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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**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 (Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\)). The oleosome yield at pH 9.5 was 64 wt.%, while the yield at pH 7 with H\(_2\)O was just 43 wt.%. The presence of cations at pH 7, significantly enhanced the yield, with 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 Ca\(^{2+}\) binding site on the N-terminal of the protein that can also bind 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 phosphatidylycerine representing 65% (wt%) of the total phospholipids, followed by phosphatidylerine, phosphatidylinositol
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, Snellenger-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 unfoldment and affects its solubility. Therefore, the aim of this work was to investigate the effect of monovalent (Na+, K+) and divalent (Ca2+, Mg2+) 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 pursued by the Division of Food Sciences, University of Nottingham, Sutton Bonington, UK. Magnesium Chloride (MgCl2) was obtained from Merck (Darmstadt, Germany). All other chemicals including the sodium chloride, potassium chloride and calcium chloride (NaCl, KCl, CaCl2) 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 (NaCl, KCl, MgCl2, CaCl2 0.2 mol/L) in ultra-pure water (MilliQ) and adjusting their pH to 7.0 with a solution of NaOH (0.1 mol/L) or HCl (0.1 mol/L). The additional aqueous solution made by 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 NaHCO3 0.1 mol/L and adjusting the pH to pH 9.5 with 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 °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 °C. After the centrifugation step, three different layers were observed: the cream, the serum and the precipitate. The oleosome cream was manually collected, dispensed in ultra-pure water (MilliQ) (1:4 w/v) and centrifuged at 10000g for 30 min at 4 °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 ± 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 & Wansnundara, 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 (d3,2) 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 ζ-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 (oleosomes) and 1.33 for the continuous phase (water). Average droplet sizes are reported using the surface weighted (d3,2) mean diameter. All measurements were conducted on fresh oleosome creams diluted in ultrapure water (1:100 w/v).

2.6. Optical microscopy analysis of oleosome emulsions

Images of the oleosome emulsions were taken with the microscope AxiosVision 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 ×.

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
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+, K+, Mg2+, and Ca2+).

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+ (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+ (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 Mg2+ (0.2 mol/L) and 55.3 wt.% with Ca2+ (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+ and K+) was expected. More precisely a slightly stronger solubilization effect from Na+ than K+ was expected, due to the order of these cations in the series, being K+ exactly to the left of Na+ on the series; however, the expected difference was not of this significant extent as extraction yield at the presence of K+ was higher than at the presence of Na+. 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+, the binding capacity of Na+ 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+ 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+ had an effect to oleosome extraction yield, a solution with higher Na+ concentration (0.3 mol/L) was also used. The oleosome extraction yield with higher concentration of Na+ (0.3 mol/L) slightly increased and resulted significantly different from the obtained with Na+ 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+ did not reach the extraction yield obtained when K+ (0.2 mol/L) was present. Therefore, besides the interactions with other components of the interface and the affect on concentration, K+ led to higher extraction yields. Furthermore, it is important to state that the yield in the presence of K+ (0.2 mol/L) at pH 7 did not significantly differ from the yield obtained when NaHCO3 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+. 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.

Table 1

| Aqueous solvent | Oleosome extraction yield (wt%) | Standard Deviation |
|----------------|--------------------------------|-------------------|
| H2O (pH 7.0)   | 42.7 ± 1.9                     |                   |
| Na+ (0.2 mol/L, pH 7.0) | 50.2 ± 2.0                   |                   |
| Na+ (0.3 mol/L, pH 7.0) | 55.3 ± 1.8                   |                   |
| K+ (0.2 mol/L, pH 7.0) | 64.2 ± 0.6                     |                   |
| Mg2+ (0.2 mol/L, pH 7.0) | 52.5 ± 4.9                    |                   |
| Ca2+ (0.2 mol/L, pH 7.0) | 55.0 ± 2.3                    |                   |
| NaHCO3 (0.1 mol/L, pH 9.5) | 63.6 ± 0.5                   |                   |

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

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, improving oleosome solubility and subsequently their extraction.
Na+ (0.2 mol/L, pH 7.0), K+ (0.2 mol/L, pH 7.0), Mg2+ (0.2 mol/L, pH 7.0), Ca2+ (0.2 mol/L, pH 7.0), NaHCO3 (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+ (0.2 mol/L, pH 7) | -21.5a              | ± 0.4             |
| K+ (0.2 mol/L, pH 7)  | -9.8a               | ± 0.5             |
| Mg2+ (0.2 mol/L, pH 7) | -9.9a             | ± 0.4             |
| Ca2+ (0.2 mol/L, pH 7) | -21.8a            | ± 0.4             |
| H2O (pH 7)           | -20.24a            | ± 0.4             |
| NaHCO3 (0.1 mol/L, pH 9.5) | -56.7d        | ± 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+, Na+ or Mg2+ had a lower oil to protein ratio compared to those that were extracted in the presence of Ca2+. On one hand the higher protein content with K+ and Na+ could explain the coalescence (Nikiforidis & Kiosseoglou, 2011; Zhao, Chen, Chen, Kong, & Hua, 2016). The oleosome creams obtained with H2O were the most stable against coalescence, as the smallest individual droplets were recovered with this medium (Fig. 2). However, at the presence of Na+ and K+, the oleosomes were less stable against coalescence, indicating an effect of the monovalent cations on the oleosome membrane 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 Ca2+ binding site on caleosins is an indication of potential interactions of caleosins with the excess of Ca2+ or in general with divalent cations. It has been reported that the exposure of caleosins to divalent cations (Ca2+ or Mg2+) 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 NaHCO3 (pH 9.5) the mechanism is completely different. The electrokinetic potential of the oleosomes at 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+ or Na+, show similar distributions, where slight coalescence was observed. The case of Ca2+ and Mg2+ 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+ and K+, 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 Ca2+ binding site on caleosins is an indication of potential interactions of caleosins with the excess of Ca2+ or in general with divalent cations. It has been reported that the exposure of caleosins to divalent cations (Ca2+ or Mg2+) 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 NaHCO3 (pH 9.5) the mechanism is completely different. The electrokinetic potential of the oleosomes at

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

|          | H2O  | Na+  | K+   | Mg2+ | Ca2+ | NaHCO3 |
|----------|------|------|------|------|------|--------|
| wt. %    | STDv | wt. %| STDv | wt. %| STDv | wt. %  |
| Wet basis|      |      |      |      |      |        |
| Lipids   | 42.8± | ± 2.8| 56.3± | ± 3.5| 52.2± | ± 0.4  |
| Protein  | 7.5±  | ± 1.2| 7.1±  | ± 0.5| 8.2±  | ± 1.3  |
| Ratio lipid:proteins | 5.7± | 7.8± | 6.3± | 7.3± | 12.8± | 17.5± |
| Dry basis|      |      |      |      |      |        |
| Lipids   | 60.6± | ± 2.8| 81.1± | ± 3.5| 73.6± | ± 0.4  |
| Protein  | 10.6± | ± 1.2| 10.3± | ± 0.5| 12.6± | ± 1.3  |
| Ratio lipid:proteins | 5.7± | 7.6± | 6.4± | 7.4± | 12.4± | 17.0± |

Values with different letters are significantly different with p < 0.05.
this pH is very high, Δ−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⁺ or Na⁺) and divalent (Ca²⁺ or Mg²⁺) 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 H₂O at pH 7, which was about 43 wt.%. More specifically, the presence of K⁺ 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 partly reduced the formation of aggregates. Divalent and the low electrokinetic potential of the system. The addition of cations at strong alkaline pH values (9.5) were used (De Chirico et al., 2018; Wang et al., 2019).

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