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

26 Oleosomes are storage vehicles of TAGs in plant seeds. They are protected with a phospholipid-
27 protein monolayer and extracted with alkaline aqueous media; however, pH adjustment intensifies
28 the extraction process. Therefore, the aim of this work was to investigate the extraction mechanism
29 of rapeseed oleosomes at pH 7 and at the presence of monovalent and divalent cations (Na^+, K^+,
30 Mg^{2+}, and Ca^{+2}). The oleosome yield at pH 9.5 was 64 wt.%, while the yield at pH 7 with H_2O was
31 just 43 wt.%. The presence of cations at pH 7, significantly enhanced the yield, with K^+ giving the
32 highest yield (64 wt.%). The cations affected the oleosome interface and their interactions. The
33 presence of monovalent cations resulted in aggregation and minor coalescence, while divalent
34 cations resulted in extensive coalescence. These results help to understand the interactions of
35 oleosomes in their native matrix and design simple extraction processes at neutral conditions.

36 **Keywords:** oil bodies, extraction, natural emulsion, rapeseed, oleosomes.

37 ## 1. Introduction

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

54 The most abundant proteins on the oleosome membrane are oleosins, which represent up to 75-
55 80% of the oleosome membrane protein content (Jolivet et al., 2011; Tzen, 2012). Oleosins are a
56 group of proteins with a low molecular weight (14-17 kDa) and are composed by a hydrophobic
57 tail that is anchored in the oil core and two short fairly hydrophilic terminals that are on the
58 oleosome surface (Lin, Liao, Yang, & Tzen, 2005). The other group of proteins present on the
59 oleosome membrane are caleosins (24-28 kDa) and steroleosins (35-60 kDa) (Lin et al., 2005).

60 Similar to oleosins, these proteins have also a hydrophobic tail, which is smaller than the one of
61 oleosins and a longer domain exposed to the bulk phase (Shimada & Hara-Nishimura, 2010). Even
62 though the exact biological functions of the membrane proteins are still to be defined (Purkrtova,
63 Jolivet, Miquel, & Chardot, 2008; Song et al., 2014), it is known that caleosins have a unique Ca^{2+}
64 binding site on the N-terminal of the protein that can also bind Mg^{2+} (Allouche, Parello, &
65 Sanejouand, 1999; Chen, Tsai, & Tzen, 1999), while steroleosins have a hydrophilic sterol-binding
66 dehydrogenase domain (Purkrtova et al., 2008). Regarding the phospholipids at the oleosome
67 interface, the main type present is phosphatidylcholine representing 65 % (wt.%) of the total
68 phospholipids, followed by phosphatidylserine, phosphatidylinositol and
69 phosphatidylethanolamine (Deleu et al., 2010; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993).

70 The understanding of the architecture of the oleosome interface, the molecular combination and
71 the forces that might occur, will help towards optimizing their extraction. Both proteins and
72 phospholipids are charged molecules and electrostatic forces can occur between neighbouring
73 oleosomes and also between oleosomes and surrounding charged material (Nikiforidis &
74 Kiosseoglou, 2011). Besides electrostatic forces, hydrophobic attractive forces might take place as
75 well. The domains of the oleosome proteins that are exposed to the bulk phase are fairly
76 hydrophilic, however, they also contain hydrophobic patches that can attract each other and lead
77 to aggregation of neighbouring oleosomes (Jolivet et al., 2017; Nikiforidis, Donsouzi, &
78 Kiosseoglou, 2016; Nikiforidis & Kiosseoglou, 2011). Furthermore, the hydrophobic domains of
79 extrinsic proteins might interact with the oleosome proteins leading to bridging flocculation (Eren,
80 Narsimhan, & Campanella, 2016). Hydrophobic attractive forces can be prevented by using
81 surfactants, like Tween or SDS (Nikiforidis et al., 2016; Nikiforidis & Kiosseoglou, 2011).

82 Nevertheless, the addition of surfactants may affect the oleosome membrane, therefore this
83 research was mostly focused on affect hydrophobic interactions by electrostatic interactions.

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

98 **2. Materials & Methods**

99 **2.1 Materials**

100 Untreated rapeseeds (Brassica napus), type Allize were kindly pursued by the Division of Food
101 Sciences, University of Nottingham, Sutton Bonington, UK. Magnesium Chloride ($MgCl_2$) was
102 obtained from Merck (Darmstadt, Germany). All other chemicals including the sodium chloride,
103 potassium chloride and calcium chloride (Nalco, KCl, $CaCl_2$) were obtained in analytical grade

104 from Sigma-Aldrich (St. Louis, MO, USA). Solutions and dispersions were made with ultrapure
105 water (MilliQ) obtained with a Merck Millipore device (Darmstadt, Germany).

106 2.2 Oleosome aqueous extraction

107 Rapeseed oleosomes were isolated using the extraction method proposed by De Chirico et al.
108 (2018), with some modifications based on the method proposed by Nikiforidis et al. (2009). The
109 different aqueous media were prepared by dissolving the different salts (NaCl, KCl, $MgCl_2$, $CaCl_2$,
110 0.2 mol/L) in ultra-pure water (MilliQ) and adjusting their pH to 7.0 with a solution of NaOH (0.1
111 mol/L) or HCl (0.1 mol/L). The additional aqueous solution made by NaCl (0.3 mol/L) was
112 elaborated in a similar way than the other salted-aqueous media. The alkaline aqueous media was
113 prepared similarly, by dissolving $NaHCO_3$ 0.1 mol/L and adjusting the pH to pH 9.5 with NaOH
114 (1.0 mol/L). A SevenMulti™ dual meter pH/conductivity (Mettler Toledo, Greifensee,
115 Switzerland) was used to monitor the pH. The seeds were soaked (1:1 w/v) in the different aqueous
116 media for 16h at 4°C. After soaking, the solid/solvent ratio was adjusted to 1:7 w/v and the
117 dispersion was blended for 60 s at 7200 rpm (Thermomix TM31, Utrecht, The Netherlands). The
118 mixture was then filtered through two layers of cheesecloth (GEFU®, Eslohe, Germany). The first
119 extract (filtrate) was centrifuged at 3000 g for 15 min at 4°C. After the centrifugation step, three
120 different layers were observed: the cream, the serum and the precipitate. The oleosome cream was
121 manually collected, dispersed in ultra-pure water (MilliQ) (1:4 w/v) and centrifuged at 10000 g
122 for 30 min at 4°C. This washing step was repeated twice. The oleosome extraction yield was
123 calculated based on the difference between lipid content remaining in the cake and the initial lipid
124 content in the seeds.

125 2.3 Compositional analysis of all streams

126 The moisture content of the retentate and oleosome cream was determined using a Moisture
127 Analyzer (MA35M, Sartorius Gottingen, Germany). Oil quantification was performed on dry
128 samples that where placed in a Soxhlet device (Buchi extractor, Büchi, Flawil, Switzerland) for 9
129 h, while the oil was extracted using petroleum ether. The oleosome extraction yield was calculated
130 based on the oil left in the solid residue after the extraction (cake) and the initial amount of oil in
131 the seeds (36.6 ± 0.5%). The protein content of the defatted samples was calculated by determining
132 the amount of Nitrogen in the samples using the Dumas method and using a conversion factor of
133 5.5 as suggested in literature(Lindeboom & Wanasundara, 2007) (Nitrogen analyzer, FlashEA 112
134 series, Thermo Scientific, Interscience, The Netherlands).

135 **2.4 Determination of oleosome particle size distribution**

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

141 **2.5 Determination of oleosome zeta potential**

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

146 **2.6 Optical microscopy analysis of oleosome emulsions**

147 Images of the oleosome emulsions were taken with the microscope AxioVision V 4.8.3.0 (Carl
148 Zeiss MicroImaging, GmbH) equipped with a digital camera (Axiocam MRc 5). The oleosome
149 cream for each treatment was diluted with ultrapure water (1:100 w/v) and one drop of the
150 emulsion was added on a glass slide and placed onto the microscope. The magnification used was
151 100x.

152 **2.7 Statistical analysis**

153 All the measurements and extractions were performed at least in triplicates. One-way analysis of
154 variance (ANOVA) test was applied to detect differences among the extraction yields as function
155 of the aqueous extraction media. Analyses were performed with the IBM SPSS statistics 23
156 software. Differences were significant at $p<0.05$.

157 **3. Results and discussion**

158 **3.1 Effect of cations on oleosome extraction yield and stability**

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

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

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

190 with higher concentration of Na^+ (0.3 mol/L) slightly increased and resulted significantly different
191 from the obtained with Na^+ at 0.2 mol/L, reaching 55.3 wt.%, these difference could mean that
192 when increasing the excess of cations not interacting with the phospholipid membrane could aid
193 the extraction; however, still this higher concentration of Na^+ did not reach the extraction yield
194 obtained when K^+ (0.2 mol/L) was present. Therefore, besides the interactions with other
195 components of the interface and the effect on concentration, K^+ leaded to higher extraction yields.
196 Furthermore, it is important to state that the yield in the presence of K^+ (0.2 mol/L) at pH 7 did not
197 significantly differ from the yield obtained when $NaHCO_3$ buffer (0.1 mol/L) at pH 9.5 was used.

198 With regards to the divalent cations, they interacted as expected with oleosome interfacial proteins
199 and significantly enhanced their extraction yield in comparison to pure water at the same pH.
200 Divalent cations can affect salt bridges in proteins causing hydration and subsequent extraction
201 (Arakawa & Timasheff, 1984). This mechanism explains the fact that divalent cations had a
202 positive effect on oleosome extraction in comparison to pure water, however, the formation of new
203 bridges resulted in a lower extraction yield in comparison to K^+. Between the effect of the two
204 divalent cations, no significantly differences were measured. According to Hofmeister series, this
205 should be expected, since their effect on protein unfolding and solubility is similar (Roberts et al.,
206 2015). The increase of the oleosome extraction yield with the aid of cations at neutral pH values
207 is an important finding proving that high extraction yields of oleosomes cannot only be achieved
208 in strongly alkaline environments.

209 Besides the effect of the cations on extraction yield, their effect on the stability against aggreation
210 of the extracted oleosomes was also investigated. Figure 1, shows the particle size distribution and
211 the optical micrographs of the initially obtained oleosome extracts. Two types of peaks are
212 observed, the first one observed from 0.1 to 2.0 μm, corresponding to individual oleosomes and

213 the second one from 5 to 50 µm, corresponding to aggregates of oleosomes. The emulsions
214 extracted at pH 9.5 ($NaHCO_3$, 0.1 mol/L) yielded oleosomes of around 1 µm, evident of native
215 individual oleosomes (De Chirico et al., 2018). The extracts with H_2O or the monovalent cations
216 at pH 7 exhibited extensive aggregation, showing a broad peak between 10 and 50 µm. The
217 oleosome aggregation when Na^+ and K^+ were present at pH 7 has been previously reported
218 (Iwanaga et al., 2007; Tzen, Lie, & Huang, 1992). This behaviour was expected due to the low
219 electrokinetic potential (< 21.5 mV) (Table 2) and resulting from low electrostatic repulsion. The
220 aggregates were probably formed due to hydrophobic forces between oleosomes and also between
221 oleosomes and co-extracted extraneous proteins that can bridge neighboring oleosomes
222 (Nikiforidis & Kiosseoglou, 2009). On the other hand, the emulsions extracted with divalent
223 cations showed bimodal distributions as some of the oleosomes extracted with these cations were
224 recovered as individual droplets with a similar distribution to those extracted at pH 9.5; however,
225 aggregation was also observed. According to Table 2, the electrokinetic potentials of the divalent
226 cations were in the same range (between -9.7 and -21.5 mV) as when the monovalent cations were
227 present and copious protein-protein hydrophobic interactions should be expected. However, the
228 presence of individual oleosomes indicates interactions of the divalent cations with the membrane
229 proteins and also with the extraneous proteins inhibiting hydrophobic attractive forces. As
230 caleosins' N-terminal containing the calcium binding site (Chen et al., 1999), is exposed to the
231 bulk phase, it has been reported that both Ca^{2+} and Mg^{2+} interact with this site affecting the protein
232 configuration and overall hydrophobicity (Allouche et al., 1999), however, more research is
233 necessary to support this hypothesis.

234 **3.2 Effect of cations on the physical stability of dense oleosome creams**

235 To investigate further the effect of the cations on oleosome stability, high-speed centrifugation
236 (10,000 g for 30 min) was applied to obtain densely packed oleosome creams. The ratio of oil and
237 proteins obtained relates to the interactions of oleosomes with extraneous proteins (Nikiforidis,
238 Kiosseoglou, & Scholten, 2013) while possible physical destabilization indicates conformational
239 changes on the membrane (Nikiforidis & Kiosseoglou, 2010). As it is presented in Table 3, the
240 oleosome creams with K^+, Na^+ or Mg^{2+} had a lower oil to protein ratio compared to those that were
241 extracted in the presence of Ca^{2+}. On one hand the higher protein content with K^+ and Na^+ could
242 explain the observed aggregates (Figure 1), where extraneous proteins bridge oleosomes through
243 hydrophobic forces and hence they are difficult to remove (Qi et al., 2017). On the other hand, the
244 lower protein content observed when Ca^{2+} was present indicates that there is less extraneous
245 protein entrapped in the cream (Nikiforidis, Matsakidou, & Kiosseoglou, 2014).

246 As it is shown in Figure 2 and as has been previously reported, extraneous proteins had a significant
247 impact on oleosome stability against coalescence (Nikiforidis & Kiosseoglou, 2011; Zhao, Chen,
248 Chen, Kong, & Hua, 2016). The oleosome creams obtained with H_2O were the most stable against
249 coalescence. Their size distribution showed a bimodal distribution with a peak corresponding to
250 small individual oleosomes from 0.05 to 0.7 μm and another peak corresponding to aggregates
251 with a size between 0.3 to 20 μm, but no coalesced droplets were observed. The oleosome creams
252 obtained with K^+ or Na^+, show similar distributions, where slight coalescence was observed. The
253 case of Ca^{2+} and Mg^{2+} was different since there was minor aggregation after the oleosome
254 extraction in comparison with the extracts recovered with monovalent cations, however, the
255 applied centrifugal forces lead to extensive coalescence and subsequent oil separation.

256 Besides the effect of extraneous proteins that can form an additional film around oleosomes and
257 prevent coalescence, interactions of the cations with the membrane molecules might also lead to

258 reconfiguration and destabilization. When pure water was used to extract oleosomes, large
259 aggregates were formed, while the droplets were very stable against coalescence, as the smallest
260 individual droplets were recovered with this medium (Figure 2). However, at the presence of Na^+
261 and K^+, the oleosomes were less stable against coalescence, indicating an effect of the monovalent
262 cations on the membrane molecular interactions. The extensive coalescence when divalent cations
263 were present (Figure 2) shows that divalent cations had a stronger effect on the membrane inter-
264 and intra-molecular interactions. The specific Ca^{2+} binding site on caleosins is an indication of
265 potential interactions of caleosins with the excess of Ca^{2+} or in general with divalent cations. It has
266 been reported that the exposure of caleosins to divalent cations (Ca^{2+} or Mg^{2+}) affects the tertiary
267 and quaternary structure (Allouche et al., 1999; Purkrtova et al., 2008) however, the type of the
268 interactions and the effect on oleosome membrane stability have to be further investigated.

269 Finally, regarding the presence of $NaHCO_3$ (pH 9.5) the mechanism is completely different. The
270 electrokinetic potential of the oleosomes at this pH is very high, -57 mV (Table 2), which creates
271 strong repulsive electrostatic forces and prevents both aggregation and coalescence. This
272 performance has reported for most cases where pH values between 9.0 and 9.5 were used (De
273 Chirico et al., 2018; Wang et al., 2019).

274 4. Conclusion

275 The presence of monovalent (K^+ or Na^+) and divalent (Ca^{2+} or Mg^{2+}) cations significantly
276 enhanced the extraction of oleosomes at pH 7. All extraction yields achieved in the presence of
277 cations were significantly different than the one with H_2O at pH 7, which was about 43 wt.%. More
278 specifically, the presence of K^+ at pH 7, reached a yield of 64 wt.% that was no significantly
279 different that the one obtained when pH 9.5 was used. Cations at specific concentrations can break

280 the salt bridges in proteins, interact their interactions and lead to an increase of their extraction
281 yield. These results show that the interactions between oleosomes and between oleosomes and co-
282 extracted proteins can be inhibited either by pH adjustment to strong alkaline environments or at
283 the presence of cations. Moreover, the interactions of the cations with the oleosome membrane
284 influenced the stability of oleosome extracts. In the absence of cations at pH 7, extensive
285 aggregation was observed, which can be attributed to hydrophobic forces and the low
286 electrokinetic potential of the system. The addition of monovalent cations caused extensive
287 aggregation as well, while the divalent cations partly reduced the formation of aggregates. Divalent
288 cations probably interacted with the oleosome membrane proteins, altering their re-configuration
289 and inhibited the protein-protein hydrophobic interactions. However, when a dense oleosome
290 cream was created, the oleosomes obtained with H_2O retained their integrity, while those obtained
291 with monovalent cations showed slightly coalescence and those obtained with divalent cations
292 where extensively coalesced. These results suggest that, membrane protein re-configuration due
293 to the presence of divalent cations has a significant negative impact on oleosome stability.

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

Aqueous solvent	Oleosome extraction yield (wt%)	Standard Deviation
H_2O pH7	42.7[a]	± 1.9
Na^+, 0.2 mol/L pH7	50.2[b]	± 2.0
Na^+, 0.3 mol/L pH7	55.3[c]	± 1.8
K^+, 0.2 mol/L pH7	64.2[d]	± 0.6
Mg^{2+}, 0.2 mol/L pH7	52.5[c]	± 4.9
Ca^{2+}, 0.2 mol/L pH7	55.0[c]	± 2.3
$NaHCO_3$, 0.1 mol/L pH 9.5	63.6[d]	± 0.5

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

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422 **Table 2.** Zeta potential of oleosomes final recovered creams.

Treatment	Zeta potential (mV)	Standard Deviation
Na^+ (0.2 mol/L, pH 7.0)	-21.5[a]	±0.4
K^+ (0.2 mol/L, pH 7.0)	-9.8[b]	±0.5
Mg^{2+} (0.2 mol/L, pH 7.0)	-9.7[b]	±0.4
Ca^{2+} (0.2 mol/L, pH 7.0)	-21.8[a]	±0.4
H_2O (pH 7.0)	-20.24[c]	±0.4
$NaHCO_3$ (0.1 mol/L, pH 9.5)	-56.7[d]	±0.3

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

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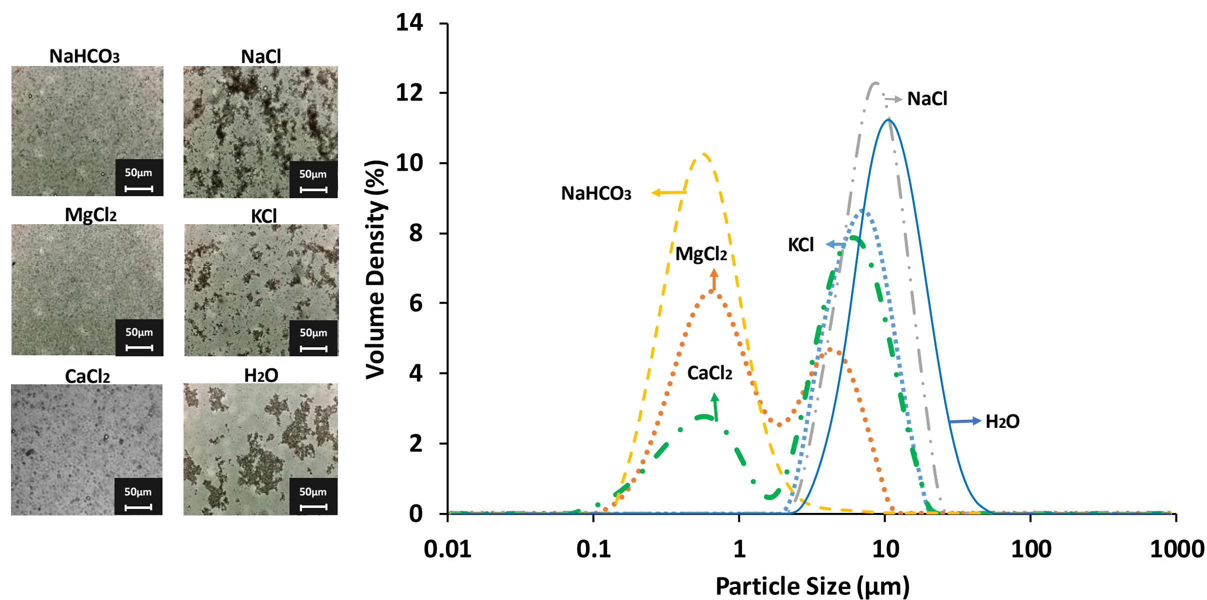
Table 3. Protein and lipid content of the recovered oleosome creams extracted with different aqueous solvents.

		H_2O		Na^+		K^+		Mg^{2+}		Ca^{2+}		$NaHCO_3$	
		wt. %	**STDv**	**wt. %**	**STDv**	**wt. %**	**STDv**	**wt. %**	**STDv**	**wt. %**	**STDv**	**wt. %**	**STDv**
Wet basis	Lipids	42.8^{a}	±2.8	56.3^{b}	±3.5	52.2^{c}	±0.4	69.2^{d}	±0.4	66.6^{e}	±0.5	70.9^{d}	±1.2
	Protein	7.5^{i}	±1.2	7.1^{i}	±0.5	8.2^{i,ii}	±1.3	9.4^{ii}	±0.5	5.1^{iii}	±0.5	3.9^{iii}	±0.1
	Ratio lipids:proteins	5.7	-	7.8	-	6.3	-	7.3	-	12.8	-	17.5	-
Dry basis	Lipids	60.6^{a}	±2.8	81.1^{b,c}	±3.5	73.6^{d}	±0.4	79.1^{b}	±0.4	85.1^{e}	±0.5	84.2^{c,e}	±1.2
	Protein	10.6	±1.2	10.3^{i}	±0.5	12.6^{i}	±1.3	11.6^{i}	±0.5	6.6^{ii}	±0.5	4.7^{iii}	±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 $p<0.05$



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460 **Caption to Figure 1**

461 Particle size distribution and microscopy images of the initial extracts obtained with (—) H_2O (pH
462 7) (-···-) Na^+ (0.2 mol/L, pH 7), (▪) K^+ (0.2 mol/L, pH 7), (•) Mg^{2+} (0.2 mol/L, pH 7), (-·-·-) Ca^{2+}
463 (0.2 mol/L, pH 7) and (---) $NaHCO_3$ (0.1 mol/L, pH 9.5). The scale bar is 50 µm.

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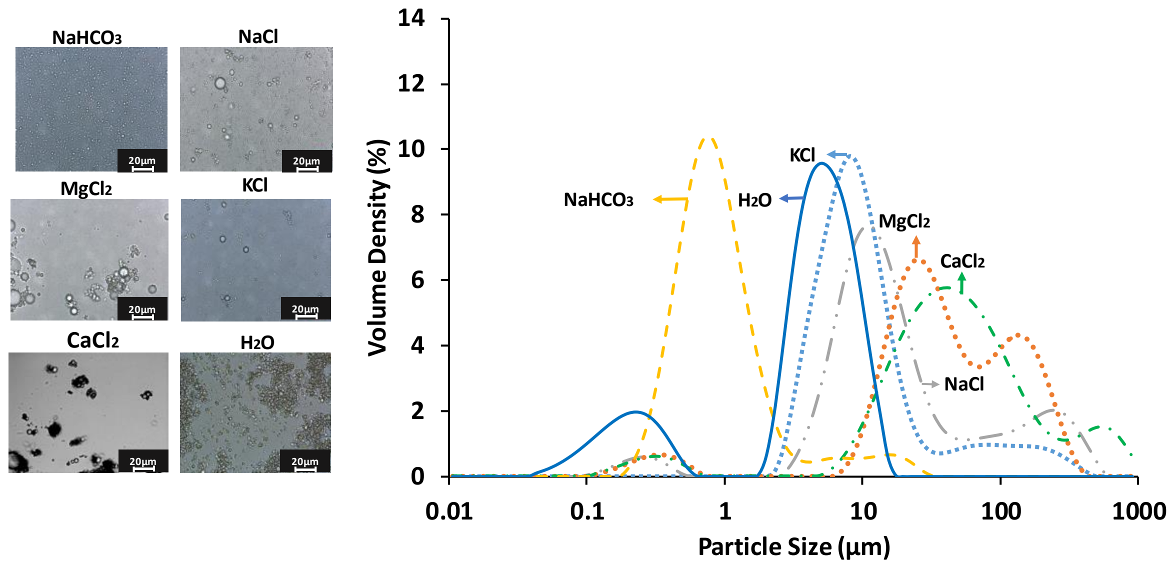

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471 **Caption to Figure 2**

472 Particle size distribution and microscopy images of the final oleosome creams after high speed
473 centrifugation obtained with (—) H_2O (pH 7) (-··-) Na^+ (0.2 mol/L, pH 7), (■) K^+ (0.2 mol/L, pH
474 7), (•) Mg^{2+} (0.2 mol/L, pH 7), (-·-·) Ca^{2+} (0.2 mol/L, pH 7) and (---) $NaHCO_3$ (0.1 mol/L, pH 9.5).
475 The scale bar is 20 µm.

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