Y., & Onuchic, J. N. (2004). Water and proteins: A love-hate relationship. *Proceedings of the National Academy of Sciences of the United States of America*, 101(10), 3325–3326.
- Lin, L. J., Liao, P. C., Yang, H. H., & Tzen, J. T. C. (2005). Determination and analyses of the N-termini of oil-body proteins, steroleosin, caleosin and oleosin. *Plant Physiology and Biochemistry*, 43(8), 770–776.

- Lindeboom, N., & Wanasundara, P. K. J. P. D. (2007). Interference of phenolic compounds in *Brassica napus*, *Brassica rapa* and *Sinapis alba* seed extracts with the Lowry protein assay. *Food Chemistry*, *104*(1), 30–38.
- Mao, Y., Du, Y., Cang, X., Wang, J., Chen, Z., Yang, H., & Jiang, H. (2013). Binding competition to the POPG lipid bilayer of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, and K<sup>+</sup> in different ion mixtures and biological implication. *Journal of Physical Chemistry B*, *117*(3), 850–858.
- Matsakidou, A., Mantzouridou, F. T., & Kiosseoglou, V. (2015). Optimization of water extraction of naturally emulsified oil from maize germ. *LWT – Food Science and Technology*, *63*(1), 206–213.
- McClements, D. J. (2004). *Food emulsions: Principles, practices, and techniques* (2nd ed.). Boca Raton, Florida, USA: CRC Press.
- Nikiforidis, C. V., Kiosseoglou, V., & Scholten, E. (2013). Oil bodies: An insight on their microstructure – Maize germ vs sunflower seed. *Food Research International*, *52*(1), 136–141.
- Nikiforidis, C. V., Matsakidou, A., & Kiosseoglou, V. (2014). Composition, properties and potential food applications of natural emulsions and cream materials based on oil bodies. *RSC Advances*, *4*(48), 25067.
- Nikiforidis, C. V., & Kiosseoglou, V. (2009). Aqueous extraction of oil bodies from maize germ (*Zea mays*) and characterization of the resulting natural oil-in-water emulsion. *Journal of Agricultural and Food Chemistry*, *57*(12), 5591–5596.
- Nikiforidis, C. V., Donsouzi, S., & Kiosseoglou, V. (2016). The interplay between diverse oil body extracts and exogenous biopolymers or surfactants. *Food Research International*, *83*(3), 14–24.
- Nikiforidis, C. V., & Kiosseoglou, V. (2009). Aqueous extraction of oil bodies from maize germ (*Zea mays*) and characterization of the resulting natural oil-in-water emulsion. *Journal of Agricultural and Food Chemistry*, *57*(12), 5591–5596.
- Nikiforidis, C. V., & Kiosseoglou, V. (2010). Physicochemical stability of maize germ oil body emulsions as influenced by oil body surface-xanthan gum interactions. *Journal of Agricultural and Food Chemistry*, *58*(1), 527–532.
- Nikiforidis, C. V., & Kiosseoglou, V. (2011). Competitive displacement of oil body surface proteins by Tween 80 – Effect on physical stability. *Food Hydrocolloids*, *25*(5), 1063–1068.
- Purkrtova, Z., Jolivet, P., Miquel, M., & Chardot, T. (2008). Structure and function of seed lipid body-associated proteins. *Comptes Rendus Biologies*, *331*(10), 746–754.
- Qi, B., Ding, J., Wang, Z., Li, Y., Ma, C., Chen, F., & Jiang, L. (2017). Deciphering the characteristics of soybean oleosome-associated protein in maintaining the stability of oleosomes as affected by pH. *Food Research International*, *100*(1), 551–557.
- Roberts, D., Keeling, R., Tracka, M., Van Der Walle, C. F., Uddin, S., Warwicker, J., & Curtis, R. (2015). Specific ion and buffer effects on protein-protein interactions of a monoclonal antibody. *Molecular Pharmaceutics*, *12*(1), 179–193.
- Shimada, T. L., & Hara-Nishimura, I. (2010). Oil-body-membrane proteins and their physiological functions in plants. *Biological & Pharmaceutical Bulletin*, *33*(3), 360–363.
- Song, W., Qin, Y., Zhu, Y., Yin, G., Wu, N., Li, Y., & Hu, Y. (2014). Delineation of plant caleosin residues critical for functional divergence, positive selection and coevolution. *BMC Evolutionary Biology*, *14*(1), 1–14.
- Thiyam-Hollaender, U., Eskin, N., & Michael, N. (2012). *Canola and rapeseed: Production, processing, food quality, and nutrition*. Boca Raton, Florida, USA: CRC Press.
- Tzen, J. T. C., Lie, G. C., & Huang, A. H. C. C. (1992). Characterization of the charged components and their topology on the surface of plant seed oil bodies. *Journal of Biological Chemistry*, *267*(22), 15626–15634.
- Tzen, J. T., & Huang, A. H. (1992). Surface structure and properties of plant seed oil bodies. *The Journal of Cell Biology*, *117*(2), 327–335.
- Tzen, J. T. C. (2012). Integral proteins in plant oil bodies. *ISRN Botany*, *2012*, 1–16.
- Tzen, J. T. C., Cao, Y., Laurent, P., Ratnayake, C., & Huang, A. (1993). Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiology*, *101*(1), 267–276.
- Wang, W., Cui, C., Wang, Q., Sun, C., Jiang, L., & Hou, J. (2019). Effect of pH on physicochemical properties of oil bodies from different oil crops. *Journal of Food Science and Technology*, *56*(1), 49–58.
- Zhang, Y., & Cremer, P. S. (2006). Interactions between macromolecules and ions: The Hofmeister series. *Current Opinion in Chemical Biology*, *10*(6), 658–663.
- Zhao, L., Chen, Y., Chen, Y., Kong, X., & Hua, Y. (2016). Effects of pH on protein components of extracted oil bodies from diverse plant seeds and endogenous protease-induced oleosin hydrolysis. *Food Chemistry*, *200*, 125–133.