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Oil body extraction from oleo-proteaginous seeds and conservation of valuable native compounds☆

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Abstract – Oil bodies, also called oleosomes have been the object of an increased interest since the last decade. Different processes of extraction and purification involve an aqueous crushing with methods to soften the cell membranes. An integrated process was used on different oilseeds to compare the different oil-body dispersions obtained. Once extracted with an aqueous crushing, oil bodies are dispersed in a creamy phase containing also an important protein content. Their stability depends on membranous proteins but also surrounding, extraneous ones. To eliminate these non-membranous proteins, the emulsion can be washed with different compounds allowing a good protein solubilization. If the fatty acid, phytosterol, tocopherol contents and distribution are compared between seeds and dispersions of oil bodies, there appears to be little significant change. These valuable compounds are hence preserved in the oil bodies. However, aqueous crushing releases phospholipase partly explaining the lower phospholipid content and the higher relative concentration of phosphatidic acid. To preserve these emulsions, it is possible to dry them either through freeze-drying or spray-drying. Spray-drying allows a better recovery of the physical structure of the emulsion after rehydration but cryo-protectants as Tris or Glycerol can limit emulsion degradation provoked by hard mechanical constraints of a freeze-drying process.

Keywords: Oleosome / sterol / protein / phospholipid / fatty acid

Résumé – Extraction des corps lipidiques de graines oléoprotéagineuses et conservation des molécules natives d'intérêt. Les corps lipidiques, également appelés oléosomes, font l'objet d'un intérêt croissant depuis la dernière décennie. Différents procédés d'extraction et de purification impliquent un broyage aqueux avec parfois des pré-traitements pour fragiliser les membranes cellulaires. Un procédé intégré a été utilisé sur différentes graines oléagineuses afin de comparer les différentes dispersions d'oléosomes obtenues. Une fois extraits par broyage aqueux, les corps lipidiques sont dispersés dans une phase crémeuse. Leur stabilité dépend de leurs protéines membranaires mais aussi des protéines environnantes. Pour éliminer ces dernières protéines, l'émulsion peut être lavée avec différents composés permettant une bonne solubilisation des protéines. Si l'on compare la teneur et la répartition des acides gras, des phytostérols et des tocophérols, il apparaît qu'il n'y a pas vraiment de changement entre les compositions dans la graine et la dispersion d'oléosomes. Ces précieux composés sont donc préservés dans les corps lipidiques. Par contre, le broyage aqueux libère de la phospholipase, ce qui explique en partie le contenu plus faible en phospholipides et la concentration relative plus élevée en acide phosphatidique. Pour conserver ces émulsions, il est possible de les sécher soit par lyophilisation, soit par atomisation. Le séchage par atomisation permet une meilleure récupération de la structure physique de l'émulsion après réhydratation et des cryoprotecteurs comme le Glycérol ou le TRIS peuvent limiter la dégradation de l'émulsion provoquée par les contraintes mécaniques d'un processus de lyophilisation.

Mots-clés : Oléosome / sterol / proteine / phospholipide / acide gras

☆ Contribution to the Topical Issue "Bioactive lipids and lipid droplets: green ressources for food and health / Lipides et gouttelettes lipidiques bioactifs : des ressources vertes pour l'alimentation et la santé".

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Highlight

- Oil-body extraction from diverse oilseeds can be achieved with a versatile aqueous process.
- Oil-body size is preserved along the extraction steps.
- Triglycerides, Tocopherols and Sterols are concentrated in oil-body dispersion.
- Extraneous proteins enhance oil-body resistance to coalescence.

1 Introduction

In oleaginous seeds, triacylglyerols are contained in spherical organelles named oil bodies or oleosomes. These structures are spherical and have a membrane constituted of a monolayer of phospholipids and proteins (Tzen *et al.*, 1993). The synergy between these membranous compounds is responsible of the high resistance of oleosomes to coalescence (Deleu *et al.*, 2010). Besides, they are able to exert high electrostatic forces to hinder flocculation. Therefore, these structures can form naturally stabilized emulsions when released in water. According to the nature of the seeds, valuable compositions of fatty acids can be found and, for example, omega-3 rich (as in flaxseed) or omega-9 rich (as in high oleic sunflower seeds) emulsions can be obtained. Substantial amount of valuable minor components as phytosterols and tocopherols can also be concentrated in these structures. That's why they are the point of interest of many cosmetic, pharmaceutical and food industries.

The first studies on oil bodies were mainly focused on investigations on their structure as a 30 yr article made on extraction and reconstitution of oil bodies (Tzen *et al.*, 1993). To release oleosomes from the seeds, solvents and shearing methods must be chosen with care to keep intact their structure. Organic solvents are obviously excluded as high temperatures. With aqueous processes, other soluble compounds from the seeds as proteins, minerals and some carbohydrates are released and it can be sometimes necessary to remove them to improve stability or potential of applications of the oleosome-rich emulsions. To get a good extraction yield, cellular membranes have to be ruptured at a high extent. Enzymes as cellulases, pectinases... can be used to remove the compounds of these membranes without being prejudicial to oleosomes (Gao *et al.*, 2022; Liu *et al.*, 2020; Loman *et al.*, 2018). Also, soaking of the seeds can be a preliminary action to soften the membrane which will exert less mechanic resistance to its rupture. These methods must be accompanied with high shearing devices to decrease the size of the particles at the level of the cells and then induce a better diffusion of internal compounds. These methods can involve twin-screw extraction which appears to give better yields (60%) than a conventional blender (43%) (Romero-Guzmán *et al.*, 2020), colloidal crushing or high pressure homogenization (Brookman, 1974; Cassen *et al.*, 2022). Some ionic linkages must also be eliminated by pH changes or salts. For example, the use of cations (monovalent or divalent) can play a role in oil-body extraction (Romero-Guzmán *et al.*, 2020).

The high shear devices can be of different natures. Some will directly exert surface shearing forces as ultrasound (Loman *et al.*, 2018), other will provoke mechanical shocks but the intensity of these forces must be limited to avoid a destruction of the structure of the oil bodies.

Even if some fractioning can be realized without water as dehulling, the separation of oil bodies from other cell constituents imposes a process in an aqueous solution where the differences in structure and densities can allow a good separation of oil bodies.

Some examples of parameters used for oil-body extraction are summarized in Table 1 This table also indicates some conditions of oil-body washing which is a required step to better isolate them from other seed constituents.

Generally, an oil body is constituted of a very large content in neutral lipids (~95%), phospholipids (1–2%) and proteins (~5%). When protein content in the obtained creamy phase is superior to 5%, non- membranous proteins are certainly present. Membranous proteins (oleosins being the major ones) have generally a low molecular weight (between 10 and 25 kDa) while surrounding proteins are of a bigger size (Huang, 1992; Lacey *et al.*, 1998a; Ntone *et al.*, 2020; Tzen *et al.*, 1993). Different methods can be used to separate oil bodies from the surroundings compounds, mainly hydrosoluble proteins and salts (Tab. 1). If these compounds have no or weak physical bonds with oleosomes they can be easily extracted with distilled water through dialysis or successive washings.

However, the links between oleosomes and non-membranous proteins can be just slightly weaker than those existing with oleosins. The removal of these proteins requires other methods. A chaotropic compound as urea can be used as it weakens the tertiary structure of proteins, decrease their intermolecular interactions and favor their solubilization. Urea is then used to extract proteins which can be anchored in the oil body at a certain extent. However, its use must be done carefully to keep intact membranous proteins that have a prominent role in the stability of oleosomes. Therefore, it will be merely used with the goal of precise scientific study of isolated oleosomes.

Some salts, as sodium chloride or ammonium sulphate can be used to induce a salting in or salting out effect. The salt, at a certain concentration can increase or decrease the ionic strength but also the water availability, inducing precipitation of proteins (poorly interacting with oil-body membrane) or solubilization of proteins (of a significant interaction with oil-body membrane).

pH is also a tool that can be used. By moving proteins backwards or forwards from their isoelectric point, it changes their number of ionized hydroxyl or amine groups and therefore their solubility. Reserve proteins of oleo-proteaginous seeds are generally highly soluble in water at elevated pH (Fig.1) and have isoelectric points generally between 4 and 6. For example, for soybean oleosomes, an isoelectric point of 5 is considered (Waschatko *et al.*, 2012) which is close to the general value for soybean proteins (between 4 and 5) (Chove *et al.*, 2001; Jaramillo *et al.*, 2011; Koshiyama, 2009; Renkema *et al.*, 2002; Virkar *et al.*, 1982; Wolf *et al.*, 1971). For rapeseed, the isoelectric point is quite variable according to the proteins with values at 3.5 (El Nockrashy *et al.*, 1977), 4.5 (Kalaydzkiev *et al.*, 2019), but most generally found between 4.5–6.5 (El Nockrashy *et al.*, 1977; Ghodsvali *et al.*, 2005;

Table 1. Different isolation procedures for the oil bodies of different seeds.

Seeds	Pre-soaking	Crushing	Ratio S:L	pH	Washing step	% proteins	Reference
Rapeseed, Cotton, Flaxseed, Maize, Peanuts, Sesame	No	Homogenization 40 s	1:4	7.5	2 M NaCl + sucrose, several cycles	0,6–3,5%	(Tzen *et al.*, 1993)
Rapeseed	4 °C, 18 h	Waring Blender (15 000 rpm)	1:10	pH 6.5-11	Sucrose (1 :4)	18%	(Zhao *et al.*, 2016)
Rapeseed (dehulled)	4 h, pH 9	Blender, 2 min	1:8	9	None	7.8%	(Ntone *et al.*, 2020)
Rapeseed	No	Mortar/Pestle + Glass Dounce homogenizer	1:3	7.5	Salts, buffer, sucrose + different methods (salt or Urea 8 M)	N.D.	(Katavic *et al.*, 2006)
Rapeseed	No	Potter/Teflon plunger with a Heidolph motor (rate 7)	1:17	7.5	Na_2CO_3 + Sucrose	Lipid/Protein (w/w) = 16.7	(Jolivet *et al.*, 2013)
Sunflower	No	Blender, 2 min	1:5	Native	Urea 9 M + H2O	7,4%	(White *et al.*, 2008)
Sunflower (dehulled)	16 h	Blender 90 s	1:7	8	pH 8, 1:4, 2 cycles	3%	(Karefyllakis *et al.*, 2019)
Soybean	20 h	Homogenization, 8min	1:5	7,5	Tris-HCl/water	8%	(Ding *et al.*, 2020)
Rapeseed	16 h	Blender 90 s + 24 H, 4 °C	1:7	9,5	$NaHCO_3$ or Urea	<3%	(De Chirico *et al.*, 2018)
Peanut	No	Blender 2 min, 18000 rpm	1:9	Native	Phosphate buffer at different pHs (7–11)	<1,5%	(Zaaboul *et al.*, 2018)
Maize	24 h	Blender 40 s x3	1:5	3–9	pH 8,5, 1:5, then pH 5	18%	(Nikiforidis and Kiosseoglou, 2009)
Safflower	12 h	Homogenization with Tris-HCl		7.5 (Tris-HCl)	Sucrose (125–500 mM), KCl (2 M), Urea (8 M) at 125, 250 mM/ 20 min, repeated 3 times		(Lacey *et al.*, 1998)

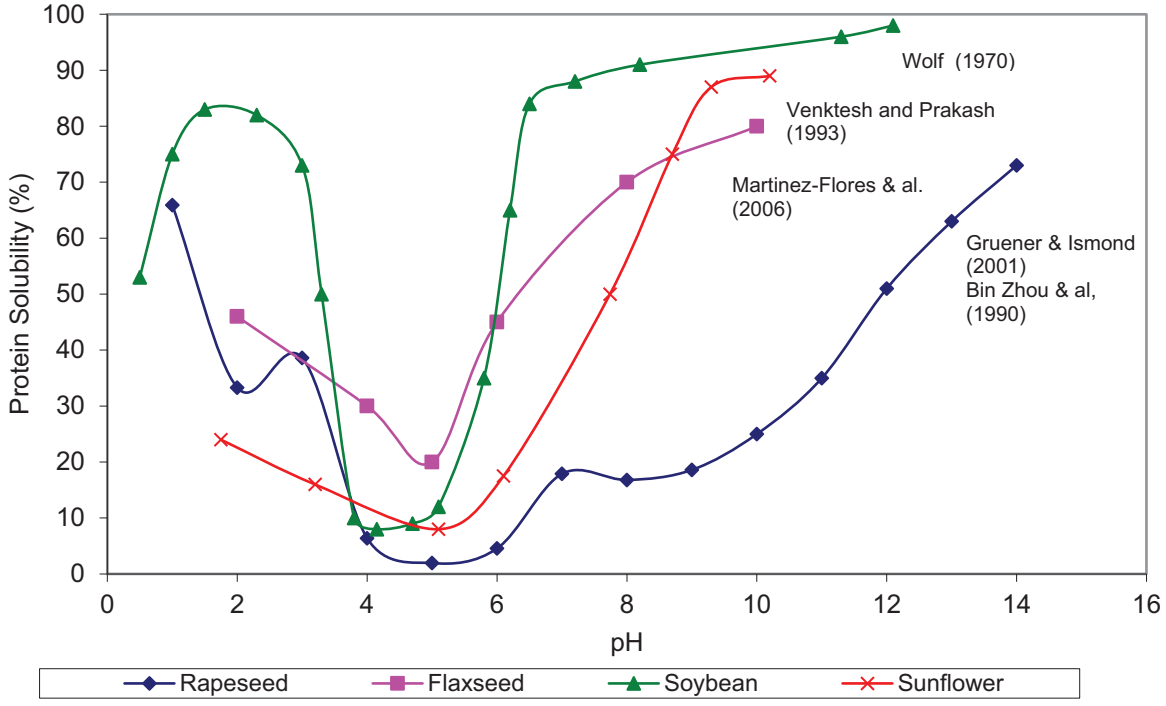

Fig. 1. Protein solubility (in percentages) of some protein concentrates with pH (blue : rapeseed – (Gruener and Ismond, 1997; Zhou *et al.*, 1990), pink : flaxseed – (Martínez-Flores *et al.*, 2006), red : Sunflower – (Venktesh and Prakash, 1993), green : soybean – (Wolf, 1970).

Table 2. Conditions of the integrated process of oil-body extraction.

	Flaxseed	Sunflower	Rapeseed
Seed Pre-Treatment	Degumming	Dehulling	None
Crushing parameters (seed concentration and maximum stirring speed reached)	15%, 7000 rpm	20%, 5200 rpm	20%, 7000 rpm
Homogenization	2 cycles 500 bars	50, 250 and 350 bars	2 cycles 350 bars
Centrifugation	10000xg, 10 min, 4 °C	10000xg, 10 min, 4 °C	10000xg, 10 min, 4 °C

Pedroche *et al.*, 2004; Von Der Haar *et al.*, 2014), but it was found at 7.2 for 12S Globulins (Schwenke *et al.*, 1981) and at higher pHs (>10) for napins (Lönnerdal and Janson, 1972; Nitecka *et al.*, 1986; Schwenke *et al.*, 1973; Von Der Haar *et al.*, 2014). Concerning oleosins, a value of 6.5 was found (Wijesundera *et al.*, 2013), quite close to the value found for oil bodies (5.5–6.5) (Tzen *et al.*, 1993). For flaxseed, values are generally found between 4 and 5 (Dev and Quensel, 1988; Hellebois *et al.*, 2021; Lan *et al.*, 2020; Malik and Saini, 2018; Sosulski and Bakal, 1969; Tirgar *et al.*, 2017; Vassel and Nesbitt, 1945) even if some authors found lower (3–3.5) (Wanasundara and Shahidi, 1994) or higher (4.7–5.6) values (Chung *et al.*, 2005). Concerning membranous proteins, an isoelectric point around 4.0 (Fabre *et al.*, 2015a) was found. For oleosomes, an isoelectric point around 3.0 was observed while it was measured earlier in a range of pH from 5.7 to 6.2 (Tzen *et al.*, 1993). However, the different purification processes of oil bodies allied with the complexity and diversity of the proteins bound to the membrane or interacting with it can explain the variability of the isoelectric points found for oil bodies.

Soaking of the seeds during several hours before crushing can also decrease the interaction between oleosomes and non-membranous proteins (Romero-Guzmán *et al.*, 2020). For soybean (Ding *et al.*, 2020), protein concentration is still high after 20 h of pre-soaking and the use of a buffer at a nearly neutral pH. For Maize, a pre-soaking of 24 h allowed a good extraction yield of oil bodies, the washing with distilled water didn't eliminate extraneous proteins but it was not the purpose of this treatment, the authors testing also the stability of urea-washed oil bodies (Nikiforidis and Kiosseoglou, 2009), a treatment already described in 1997 (Tzen *et al.*, 1997). Regarding Safflower, the links seem weaker than for sunflower as the use of a pre-soaking overnight and several salt-washings may be sufficient for the first while urea was necessary for sunflower (Lacey *et al.*, 1998). Another study gave different results as pre-soaking sunflower seeds during 16 h and a simple washing at pH 8 (Karefyllakis *et al.*, 2019) appeared more efficient than Urea washing (White *et al.*, 2008) with a final lower concentration of proteins. For sesame and peanuts, an alkaline solution as for sunflower (pH 11) can be efficient to solubilize proteins (Zaaboul *et al.*, 2018). At this pH 11, if extraneous proteins are mostly extracted for jicama, sunflower, peanut and sesame, it remains enzymes in castor bean and still an elevated number of proteins in the extract of rapeseed oil bodies. It is particularly complicated to break here the link between some enzymes and the oil body (Zhao *et al.*, 2016) which could be beneficial to avoid degradation of membranous proteins and phospholipids. In rapeseed, it appears that several washings with alkaline solution (sodium carbonate) or urea with good buffering conditions are required (De Chirico *et al.*, 2018).

Therefore, washing of emulsion is generally a multi-step process that can have an important effect on the chemical stability of oil bodies, decreasing the presence of enzymes at their surface. However, the interfacial layer is thinner and may give a weaker resistance to oxidation. It has also an effect on the physical stability of oil bodies. Generally, washing the emulsion induces a decrease of the concentration of high molecular weight proteins, reducing therefore depletion forces and slowing down flocculation if pH stays away from the isoelectric point. However, with denaturation of membranous proteins at high pH and the solubilization of extraneous proteins, a thinning of the interfacial zones occurs and possibly a decrease of the interactions oleosins/phospholipids. The steric hindrance is lowered and coalescence phenomenon can be enhanced (Fabre *et al.*, 2015a; Nikiforidis and Kiosseoglou, 2009). The conservation of valuable compounds of the seeds as triglycerides, phospholipids, tocopherols and sterols in the extracted oil bodies and the conservation of their structure is therefore a challenge that involves the choice of mechanical and chemical solutions adapted to the studied matrixes. Rapeseed, Flaxseed and Sunflower are three common species that are here chosen to demonstrate the versatility of an aqueous integrated process and its effects on the physical and chemical properties of the extracted enriched oil-body phase.

2 Materials and methods

Rapeseed (*Brassica napus*) was obtained from the MOMONT cooperative (Mons-en-Pevele, France), it is the KOSTO variety. Flaxseed (*Linum usitatissimum*), NIAGARA variety, comes from the "Linéa/SCA LIN 2000" cooperative (Grandvilliers, France). The sunflower (*Helianthus annuus*) seeds are of High Oleic variety and were supplied by Toulousaine de Céréales (Lespinasse, France)

The aqueous integrated process applied to flaxseed, sunflower and rapeseed is summarized (Tab. 2).

It has already been described in the case of sunflower seeds (Cassen *et al.*, 2022) and flaxseed (Fabre *et al.*, 2015a) and adapted here to the three different seeds processed (Fig. 2).

Dehulling step is only necessary for sunflower. It is realized by an industrial ripple-mill process (Terres Innovia, Pessac, France). However, for the mucilaginous flaxseed, an ultrasound-assisted mucilage extraction is first realized to avoid a thickening of the crushing medium. This step has already been described (Fabre *et al.*, 2015a, Fabre *et al.*, 2015b).

To remove mucilage from flaxseeds, a suspension of seeds in distilled water is treated 30 min with an ultrasound probe

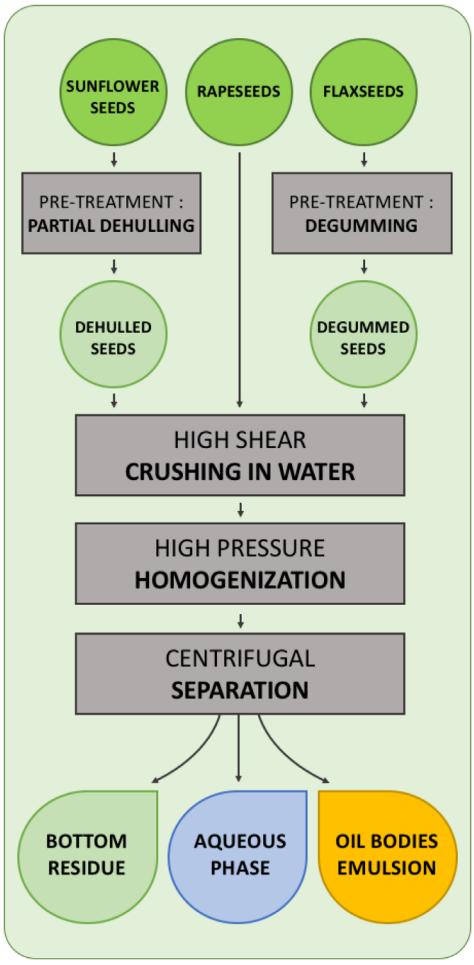

Fig. 2. Schematic diagram of the integrated processes adapted to sunflower, rapeseed and flaxseed.

(Sonics Vibracell – 500 W, 22 kHz) of a diameter of 13 mm, used at an amplitude of 60% and a pulsation of 5s ON, 10s OFF. Complete (rapeseed), dehulled (sunflower) or degummed (flaxseed) seeds are crushed in distilled water with a high-shear rate device equipped with a double rotor/stator with 8 mm round holes and 2 mm square holes (Silverson L4TRT, Silverson Machines LTd). The treatment is performed during 10 min at different speeds and seed concentrations to remain in a low viscous state allowing shear-type milling in aqueous phase. The medium is then treated in a high-pressure homogenizer to better break the oleaginous cells.

At the end of the process, three phases are obtained after centrifugation. A creamy upper phase in the form of a direct emulsion that comprises the oil bodies (OBs), a medium aqueous phase and a pellet.

The size distribution of the OBs in emulsions was determined with a Malvern Mastersizer 2000 laser light scattering instrument coupled with a Hydro2000S sample-handling unit (Malvern, UK). The viscosity considered was the viscosity of water and the refractive index was chosen as RI = 1.52 which is a default value for vegetable oil also in the range accepted (1.35–1.6) in the literature for adsorbed proteins (Vörös, 2004). Absorption is also related to the nature of what is analyzed. Here, the medium contains both opaque and non-spherical solid particles and oil droplets of a complex structure, so we use absorption = 1. A fraction of the sample was diluted in water and stirred at 3000 rpm. Continuous ultrasound treatment at a tip displacement of 80% was performed until the droplet aggregates were disrupted. Various measurements were made during the ultrasound treatment. The mean value obtained is calculated with the final stabilized measurements.

Observation of dried emulsions was performed with a scanning electron microscope LEO 435 VP, LEO Electron Microscopy Ltd. (Thornwood, NY, USA). Samples are further dehydrated with desiccants under vacuum then metallized with silver under an argon plasma. Photographs are recorded with the LEO 32 software.

Protein content was indirectly determined with the Kjeldahl method (French standard NF V 18-100) which gives the nitrogen amount which can be converted to a protein amount with a standard conversion factor of 6.25 for comparison purpose.

Mineral content was estimated using the ash remaining after calcining the dried matter in an oven at a temperature of 550 °C until no further change in weight was observed (French standard NF V 03-322).

The DIONEX THERMO ASE 350 apparatus was used to determine the lipid content of the analyzed phases. For the particular case of emulsions, it was necessary to dry them (103 °C, 24 h) before grinding them. The extraction of lipids was then carried out in the ASE 350 with cyclohexane on a mixture of dried and grinded emulsion with an inert material (Fontainebleau sand) favoring the flow of the solvent during extraction. Extraction was carried out over 4 static cycles of 10 min, at 100 °C and 100 bar. The lipid content is then determined by gravimetric measurement after evaporation of the extraction solvent.

Fatty acid composition was determined after trans-methylation with TMSH (0.2 M trimethylsulfonium hydroxide in methanol) according to AFNOR Method NF EN ISO 12966-3. The fatty acids methyl esters (FAME) obtained by this transesterification reaction were analyzed with a gas chromatograph (Fabre *et al.*, 2015a; Roche *et al.*, 2006) equipped with a CP-select CB column (50 m long, 0.32 mm i.d., 0.25 µm film thickness).

Tocopherol content was determined by high-performance liquid chromatography as previously described (Fabre *et al.*, 2015a). 20 mg of oil are diluted in 1 ml of cyclohexane. 20 µl of the resulting solution are then injected in a liquid chromatography system (Dionex) equipped with a Kromasil 100 SIL 5µ column (250 mm long, with an internal diameter of 4 mm). A mixture of 99.5% isooctane and 0.5% isopropanol (v/v) was used as the mobile phase, at a flow rate of 1.1 ml/min. Tocopherols were detected with a fluorimeter (Dionex), with an excitation wavelength of 290 nm and an emission wavelength of 317 nm. Quantification of tocopherols was obtained through external calibration.

Sterol content was determined by gas chromatography as explained in preceding articles (Fabre *et al.*, 2015a; Roche *et al.*, 2010) with silylation with BSTA/TMCS. A Perkin-Elmer gas chromatograph equipped with an Agilent VF-5ms column (30 m long, diameter of 0.25 mm, film thickness of 0.25 µm) was used. It is coupled to a flame ionization detector

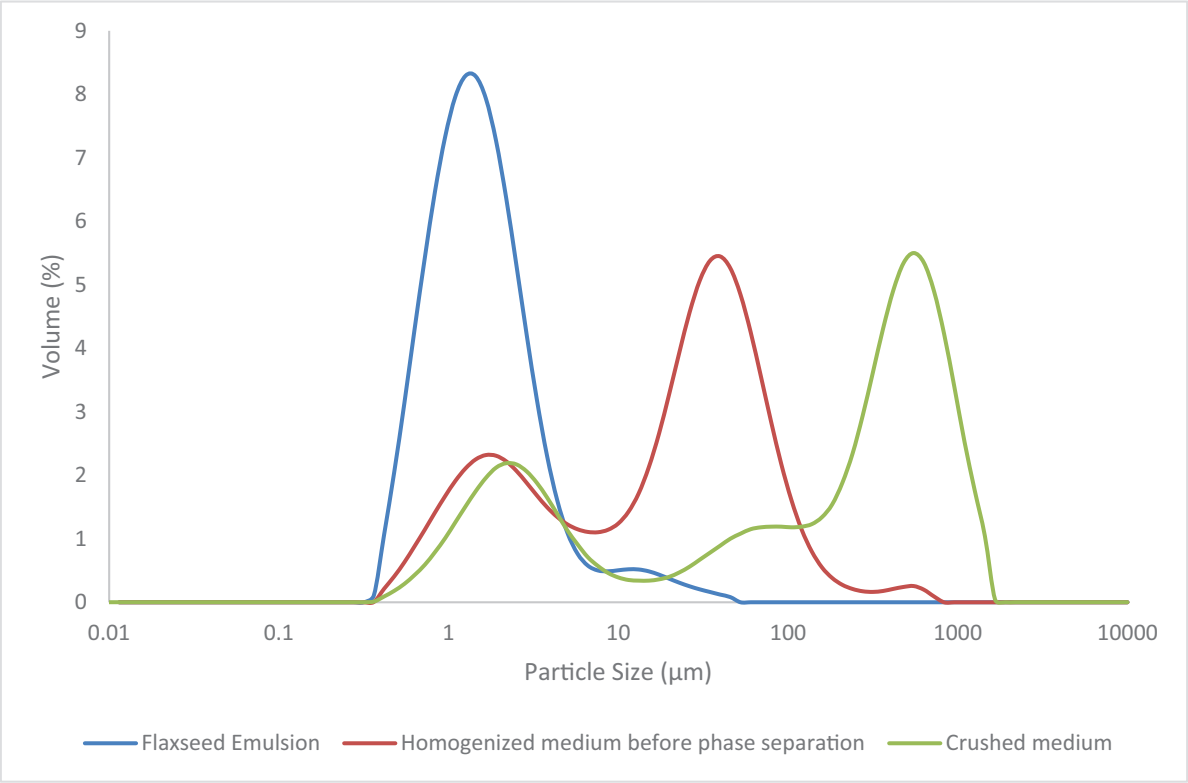

Fig. 3. Granulometric volume distribution of the medium obtained after flaxseed crushing (green), high pressure homogenization (red) and of the cream obtained after centrifugation (blue).

operating at 355 °C. Quantification was obtained through internal calibration.

Phospholipid content and classes were obtained after solid phase extraction and HPLC/ELSD chromatography (Cassen *et al.*, 2022; Mills and Goldhaber, 2010; Silversand and Haux, 1997). SPE was performed with Supelclean LC-SiSPE 500 mg/6mL cartridge (Supelco, Bellefonte, USA). HPLC was performed with column of type Lichrospher 100 diol (5 µ) 150 × 3 mm and the detector is a Alltech 3300 (Büchi, Postfach, Switzerland). Quantification is obtained through external calibration with commercial standards.

All analytical measurements are presented with the average of triplicates and the standard deviation.

3 Results and discussion

A granulometry measurement was performed on the different emulsions obtained and is illustrated for flaxseed (Fig. 3). The results obtained after crushing, high pressure homogenization and the cream obtained after centrifugation (Flaxseed emulsion) can be compared. They all reveal the presence of particles smaller than 10 µm corresponding to oil bodies, indicating that the different stages have little effect on the size of the lipid droplets. For flaxseed emulsion, after crushing, the volume of particles between 100 and 1000 µm predominates while after homogenization the range is between 10 and 100 µm, indicating a strong decrease of the size of solid particles. In the total medium after phase separation, the volume of oil bodies is largely predominant with sizes between 1–3 µm (D[4:3] = 2.4 µm). Larger particles corresponding to solid fragments or aggregates are still measured in the range of 30–40 µm but their number is neglectable, even if they can represent a significant volume.

The different results for the obtained emulsions are summarized with the volume mean diameter (D[4.3]) and the median diameter d(0.5) (Tab. 3).

The results are in the range of literature values for these seeds. The lower values obtained by optical microscopy (Boulard *et al.*, 2015; Tzen *et al.*, 1993) could be explained by the number-average diameter given by this method which gives a higher consideration of small particles than volume-average diameters obtained by laser granulometry but also from different genotypes and days after pollination (Boulard *et al.*, 2015). For sunflower the literature value of 2.58 µm was obtained by PFNMR (pulsed field gradient nuclear magnetic resonance) which also gives larger diameters than number-average values obtained by optical microscopy (Boulard *et al.*, 2015; Gromova *et al.*, 2015).

Emulsion composition is similar between the three seeds (Flaxseed, Sunflower and Rapeseed) which have nearly the same lipid content (Tab. 4). The mucilage content of flaxseed, from 3 to 9% (Fabre *et al.*, 2015b; Fedeniuk and Biliaderis, 1994) is mainly detrimental to protein one in the emulsion.

It is important to keep intact in the oil body dispersion the highly nutritional elements or valuable lipophilic compounds

Table 3. Oil-body diameters in the different oil seeds (obtained through laser granulometry) compared with literature values.

Seeds	**D[4.3]**	**d(0.5)**	**Literature**
Flaxseed	2.4 ± 0.1 μm	1.4 ± 0.1 μm	1.34 μm*
Rapeseed	1.6 ± 0.0 μm	1.0 ± 0.0 μm	0.65 μm* 0.4–2 μm**
Sunflower	2.6 ± 0.1 μm	2.0 ± 0.0 μm	2.58 μm***

* (Tzen *et al.*, 1993),
**(Boulard *et al.*, 2015),
****(Gromova *et al.*, 2015)

Table 4. Composition of flaxseed, sunflower and rapeseed emulsions expressed relatively to their Dry Matter (% DM).

	Flaxseed	Sunflower	Rapeseed
Protein (% DM)	9.5	13.1	14.7
Lipid (% DM)	75.0	73.9	75.8
Minerals (% DM)	0.6	1.9	1.7
Other (% DM)	15.0	11.0	7.8

Table 5. Phytosterol concentration and relative (rel %) distribution in rapeseed, flaxseed and sunflower.

	Rapeseed		Flaxseed		Sunflower	
	Seed (rel %)	Emulsion (rel %)	Seed (rel %)	Emulsion (rel%)	Seed (rel%)	Emulsion (rel%)
Cholesterol	0.36 ± 0.06	0.36 ± 0.03	0.74 ± 0.03	1.62 ± 0.18	N.D.	N.D.
2,4methylene cholestérol	N.D.	N.D.	1.74 ± 0.03	N.D.	N.D.	N.D.
Brassicasterol	16.45 ± 0.22	16.49 ± 0.12	N.D.	N.D.	N.D.	N.D.
Campesterol	32.99 ± 0.13	33.49 ± 0.48	24.96 ± 0.05	32.37 ± 0.76	12.98 ± 0.13	12.51 ± 0.47
Stigmasterol	0.72 ± 0.06	0.70 ± 0.06	3.60 ± 0.05	5.42 ± 0.23	8.94 ± 0.11	9.01 ± 0.34
β-Sitosterol	47.30 ± 0.15	46.65 ± 0.09	53.10 ± 0.32	56.72 ± 0.54	60.52 ± 0.28	62.50 ± 0.38
Δ5-Avenasterol	2.18 ± 0.05	2.32 ± 0.30	18.34 ± 0.11	3.87 ± 0.92	4.41 ± 0.14	4.63 ± 1.43
Δ7-Stigmastenol	N.D.	N.D.	N.D.	N.D.	8.97 ± 0.08	7.17 ± 0.37
Δ7-Avenasterol	N.D.	N.D.	N.D.	N.D.	4.18 ± 0.13	4.18 ± 0.43
Total (mg/100g lipids)	705.40 ± 11.20	550.16 ± 21.82	376.97 ± 1.71	178.97 ± 5.00	338.43 ± 17.77	282.98 ± 25.60

of the seed. The content and composition in minor components of the emulsion (mg/100 g lipids) obtained for different seeds can be compared with the values initially found in the seed. Concerning phytosterols (Tab. 5), *β*−sitosterol is the most concentrated phytosterol and is at similar relative concentration in all the seeds (around 50–60% of total sterol content). Campesterol is at a high concentration in rapeseed and flaxseed (around 33%) while it only represents 13% in sunflower seeds. Brassicasterol is only found in rapeseed. Δ7-Stigmastenol, Δ7-Avenasterol are only found in sunflower.

The sterol distribution in the seeds is similar as the distribution in the emulsion. However, the sterol concentration relative to oil content is lower in the emulsion (from 47% to 78% of the sterols found in the seed) which can be due to their non-exclusive presence in the oil bodies with sterols linked to a certain extent to some parts of the seed matrix. One sterol, Δ5-Avenasterol, is little extracted in the flaxseed emulsion (<4% of the sterols) while it represents nearly 20% of the sterols found in the seed.

In the example of sunflower and rapeseed, the fatty acid composition of the seed phospholipids is similar as for the triglycerides (Tab. 6) with sunflower seed rich in oleic acid and rapeseed richer in vaccenic and polyunsaturated fatty acids. Phospholipid have a higher concentration of saturated chains than triglycerides as already reported (Cansell *et al.*, 2017), phospholipid preferably associating a saturated chain with an unsaturated one. This can explain a more equilibrate ratio saturated/unsaturated fatty acids for phospholipids than for triglycerides.

The ratio lipid/phospholipid in the seeds is not found in the emulsion but many phospholipids are not extracted in the emulsion, being part of other membranes than oil-body ones

Table 6. Fatty acid (FA) chains of phospholipids (PL) and triglycerides (TAG) of sunflower and rapeseed.

Fatty acid chain	Sunflower seed		Rapeseed	
	PL (rel. %)	TAG (rel. %)	PL (rel. %)	TAG (rel. %)
C16:0	8.5 ± 0.6	3.3 ± 0.0	10.5 ± 0.1	5.0 ± 0.0
C18:0	2.0 ± 0.3	1.5 ± 0.0	0.9 ± 0.1	1.6 ± 0.0
C20:0	N.D.	N.D.	N.D.	0.5 ± 0.0
C22:0	N.D.	N.D.	0.3 ± 0.0	0.5 ± 0.0
C18:1n-9	84.2 ± 0.9	91.5 ± 0.2	0.4 ± 0.0	0.2 ± 0.1
C18:1n-7	0.9 ± 0.0	1.1 ± 0.0	51.5 ± 0.2	59.1 ± 0.0
C20:1n-9	N.D.	N.D.	2.7 ± 0.0	3.6 ± 0.0
C22:1n-9	N.D.	N.D.	0.2 ± 0.	0.9 ± 0.1
C18:2	4.3 ± 0.0	2.6 ± 0.1	29.3 ± 0.2	19.9 ± 0.0
C18:3	N.D.	N.D.	4.2 ± 0.1	8.8 ± 0.3
Ratio Unsaturated/Saturated FA	8.5 ± 0.8	19.8 ± 0.1	7.5 ± 0.2	12.1 ± 0.1

Table 7. Phospholipid concentration and distribution in sunflower and rapeseed seeds and associated emulsions.

Relative concentration (%)	Sunflower seed	Sunflower emulsion	Rapeseed	Rapeseed emulsion
Phosphatidylcholine	68.1 ± 11.7	61.3 ± 8.1	83.8 ± 8.7	33.6 ± 15.7
Phosphatidylinositol	15.1 ± 2.8	15.0 ± 1.8	8.7 ± 1.0	10.8 ± 3.2
Phosphatidylethanolamine	11.6 ± 2.4	4.5 ± 0.5	7.5 ± 0.6	6.0 ± 2.3
Phosphatidic acid	5.2 ± 1.2	18.3 ± 3.8	N.D.	49.7 ± 17.8
Total (mg/100g lipids)	1811.2 ± 122.2	488.2 ± 32.1	2281.9 ±41.0	1002.7 ± 187.9

Table 8. Composition and relative) distribution (rel%) of tocopherols in rapeseed and derived emulsion.

Composition	Seed	Emulsion
α-tocopherol (rel%)	43.5 ± 0.5	35.8 ± 0.1
γ-tocopherol (rel%)	53.9 ± 0.7	64.2 ± 0.1
Total (mg/100g lipids)	74.6 ± 1.1	67.3 ± 6.7

(Tab. 7). Phosphatidic acid is more concentrated in the emulsion and this is more pronounced for rapeseed with a strong diminution of phosphatidylcholine (PC) concentration to the profit of Phosphatidic acid (PA) due to the release of phospholipase during the aqueous crushing of the seeds (Ambrosewicz-Walacik *et al.*, 2015; Pasker *et al.*, 2015). Allied with phosphatidic acid, the presence of phosphatidylserine was detected by Liquid chromatography/Mass spectrometry in a study on purified rapeseed oil bodies (Boulard *et al.*, 2015).

If the content and distribution of tocopherols in the specific case of rapeseed are examined, similar values are found between seeds and emulsion with a higher prevalence of γ-Tocopherol in the emulsion (Tab. 8). This higher content in γ-Tocopherol is coherent with the values found in another study on the oil bodies isolated from two rapeseed genotypes (γ-Tocopherol relative percentages of 57 and 71%) (Boulard *et al.*, 2015). Tocopherol yield is then well correlated with oil-body one so that its antioxidant properties are retained in the released oil bodies of the seed. It has been shown for sunflower (Fisk *et al.*, 2006) that tocopherol concentration in lipids from oil bodies washed with a sodium phosphate buffer (mg/g lipid) is even higher than in total seed lipids (57 mg/100 g lipid *vs.* 36 mg/100 g lipid). There is also a remarkably high prevalence of alpha form compared to gamma form, which does not significantly change between the seed and washed oil bodies.

4 Emulsion purification and conservation

To help conservation of the emulsion, different solutions can be used. The emulsion can be formulated with natural (*e.g.*, essential oils), bio-sourced (*e.g.*, monoglycerides) or synthetic preservatives (*e.g.*, sodium benzoate). The emulsion can also be

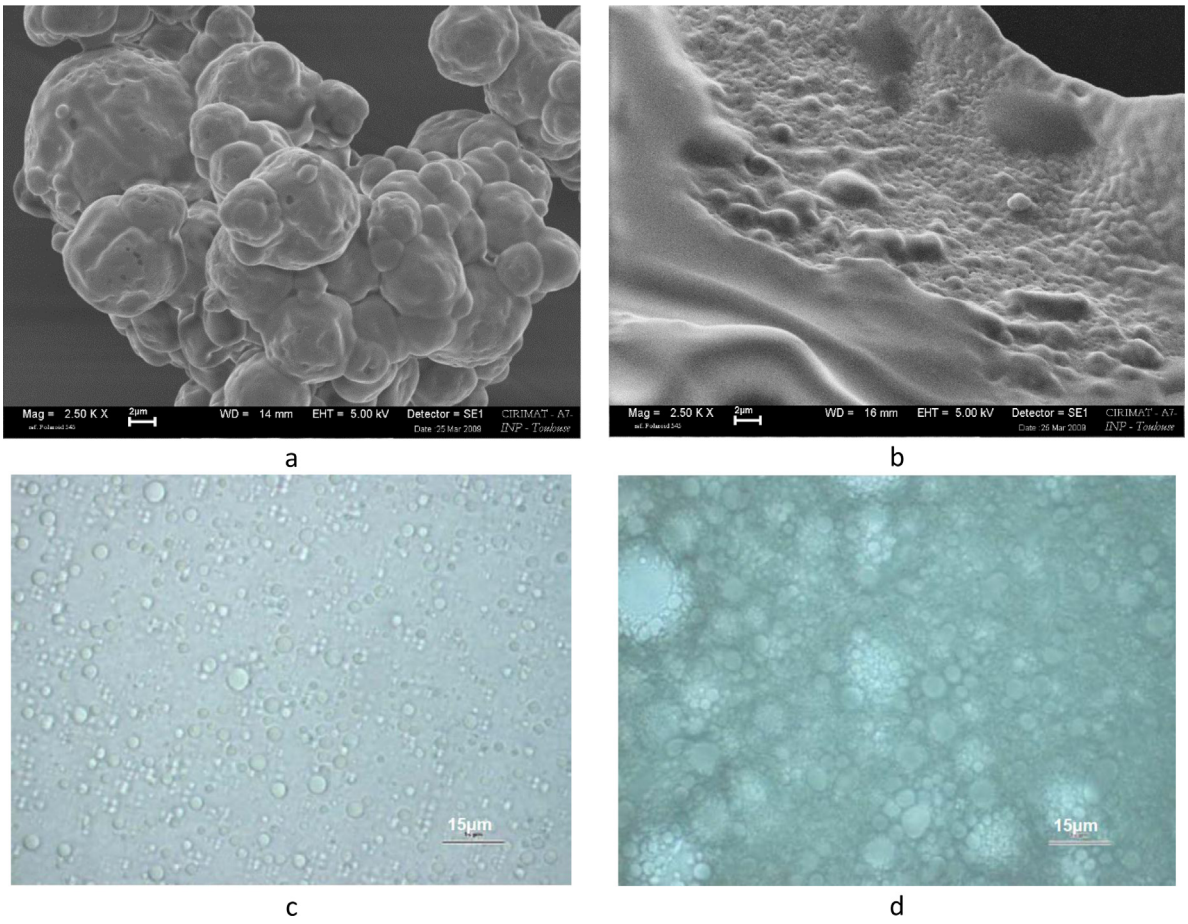

Fig. 4. Scanning Electron Microscopic observations of freeze-dried (A) and spray-dried emulsion (B) (Magnification of 2500x) and Optical Microscopic observation of rehydrated spray-dried emulsion (C) and freeze-dried emulsion (D) (Magnification of 1000x).

dried to decrease water activity. In this case, it can be freeze-dried or spray-dried. Even if oleosins have proven a certain protection against freezing for *Arabidopsis thaliana* (Shimada *et al.*, 2008), they seem insufficient for rapeseed. Indeed, freeze-drying strongly alters the structure of the emulsion (Fig. 3b compared to Fig. 3a) and rehydrating it leads to larger droplets (Fig. 3d compared to Fig. 3c). The presence of a cryo-protectant would be then recommended while with spray-drying, the structure seems more preserved even if some coalescence still occurs Fig. 4a and Fig. 4c

It can be important to determine if purification of rapeseed oil bodies through different washing steps aiming at eliminating non-membranous proteins could foster the stability and cryo-resistance of the emulsion.

Considering different washing solutions applied (Fig. 5) (v/v), dilution of the emulsion with sodium bicarbonate or sodium chloride then two other successive washings with distilled water allow the decrease of protein concentration by around 50% and an increase of temperature does not increase protein solubilisation. Tris-HCl at 0.1 M allows a further decrease of protein concentration (60%) while washing in a non-buffered medium doesn't give better results than distilled water (20%). To test the importance of loosely linked proteins, emulsions were washed with NaCl 3% prior to freeze-drying or spray-drying.

If the resistances of different formulated emulsions (Tab. 9) are compared, it appears that the vanishing of proteins vicinal to oil bodies strongly weakens the oil-body structures with the impossibility to obtain a dried emulsion. Droplet diameter was estimated based on microscopic observations with a number average mean D[1.0]. If the emulsion is formulated with 1% glycerol, the droplet size is doubled but this treatment is more efficient than mannitol which doesn't significantly increase the stability. The addition of Tris at a concentration of 0.075 M seems to allow the recovery of the initial droplet size of the emulsion even if the real extent of the preservation of emulsion structure would deserve deeper analysis. The primary amine and high susceptibility to hydrogen bonding of Tris certainly plays a role on protein configuration so that mechanical constraints due to water freezing and droplet concentration due to dehydration do not induce high coalescence.

With the results observed (Tab. 9), it appears that washing the emulsion provokes the disappearance of large proteins and the associated steric effect which leads to a very important coalescence, oil exuding for the dried extract after either freeze-drying or spray-drying.

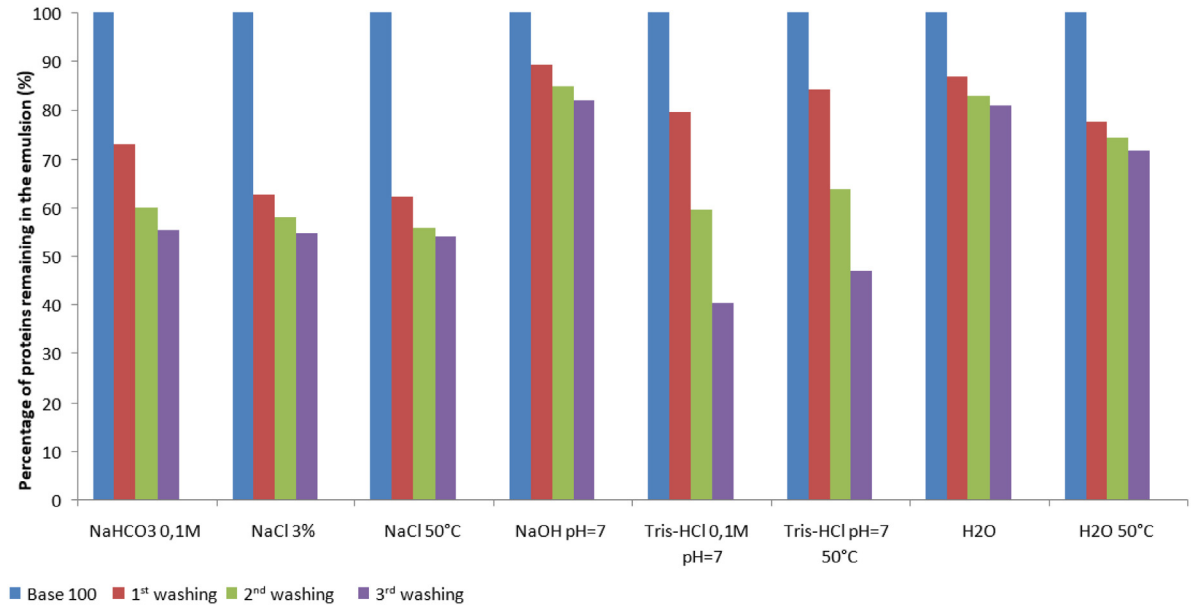

Fig. 5. Percentage of proteins remaining in the extracted emulsion after one, two and three cycles of different washing protocols applied.

Table 9. Formulation of emulsions prior to freeze-drying.

# Experiment	Formulation	Concentration	Droplet Size after rehydration and 5' stirring at 13000 rpm
1	Addition of glycerol	1%	D[1.0] ≈ 2xD[1.0] native
2	Addition of mannitol	1%	D[1.0] ≈ 4xD[1.0] native
3	Addition of Tris	0,075 M	D[1.0] ≈ D[1.0] native
4	NaCl 3% washing of the emulsion	–	No powder obtained but free oil exuding

5 Conclusion

Oil body extraction from oleoproteaginous seeds can be realized with a versatile integrated process to adapt process parameters to the structure and composition of the seed. A compromise must be found between yield, purity and stability. Removing extraneous proteins provides a better stability against flocculation as these proteins are little ionized at the native pH of the extracted media and exert depletion forces increasing flocculation. However, they increase steric repulsion and allow a better resistance to coalescence when droplets densification is favored by desiccation. The obtained oil-body emulsion contains valuable major and minor lipophilic compounds of the seeds which can help the physical and chemical stability of these oil bodies but also their valorization in food, cosmetic or more technical applications.

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Conflicts of interest

The authors have no conflict of interest to declare.

Authors contributions

Jean-François Fabre : Conceptualization, Investigation, Methodology, Visualization, Writing – Original Draft Eric Lacroux: Investigation, Conceptualization, Methodology, Writing – Review & Editing Muriel Cerny: Investigation, Methodology Guadalupe Vaca-Medina: Investigation Audrey Cassen: Investigation Zephirin Mouloungui: Project Administration, Supervision, Funding Acquisition Othmane Merah: Supervision Romain Valentin: Supervision, Validation

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