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FLAX AND HEMP LIN ET CHANVRE

Barriers to the release of flaxseed oil bodies and ways of overcoming them

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Abstract – The outermost mucilaginous layer of the seed is a major, valuable component of flaxseed, but it induces a rapid thickening of the medium when the seeds are immersed in water, reducing the efficiency of oil body extraction. Ultrasound can be used to extract this mucilage rapidly before the seeds are ground in water. This makes it possible to extract the oil bodies as an emulsion, with the proteins present acting as dispersing and stabilizing agents. This emulsion is a thick fluid displaying shear-thinning and predominantly elastic behavior. This phase can be valorized directly due to its high concentration in polyunsaturated fatty acids, high content of valuable sterols, and high arginine content of its proteins.

Keywords: Flaxseed / mucilage / zeta potential / ultrasound / oil bodies

1 Introduction

Linum usitatissimum has been widely used since prehistoric times for the production of material and for medicinal purposes and new uses are still being found.

The external fibers of the plant contain lignans, antioxidant compounds that may contribute to prevent certain cancers (Thompson and Cunnane, 2003). Flaxseed mucilage has anti-inflammatory effects on the intestine and seems to decrease cholesterol and glucose levels in the blood (Mitra and Bhattacharya, 2009; Thakur *et al.*, 2009). Flaxseed proteins, rich in arginine, are thought to have antioxidant (Bourdon *et al.*, 2005; Elias *et al.*, 2005) and antiatherogenic (Marambe *et al.*, 2011; Wallner *et al.*, 2001) properties.

However, the principal valuable feature of flaxseeds is their high oil content, rich in polyunsaturated fatty acids, including omega-3 and omega-6 fatty acids in particular. It has been suggested that the addition of flaxseeds to the feed of ruminants and poultry results in meat of a higher quality than that obtained from animals fed exclusively on maize and soybean (Kouba and Mourot, 2011). The consumption of omega-3 fatty acids can also have diverse beneficial effects on human health (Abozid and Ayimba, 2014) and this has led to the use of flaxseed oil in pharmaceutical products, cosmetics and food supplements. These fatty acids are subject to oxidation and

polymerization, conferring drying properties on flaxseed oil, which is used in diverse technical applications, including the manufacture of paints and mastic and in the treatment of wood (Jhala and Hall, 2010; Humar and Lesar, 2013; Ozgenc *et al.*, 2013). Within the seed, this oil is stored in oil bodies, which protect the triglycerides (97.6% of the mass of the oil body) by surrounding them with a membrane composed of phospholipids (0.9%) and proteins (1.34%) (Tzen *et al.*, 1993). The oil bodies are stabilized and protected against coalescence by the proteins present in the membrane; they can be directly incorporated into formulations and this process is facilitated in some cases by the emulsifying compounds present in the seed. They can thus be used as they are, or as vectors for the delivery of active ingredients, in various domains, including nutrition, cosmetics and paints (Deckers *et al.*, 2000; Guth and Cappabianca, 2006; Voultoiry and Flavigny, 1995).

Oil bodies and tensioactive proteins are generally extracted in water. However, unlike many other oleaginous and proteinaceous seeds (*e.g.* rapeseed, soybean, sunflower), the seeds of the flaxseed plant have large amounts of mucilaginous polysaccharide at their surface. These polysaccharides thicken the medium when the seeds are placed in water, rendering the mechanical processing of the seeds difficult.

The determination of the composition of the oil bodies and the role of the proteins and mucilage in their release and stability would therefore facilitate the valorization of these organelles.

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2 Materials and methods

2.1 Reagents

The reagents and standards used were all of analytical quality and were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France), with the exception of ninhydrin and buffers, which were provided by Biochrom Ltd. (Cambridge, United Kingdom).

2.2 Seeds

We used the flaxseed variety Niagara, provided by Linéa, France. The seeds of this variety are particularly rich in lipids (47.4%) and have a protein content of 23.3%.

2.3 Analysis

Lipid content was determined on a dry-matter basis, with a DIONEX ASE 200 Accelerated Solvent Extractor (Idstein, Germany), with cyclohexane as the solvent (Valentin *et al.*, 2013). The extraction conditions were as follows: oven temperature, 105 °C; cartridge pressure, 90 bars; three 10-minute extraction cycles.

The fatty acid profile of the triglycerides present in the oil 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 (Roche *et al.*, 2006) equipped with a CP-select CB column (50 m long, 0.32 mm i.d., 0.25 µm film thickness); helium was used as the carrier gas, at a flow rate of 1.2 ml/min; split injector (1:100) and FID were maintained at 250 °C; the initial oven temperature was set at 185 °C for 40 min, increased to 250 °C at a rate of 15 °C/min and maintained at this temperature for 10 min.

Sterol content was determined by gas chromatography (Roche *et al.*, 2010). We introduced 50 µl of a 2 mg/ml solution of cholestanol in chloroform into a 15 ml screwtop tube. The chloroform was then eliminated by evaporation. A test run was then carried out with the addition of 100 mg of oil to the tube, followed by 2 ml of 1 M KOH in ethanol. The mixture was vortexed and heated at 75 °C in a water bath for 30 min. It was then allowed to cool to room temperature; 1 ml of distilled water was added and the mixture was vortexed. The unsaponifiable material was then extracted in 6 ml of cyclohexane. We collected 160 µl of the cyclohexane phase, which we then silylated by adding 40 µl of BSTFA/TMCS (99/1 mixture). The sample was heated for a few minutes at 103 °C and then 1 µl was injected into 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) and coupled to a flame ionization detector operating at 355 °C. The carrier gas was helium, at a column head pressure of 100 kPa in the headspace. The thermal conditions were as follows: 55 °C for 0.5 min, an increase in temperature of 200 °C/min to 340 °C, then maintenance at 340 °C for 30 min for the injector; 160 °C for 0.5 min, then an increase of 20 °C/min to 260 °C, then of

2 °C/min to 300 °C and 45 °C/min to 350 °C for the oven. The various sterols were identified by comparing their retention times with those of commercial standards or reference values. The sterols were quantified against an internal standard.

Tocopherol content was determined by high-performance liquid chromatography. We solubilized 20 mg of oil in 1 ml of cyclohexane. We then injected 20 µl of the resulting solution into 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. The tocopherols present in the oil were quantified against an external standard, and were identified by a comparison of retention times with those of reference standards.

Protein content was determined by assaying the nitrogen by the Kjeldahl method (French standard NF V 18-100) and converting the values obtained to protein levels by applying a conversion coefficient of 6.25. Mineral content was determined by calcination in an oven at a temperature of 550 °C until no further change in weight was observed (French standard NF V 03-322).

The amino-acid composition of the proteins was determined by the method of Moore and Stein (Moore and Stein, 1948). The samples were hydrolyzed with 6 N hydrochloric acid at 103 °C for 24 h. The amino acids released were then separated by ion exchange chromatography. The analysis was carried out with a Biochrom 20+ amino-acid analyzer (Biochrom Ltd., Cambridge, United Kingdom) equipped with a 200 × 4.6 mm column + precolumn system with sodium-based ion exchange resins. The amino acids were separated by elution with buffers of different pH at various defined temperatures. They were reacted with ninhydrin and detected at a wavelength of 570 nm, with the exception of proline, which was detected at 440 nm.

The mucilage and grains were freeze-dried in a Cryo-Rivoire machine: the samples were first cooled to -40 °C at a rate of 0.5 °C and then maintained under a vacuum of 0.3 mbar until being returned to room temperature.

2.4 Determination of the size of solid particles and droplets

The sizes of particles and droplets were determined by laser granulometry in both the wet and dry modes, with a Mastersizer 2000 machine (Malvern, Worcestershire, United Kingdom). In the wet mode, the medium was diluted and a slight ultrasound treatment was performed until the breaking up of droplets aggregates.

2.5 Zeta potential determination

The zeta potential of particles in solution was determined with a Malvern Zetasizer Nano ZS. It was calculated from the electrophoretic mobility, with the Smoluchowski equation (Smoluchowski, 1903).

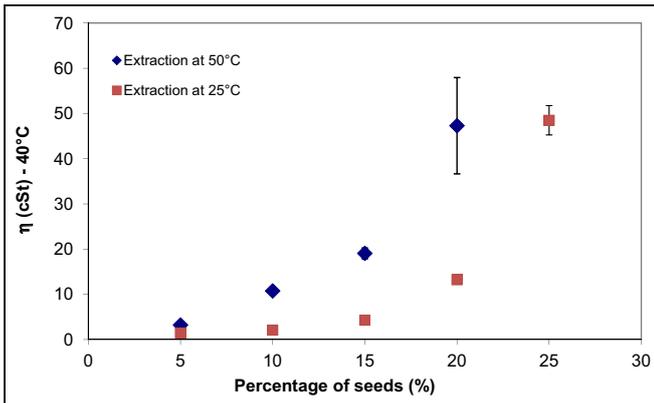


Fig. 1. The kinematic viscosity (at 40 °C) of the aqueous phase recovered after stirring flaxseeds in water for 30 min at 25 °C and 50 °C, as a function of the percentage of seeds in the mixture.

2.6 Analysis of rheological behavior

Viscosity was analyzed with a Cannon-Fenske capillary viscosimeter in a water bath at 40 °C. Rheometric analysis was carried out with an Anton-Paar MCR302 rheometer with a cone-plane geometry at 25 °C. A rotational analysis was performed by scanning with a 0 to 2.5 N/m² constraint. An analysis in the oscillatory mode was carried out, beginning with an amplitude scan at 25 °C and a frequency of 1 Hz, until 100% deformation. A frequency scan was then carried out for the linear part of the viscosity curve.

3 Results and discussion

3.1 Aqueous-phase mucilage removal from the flaxseeds

Mixtures of seeds and water were magnetically stirred at 500 rpm at a temperature of 25 to 50 °C: the viscosity of the aqueous phase increased substantially with the seed density (Fig. 1).

This viscosity limits the possibilities for grinding the seeds and separating out compounds of interest. Consequently, it would be useful to extract the mucilage responsible before processing the seeds. The mucilage is highly soluble in water and aqueous extraction is, therefore, feasible. In the presence of water, the mucilage hydrates and expands until it splits the cells containing it. It then spreads throughout the aqueous phase. At high seed densities and room temperature, the diffusion of the mucilage into the water may be slow and is rapidly impeded by the increase in viscosity.

A magnetic stirrer can accelerate this process somewhat, but does not allow rapid elimination of sufficient quantities of mucilage; ultrasound has been shown to be much more effective (Fabre *et al.*, 2015). We found that an ultrasound probe or column was sufficiently powerful to lyse the mucilaginous cells, hydrate the mucilage and detach much of it from the seeds.

We used a laboratory ultrasound probe (Sonics Vibracell – 500) at various amplitudes, with 5 s pulses of activity,

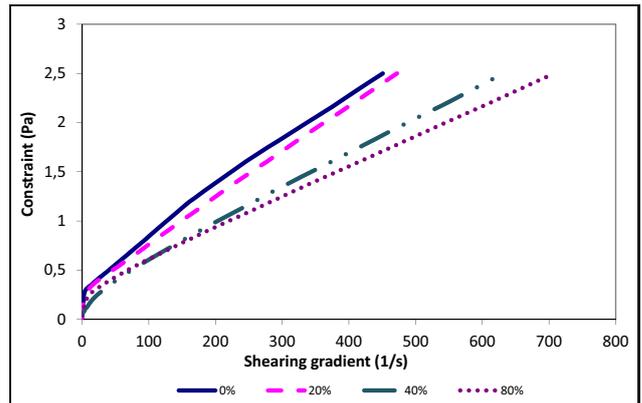


Fig. 2. Flow curves for solutions of 1% freeze-dried mucilage in water, as a function of the ultrasound amplitude used for mucilage extraction.

separated by intervals of 10 s, over a period of 30 min. The extraction mediated by the ultrasound pulses and the hydrolysis induced by the polysaccharides made it possible to ensure that the viscosity of the medium remained low.

The seeds were separated on a metallic filter with pores of 0.5 cm in diameter. Both the seeds and the aqueous extract obtained were then freeze-dried, and about 7% of the total seed mass was recovered in the aqueous phase. The mucilage had low protein (1.3 ± 0.40%) and mineral (7.0 ± 0.5%) contents. This procedure is, therefore, non-destructive. It limits the extraction to the components present at the surface of the seed. It also extracts little, if any, cyanogenic glycoside or phytic acid (Kadivar, 2001).

3.2 Rheological properties of the mucilage

The freeze-dried mucilage recovered retained its thickening properties and could therefore potentially be used as a stabilizer of aqueous emulsions (Fig. 2). As previously, it displayed shear-thinning behavior at a concentration of 1% in water, due in particular to the high molecular weight of the neutral polysaccharides (Cui *et al.*, 1994). The flow curve can be modeled by laws with different levels of precision. A simple power law (or Ostwald law), generally used for solutions of mucilage (Fedeniuk and Biliaderis, 1994; Wang *et al.*, 2011) can be improved by adding an affine function (Tscheuschner's law). If the behavior of the mucilage is modeled in this way, the regression coefficients are good ($r^2 = 0.99994$, based on mucilage extracted at an ultrasound amplitude of 60%):

$$\tau = a + \eta_{\infty}\dot{\gamma} + \eta_0\dot{\gamma}^p, \quad (1)$$

where τ is the shearing constraint (Pa), $\dot{\gamma}$ is the shearing gradient (1/s), η_{∞} is the viscosity for an infinite gradient (plastic viscosity) Pa.s, and η_0 is the viscosity for a zero gradient (at an ultrasound amplitude of 60%, $\eta_0 = 222.13$, $\eta_{\infty} = 2.97$ mPa.s and $p = 0.17$).

At a mucilage concentration of 1% in water, the rheological tests in oscillatory mode with a 5 Pa constraint showed that the elastic component of the mucilage increased relative

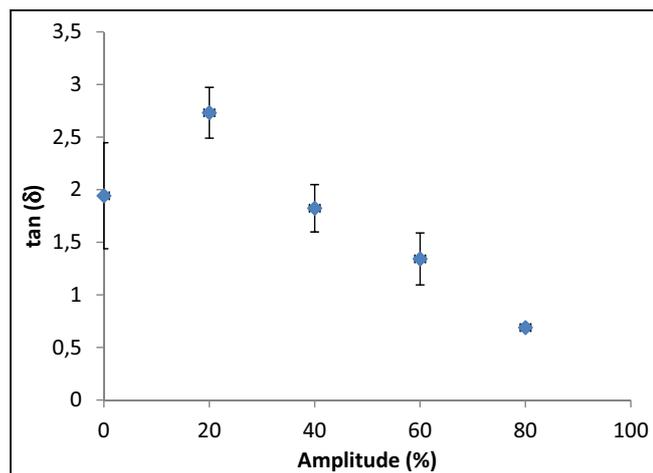


Fig. 3. Change in the G''/G' ratio ($\tan \delta$) of solutions of 1% freeze-dried mucilage in water, as a function of the ultrasound amplitude used for mucilage extraction.

to the viscous component with increasing ultrasound amplitude (Fig. 3). This pattern of behavior is undoubtedly linked to the extraction of larger amounts of neutral polysaccharides and structural proteins.

Once their mucilaginous outer film has been removed, the seeds can be ground in water to extract the oil bodies.

3.3 Release of the oil bodies by grinding the seeds in water after the removal of their mucilage

We extracted the oil bodies, by grinding seeds from which the mucilage had been removed in distilled water, at a concentration of 15% by mass. A high-shearing force homogenizer (Silverson L4RT with a double rotor/stator with square holes) was used for 10 min, at 7000 rpm. The homogenate was then treated twice in a high-pressure homogenizer, at 500 bars. The homogenate obtained was then centrifuged at $15000 \times g$ at 6 °C for 10 min. An emulsion, in the form of a lipid-rich cream, was recovered, together with an aqueous phase and a pellet of fibrous sediment. This emulsion contains about 70% water, 25% lipids and 3.5% proteins. Around two-thirds of the oil content of the seed were hence extracted as dispersed oil bodies but higher yields may be achieved with low solid ratios, higher shear rates and the use of enzymes (Campbell *et al.*, 2011). Most of the proteins were non-membrane proteins (87%). The lipids present were principally triglycerides of unsaturated fatty acids (90%): linolenic acid (54.1%), linoleic acid (15.7%) and oleic acid (20.2%). The saturated fatty acids were mostly stearic acid (5.3%) and palmitic acid (4.7%).

Minor compounds playing a role in emulsion stabilization and/or valorization were also identified. Hence, membrane phospholipids are surfactants acting synergistically with membrane proteins to stabilize oleosomes (Deleu *et al.*, 2010; Roux *et al.*, 2004). The lipids also contained 0.28% sterols, concomitantly extracted by this aqueous and milder process than classical refining or trituration processes (Mouloungui *et al.*, 2006). They are principally in the form

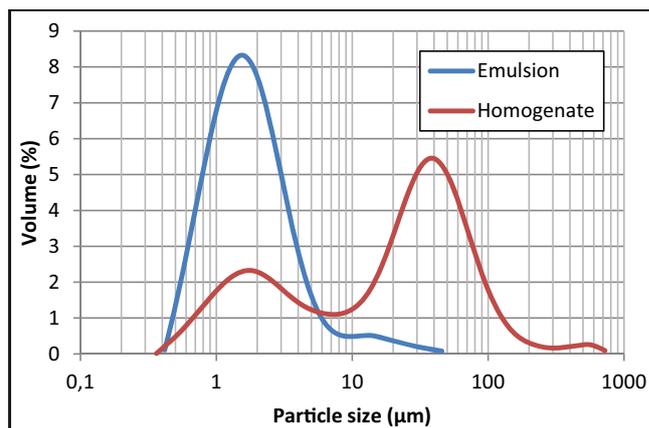


Fig. 4. Granulometric analysis of the aqueous homogenate of flaxseeds and of the emulsion recovered after centrifugation.

of β -sitosterol (134.7 ± 1.0 mg/100 g of lipid), campesterol (74.4 ± 0.3 mg/100 g of lipid), Δ^5 -avenasterol (46.2 ± 0.1 mg/100 g of lipid) and stigmasterol (25.3 ± 0.1 mg/100 g of lipid). This distribution is similar to those generally observed in the literature (Ciftci *et al.*, 2012; Itoh *et al.*, 1973; Schwartz *et al.*, 2008).

Similarly, the oil contained tocopherols, at a concentration of 36.29 mg/100 g of oil, almost all in the gamma form (97.75%), as it has ever been observed for other flaxseed varieties (Oomah *et al.*, 1997; Schwartz *et al.*, 2008). This content of gamma tocopherol is in the high range of those observed for other species (soybean oil: 27 mg/100 g (Grilo *et al.*, 2014), soybean oil bodies: 12–23 mg/100 g (Fisk and Gray, 2011), rapeseed oil: 9 mg/100 g (Şeker *et al.*, 2008) 39 mg/100 g (Schwartz *et al.*, 2008), sunflower oil: 1.4 mg/100 g (Schwartz *et al.*, 2008), 9 mg/100 mg (Grilo *et al.*, 2014)). The presence of tocopherols, which work together with phospholipids which, as the neutral lipids, are mainly unsaturated (Herchi *et al.*, 2012; Wanasundara *et al.*, 1999), may improve the storage properties of freeze-dried oleosomes (Kamal-Eldin and Appelqvist, 1996; Doert *et al.*, 2012).

Both sterols and tocopherols have potential medical applications, based on their cholesterol-lowering and antioxidant activities, respectively, contributing substantially to the nutraceutical value of the oil (Kritchevsky and Chen, 2005).

A granulometric analysis of the aqueous homogenate and of the emulsion recovered showed that centrifugation did not increase the mean size of the droplets, which remained at about 1.3 μm (Fig. 4). Thus, no coalescence appeared to have occurred during this concentration process, demonstrating the resistance of the oil body membrane.

Rheometry showed that the emulsion was a power-law fluid (Fig. 5), with shear-thinning behavior, to which it was possible to apply a Sisko law with a regression coefficient of $r^2 = 0.99678$:

$$\tau = a\dot{\gamma} + b\dot{\gamma}^p \quad (2)$$

where τ is the shearing constraint (Pa), $\dot{\gamma}$ is the shearing gradient (1/s), and a , b and p are constants ($a = 0.13$; $b = 304$; $p = 0.01$).

A rheometric analysis in the oscillatory mode, with low-level deformation (0.2%) revealed that the emulsion behaved

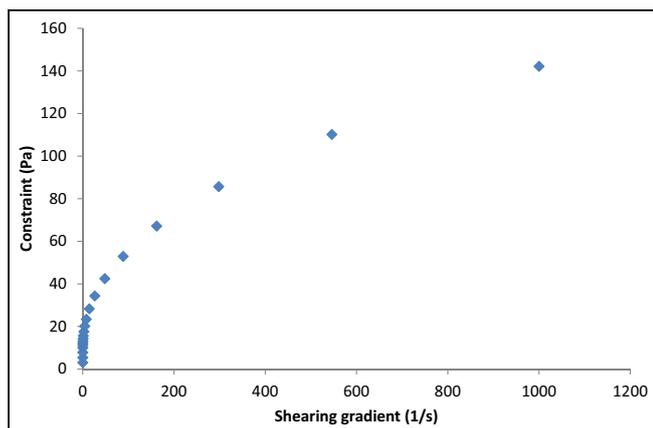


Fig. 5. Flow curve for the emulsion at 25 °C, with shear gradient scanning.

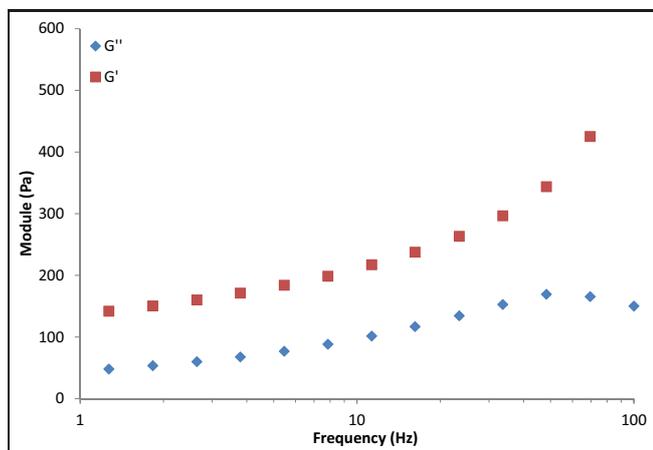


Fig. 6. Changes in the elastic and viscous modules as a function of the frequency at 0.2% deformation, at 25 °C.

like a viscoelastic solid with no flow over a broad range of frequencies (Fig. 6). The rigidity of its structure is therefore likely to be maintained during storage.

The emulsion was a direct emulsion (oil/water) with a conductivity of 0.84 mS/cm and a native pH of 6 ± 0.5 . It had a low zeta potential (-9 mV), which may account for the strongly flocculated state of the oil bodies. To facilitate the valorization of this oil body concentrate, we need to understand and be able to predict its behavior following dilution and changes in pH.

Oil bodies are not very susceptible to coalescence, due in particular to the presence of membrane proteins (Huang, 1996). The physical stability of the medium is therefore linked principally to the resistance of the oil bodies to flocculation. Phospholipids have low acidity constants (Moncelli *et al.*, 1994; Tsui *et al.*, 1986), resulting in a negative charge on the oil body surface. This charge is partly counterbalanced by the negative charge on the membrane proteins (Tzen *et al.*, 1992). It is thus important to determine the influence of pH and the electrostatic repulsion capacity of the membrane proteins and extracted water-soluble compounds.

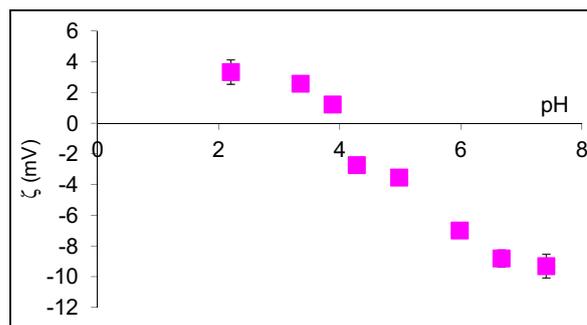


Fig. 7. Changes in the zeta potential of the membrane protein extract as a function of pH.

3.4 Membrane proteins

The membrane proteins (oleosins) had to be separated from the phospholipids and the other proteins present in the cream. We added two volumes of sodium chloride solution (3% w/w) per volume of emulsion, so as to dissolve the non-membrane components associated with the oil bodies. The solution was gently shaken and then centrifuged at $10\,000 \times g$ for 10 min at 0 °C. A compact cream was thus obtained. This new cream was washed several times in distilled water.

The preparation was then delipidated several times, with diethyl ether, and the phospholipids were extracted in a chloroform/methanol (2:1 by volume) mixture (Deleu *et al.*, 2010). We thus obtained a protein concentrate with a purity of $80 \pm 2\%$ (based on a nitrogen to amino acid conversion factor of 6.25).

The zeta potential of the membrane proteins recovered varied with pH, and the electrostatic repulsion properties of these proteins were clearly favored by neutral pH conditions (Fig. 7).

3.5 Non-membrane compounds in the aqueous phase

The aqueous phase was separated from the emulsion rich in oil bodies by centrifugation. It was then freeze-dried to obtain flakes with a protein content of 41% (termed contaminants hereafter).

The isoelectric point of these contaminants was lower than that of the membrane components (Fig. 8). Again, the electrostatic repulsion mediated by these proteins was weak, and they probably acted as flocculation agents through depletion forces. However, the use of a neutral pH again limited flocculation.

Once the contaminants had been removed by extraction in salt solution, the emulsion showed a lesser tendency to display flocculation, but it was also less resistant to coalescence. A gel/fusion test (1 min in liquid nitrogen, immediately followed by 15 min in an oven at 103 °C) on the oil bodies partially depleted of their “non-membrane” components showed that this treatment resulted in the release of twice as much free oil after centrifugation ($10\,000 \times g$, 10 min, room temperature) as was obtained with the unwashed emulsion. The role of oleosins in the prevention of coalescence has been clearly demonstrated and may underlie the resistance of the oil bodies to gel formation (Shimada *et al.*, 2008), but it seems to be important to retain these “non-membrane” components for the freeze-drying of the emulsion.

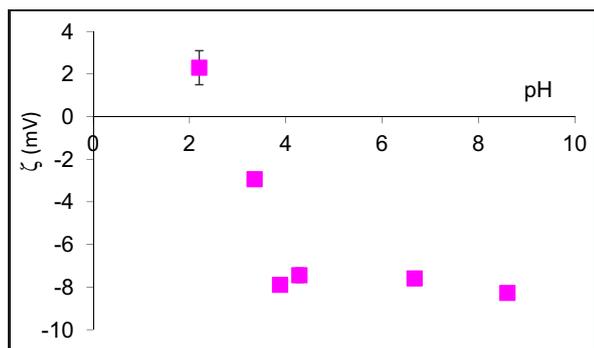


Fig. 8. Zeta potential of the “contaminants” as a function of pH.

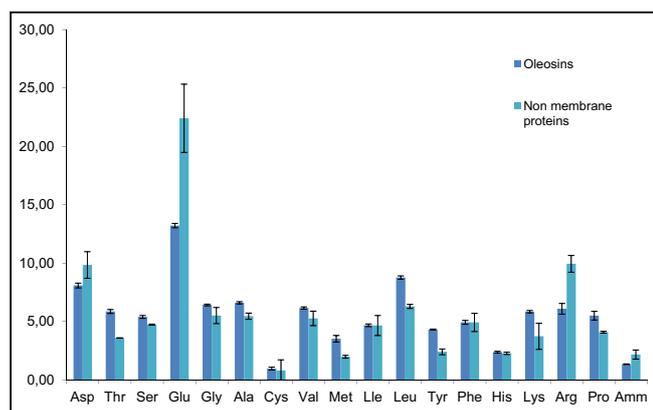


Fig. 9. Comparison of the amino-acid compositions of the membrane and non-membrane proteins.

The amino-acid profile of these non-membrane proteins differed little from that of the membrane proteins (Fig. 9).

In terms of antioxidant potential, the higher proportion of arginine residues in these preparations (Wallner *et al.*, 2001) counterbalances the lower proportion of methionine (Bourdon *et al.*, 2005), lysine and serine residues, all of which may have antioxidant activity. This activity is dependent on pH, with some amino acids having antioxidant activity at high pH and oxidant activity at low pH (Marcuse, 1960). In any case, the membrane and contaminating proteins may play a role in the oxidative stabilization of the oil bodies.

4 Conclusion

The mucilaginous polysaccharides and interfacial proteins in flaxseed are largely determinant of the results of extraction and stabilization of oil bodies. The viscosity provided by the mucilage hinders the release of oil bodies during aqueous extraction, but may help to stabilize formulations containing oil bodies. The interfacial proteins help to control flocculation and coalescence and may even slow triglyceride oxidation. The expression of these different properties can be modulated by mechanical (sonication → molar mass → viscosity) and/or chemical (pH → zeta potential, potential for oxidoreduction → electrostatic repulsion, oxidation) processes. The oil body concentrate, consisting mostly of triglycerides and proteins, but also containing minor components, such as phospholipids, sterols and tocopherols, could be valorized for nutraceutical and technical applications.

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