

**Optimising the recovery and stabilisation
of intact oil bodies from oilseed rape**

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Abstract

Oil bodies (oleosomes) are sub-cellular droplets representing the main form of energy storage in oleaginous plant seeds. The release of intact oil bodies by wet milling oilseeds results in a natural emulsion (no need for additional emulsifiers) and is likely to have a lower environmental impact than conventional oilseed processing. During the aqueous recovery of oil bodies, other water-soluble seed material is solubilised and carried-over in the final product. The aims presented in this thesis were:

1. Recover intact oil bodies free from exogenous seed materials
2. Understand which the mechanisms of oil body instability during storage are
3. Stabilise the oil body material
4. Assess emulsion physicochemical stability during storage

The degree to which recovered oil bodies remained intact was established by exposing the recovered crude cream to urea (9M) and measuring any changes to droplet size; no change indicates a high degree of intact oil bodies. A lipase assay was used to monitor enzyme carry-over in oil body preparations and to measure thermal process effectiveness. Chemical (hydrolysis of lipids and integral proteins) and physical (particle size and zeta potential) stability were measured during oil body storage. The correlation between thermal treatment, lipase activity, and oil body stability was tested.

The use of alkaline pH solutions (>8.5) to soak and grind oilseed rapeseed were more effective against the contamination of oil body material

with seed proteins/enzymes, compared with neutral pHs. Soaking and grinding seeds with a NaHCO_3 solution (0.1 M, pH 9.5) yielded oil bodies with a similar composition to those prepared in urea (9 M); despite this 'clean up' by bicarbonate solutions, the physical stability over storage was compromised, due to the presence of hydrolytic enzymes. Heating a dispersion of oil bodies (milk or resuspended cream) for 6 minutes at 95°C significantly reduced lipolytic activity (>90% enzyme inactivation) and resulted in oil bodies that were stable physically and chemically.

Using the optimized recovery protocol and thermal treatment, oil bodies retained during storage (1 month, 20 °C) a particle size ($D_{4,3}$) of about 1.4 μm ; as the pH was increased from 3 to 10 the zeta potential changed from around +55 mV to -65 mV (pI of 6). In terms of chemical stability, thermally treated emulsions did not show signs of either proteolytic or lipolytic activity. The oxidative stability of oil bodies was significantly improved after thermal treatment.

To my family,

To Maria.

Per aspera ad astra

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Being grateful is to understand our limitations and recognize the beautiful capacity of others to contribute to our good and personal growth.

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List of Abbreviations

BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
COB	Crude oil bodies
DAG	Diacylglycerol
DWB	Dry weight basis
ER	Endoplasmic reticulum
FFA	Free fatty acid
FS	Free Sterols
GC-MS	Gas Chromatography Mass Spectrometry
MAG	Monoacylglycerol
NL	Neutral Lipid
OSR	Oilseed rape
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
pI	Isoelectric point
PI	Phosphatidylinositol
PL	Phospholipid

PS	Phosphatidylserine
PSD	Particle Size Distribution
PV	Peroxide value
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SE	Sterol Esters
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TLC	Thin layer chromatography
Urea-WOB	Urea washed oil bodies
WOB I	Washed oil bodies (cream/emulsion after first wash)
WOB II	Washed oil bodies (cream/emulsion after second wash)

CHAPTER 1

INTRODUCTION

1.1. Background

Emulsions are mixtures that are prepared from two mutually insoluble components. The most common type of emulsions are those in which an aqueous and a lipophilic component are employed (usually water and oil); these systems are frequently referred as oil-in-water and water-in-oil emulsions (McClements, 2004). A considerable number of natural and processed food consist either partly or wholly as emulsions, or have been in some stage of their production emulsified, including mayonnaise, milk, cream, soups, salad dressing, sauces, ice cream, margarine (McClements, 2004). Generally, emulsions are prepared in the presence of a multiplicity of other natural or synthetic components (e.g., emulsifiers), in order to achieve a desirable balance of emulsification, viscosity, stability, and appearance.

In the seeds of oil crops, including some economically important crops, such as soybean, rapeseed and sunflower, oil in the form of triacylglycerol (TAG), is the major seed storage reserve. The oil fraction is stored in discrete subcellular structures known as oil bodies, also called oleosomes or lipid bodies (Huang, 1992). By partitioning TAGs into small lipid droplets, the lipids, and so energy reserve of the plant, are preserved. These droplets are stabilised by a layer of phospholipids and proteins, called oleosins, which exhibit a unique structure and are crucial for oil body stability. Modeling and biochemical evidence suggest that a central hydrophobic domain anchors the oleosin in the oil body through interaction with TAGs molecules, and two N-

and C- terminal amphipathic domain reside on the surface of the droplet facing the exterior (Huang, 1992; Tzen et al., 1993).

During the conventional process of oil extraction from oleaginous seeds, oil bodies are destroyed through grinding of the seed, which are then pressed to extract the oil (Daun et al., 2011). To maximise the yield, the use of organic solvents (e.g., hexane) is sometimes applied (Bockisch, 1998). Oil extraction and the subsequent refining step has a high environmental impact, and it is hazardous due to flammability and risk of explosion. It is estimated that the production of oil from vegetable sources in 2017/2018 was approximately 195 million tonnes per annum (www.usda.gov), of which 30% is processed into emulsions using high-energy homogenisation and the addition of emulsifiers/surface active agents.

The release (recovery) of oil bodies from the cells of oilseeds into an aqueous medium (a relatively benign wet milling process) leads to a stable oil-in-water emulsion. Compared with conventional oilseed processing, the recovery of oil bodies as a natural source of pre-emulsified oil, to be used in a range of food applications, is likely to reduce the environmental impact.

Oil bodies have been extensively studied from a wide range of oil crops and cereal, such as soybean (Cao et al., 2015; Chen and Ono, 2010; Fisk and Gray, 2011; Wang et al., 2017), maize (Nikiforidis and Kiosseoglou, 2010, 2009; Tzen et al., 1993), rapeseed (Katavic et al., 2006; Tzen et al., 1993; Zhao et al., 2016b), peanuts (Tzen et al., 1993; Zaaboul et al., 2018) and sunflower (Fisk et al., 2008; Sadeghipour and Bhatla, 2002; White et al., 2008). However, differences in the recovery protocol affect the physicochemical

properties of the oil body material and thus its functionality. Moreover, the ambient shelf life of oil bodies is usually limited to a few days, limiting their application for food purposes; recent works revealed the presence of seed enzymes in the oil body cream (Zhao et al., 2016b), but the magnitude of these activities and how they affect product stability over storage is yet to be discovered.

The aims of this thesis were to (I) develop an effective protocol for the recovery of oil bodies from oilseed seeds, (II) keeping their integrity and (III) natural composition *ex-vivo*, with (IV) enhanced storage stability. A deep understanding of the driving factors affecting the physicochemical properties and shelf life of recovered material was necessary to achieve this purpose. The oil bodies were recovered from rapeseed, an important agricultural commodity ranking second in the world production of oil crops after soybean. In the United Kingdom, rapeseed is widely cultivated, reaching 550,000 ha in 2017 (<https://cereals.ahdb.org.uk/markets>).

The results in this thesis are divided into three main chapters, according to three major hypotheses that were tested. **Chapter 3** gives a deep understanding of the factors in the recovery protocol that affect the quality of the final material. Considering that oil bodies in seed are protected from coalescence by the oleosin coat (Huang, 1996; Maurer et al., 2013; Schmidt and Herman, 2008; Tzen and Huang, 1992), it was hypothesised that intact oil bodies *ex-vivo* having a surface composition similar to the one *in-vivo*, would create a natural emulsion with high physical stability. Moreover, it was also hypothesised that a clean extract from exogenous seed material would have lower enzymatic load, thus enhanced shelf life. Particle size, ζ -potential and

compositional analyses were used to study the effect of a range of buffers at different pH values on the quality (integrity) and purity (in terms of exogenous seed proteins) in the final extract. By this way, it was possible to gain the necessary knowledge to develop a protocol for the recovery of oil bodies to be used in this thesis. **Chapter 4** explores for the first time the magnitude of enzymatic activity in oil bodies *ex-vivo*, as affected by extraction procedures. The assessment of enzymatic activity was used not only to study the correlation between exogenous protein content and enzymatic activity in oil bodies, but also as a marker to develop the thermal process that would allow the inactivation of enzymes carried over from the seed matrix. Furthermore, physical analyses were used here to test if the oil bodies would withstand the thermal process applied. **Chapter 5** gives an understanding of the factors that affect the physical and chemical stability of oil bodies *ex-vivo* over storage; thus, the effect of thermal treatment (developed in Chapter 4) on shelf life was tested. Physical (particle size, ζ -potential) and chemical stability (lipid composition and oxidation) over time were studied. To begin with, an overview of the current research on oil bodies is given in **Chapter 1**, with principles of the methodologies used to characterise the material. The materials and methods used in this thesis are presented in **Chapter 2**.

1.2. Rapeseed seed composition

The increasing demand for edible oils and the continuous growth of the bio-fuel industry has been the driving factor for the remarkable increase of oilseed production. Between early 1970's and late 1990's the cultivation of oil crops has increased by 70 million hectares, and is expected to increase further up to 150 million hectares by 2030 (Alexandratos and Bruinsma, 2012).

Canola and rapeseed belong to one of the most widespread family of cultivated plants: the *Brassicaceae* (or *Cruciferae*). Cultivation and selection led to the development of the common brassica vegetables of today, including cabbage, broccoli, cauliflower, turnip and their relatives (Daun et al., 2011).

All brassicas cultivated for use of the seed oil are sometimes referred as oilseed rape (OSR), whereas the brassica plants cultivated for using the seeds as spice are often named as "mustard". Initially cultivated in India about 4000 years ago, rapeseed seeds appeared in Europe only from the 13th century. This oil was rich in erucic acid and was mainly used for non-food purposes. However, following health concerns in the late 1960's Canadian breeders produced a variety of rapeseed with less than 2% of erucic acid, and low content (<30 $\mu\text{mol/g}$ oil-free seed) of glucosinolates (anti-feedant) (Gunstone, 2013). These varieties are often described as double zero, and in 1974 were licenced under the name of Canola. The wide adaptation to extremes of temperate zones made these varieties suitable to be grown in Nord America, Europe, Asia and Australia (Figure 1.1).

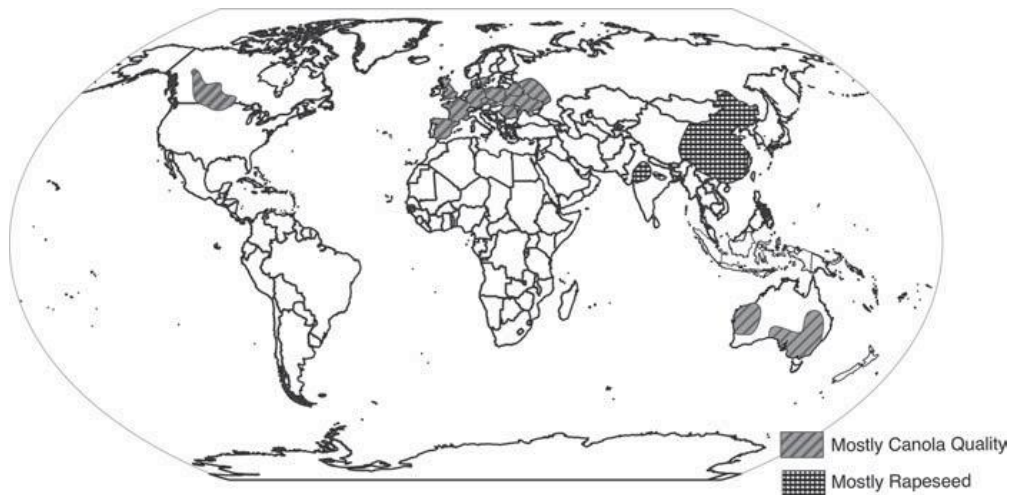


Figure 1.1: Major rapeseed and canola production area in the world. Areas where canola and rapeseed are predominantly grown are shown in grey and black, respectively. (Daun et al., 2011).

The oil content of rapeseed seeds is usually about 43%, but varies between 40 and 50% (Hu et al., 2017) depending on specie, variety and environmental condition. Moreover, changes to seed characteristic such as seed size (Marquard and Arnholdt, 1978) and seed coat (Rashid et al., 1994) also affect oil content in seed. Canola oil has a low amount of saturated fatty acids (palmitic and stearic acid), which is about 6% of the total fatty acid composition, lower than any other commodity oil. The content in linoleic and linolenic acid is typically about 20 and 10%, respectively; oleic acid is usually above 60%.

Protein content in canola seeds may vary between 18 to 30%, and are localised mainly in the protein storage vacuoles (PVS) (Wanasundara et al., 2016). In canola, two classes of seed storage proteins are predominant: 11S (or 12S) globulin (legumin type), cruciferin (300–350 kDa) and 2S (or 1.7S) albumin, napin (12–16 kDa), representing up to 90% of the total proteins

(Campbell et al., 2016). Besides that, structural protein such as oil body protein and lipid transfer protein are also found.

1.3. Rapeseed seed processing

The extraction of rapeseed oil typically requires a two-step process comprising three overall stages; seed pre-treatment, mechanical pressing followed by solvent extraction, producing crude oil and a by-product meal, which is utilised as animal feed. Oil extraction process from rapeseed seeds is summarised in Figure 1.2. A critical step before any physical processing is the adjustment of moisture between 7-7.5%, which enhances oil extraction, solvent recovery from the meal, oil quality, and degumming efficiency (Daun et al., 2011). Contrary to sunflower seeds, de-hulling of rapeseed is not a common procedure, due to the small size of the seed which makes the process expensive (Matthäus, 2007). After conditioning, the rupturing of the cell wall (flaking) exposes the lipid droplets, providing an open pathway for the oil particles to migrate away from the cellular structure during pressing. Moreover, flaking helps the solvent to penetrate the broken cellular structure leaching the oil, and then migrate out from the meal leaving the structure solvent-free. The flaked-seeds are conveyed to the cooker. Heating the seeds (90-105 °C) decreases oil viscosity, easing its removal from the ground matrix, plus inactivates enzymes and prevents growth of mould and bacteria. A screw press extracts up to 70% of the oil, while the rest is extracted through exposure of the seeds to organic solvents, with hexane being the most commonly used

(Bockisch, 1998). After removal of the solvent, crude oil is produced, while the meal is commercialised as animal feed.

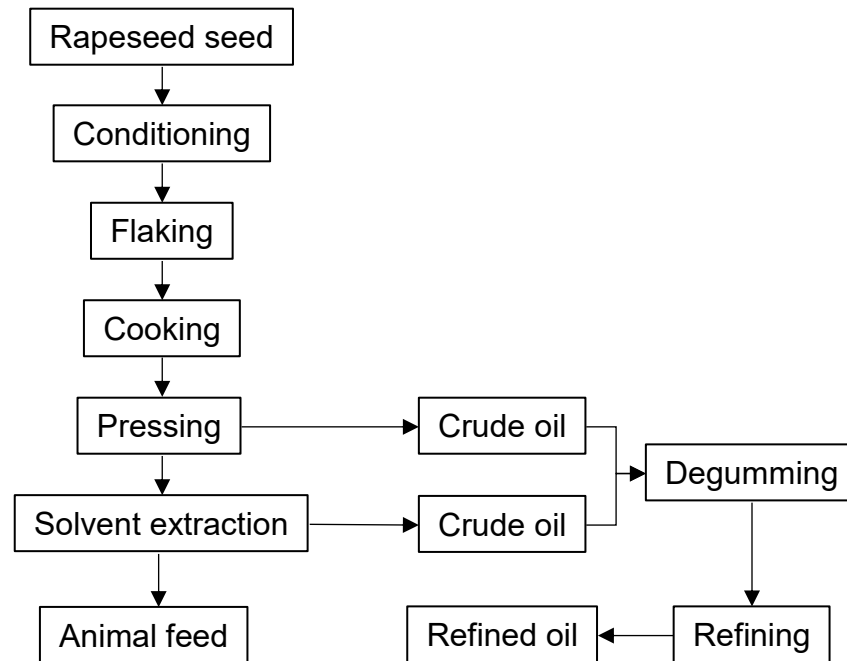


Figure 1.2: General overview of oil extraction from rapeseed seeds

The crude oil contains a variety of undesirable compounds such as free fatty acids, phospholipids, and pigments that need to be removed (through refining steps) to improve oil quality for human consumption (Matthäus, 2007). Refining is a multistage process including degumming, neutralising, bleaching and deodorising; Figure 1.3 shows a detailed flowchart for this process, which can be distinguished in “chemical” and “physical” refining.

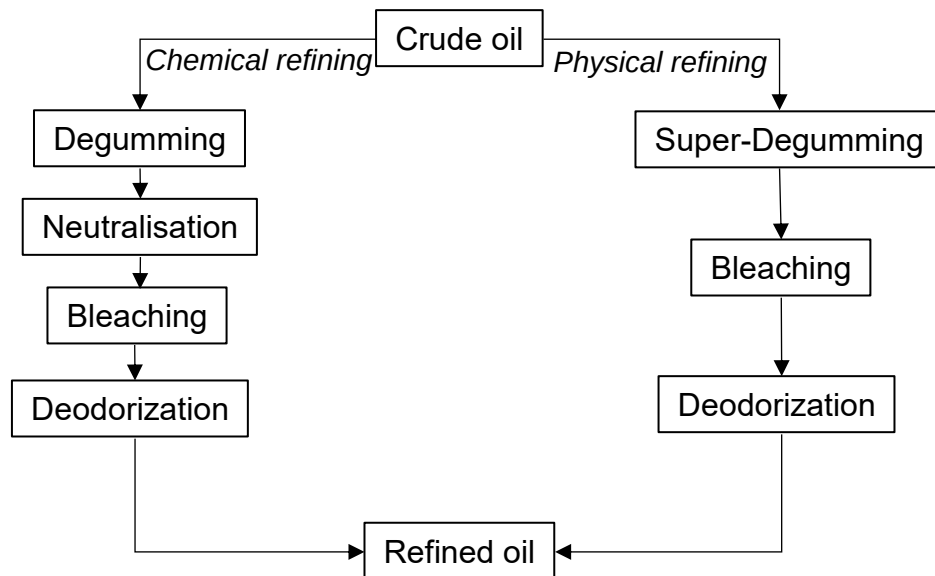


Figure 1.3: Refining of edible oil.

During the extraction process, some mineral salt are extracted with the oil. These ions, when attached to the phospholipids, will render the phospholipids non-hydratable in a water-degumming process. Most canola oil degumming uses a so-called “super-degumming” process, where a mild acid (e.g., citric acid) chelates phosphatides, which will be removed from the oil along with hydratable phosphatides, with the addition of water. This type of oil does not require an alkali refining, and can proceed directly to bleaching into a process called “physical refining” (Daun et al., 2011).

1.4. Oil bodies

Plants store energy as polysaccharide or lipids to support periods of active metabolism such as seedling establishment during germination (Murphy and Vance, 1999). Lipids are stored in subcellular particles, which can be found in a number of tissue and organs, but the highest concentration is found in oilseed seeds (Frandsen et al., 2001; Murphy, 2001). In most plants, lipids are in the form of triacylglycerols (TAGs) which are pre-dispersed by phospholipids (PLs) and proteins (oleosins) as small hydrophilic droplets (oil bodies, also called oleosomes or lipid bodies) (Huang, 1992).

Oleosins are proteins of low molecular mass (15-26 kDa) and have been proposed to serve as a structural role in oil body stabilization (Vance and Huang, 1987) but also to provide a specific binding site for lipase during germination (Huang, 1992). Oleosins are unique proteins found only in oil bodies, anchored to the oil matrix by a central hydrophobic domain (Huang, 1992; Tzen and Huang, 1992) (Figure 1.4). Oil body size varies among oilseed species, typically between 0.2 and 2.5 μm (Tzen et al., 1993). However, environmental and nutritional conditions, as well as amount of oleosin synthesised by the plant, influence oil body size (Siloto et al., 2006; Ting et al., 1996; Tzen et al., 1993). Oil bodies isolated from seed consist of approximately 95-98% TAGs, 0.5-2% PLs and 0.5-3% proteins (Tzen et al., 1993).

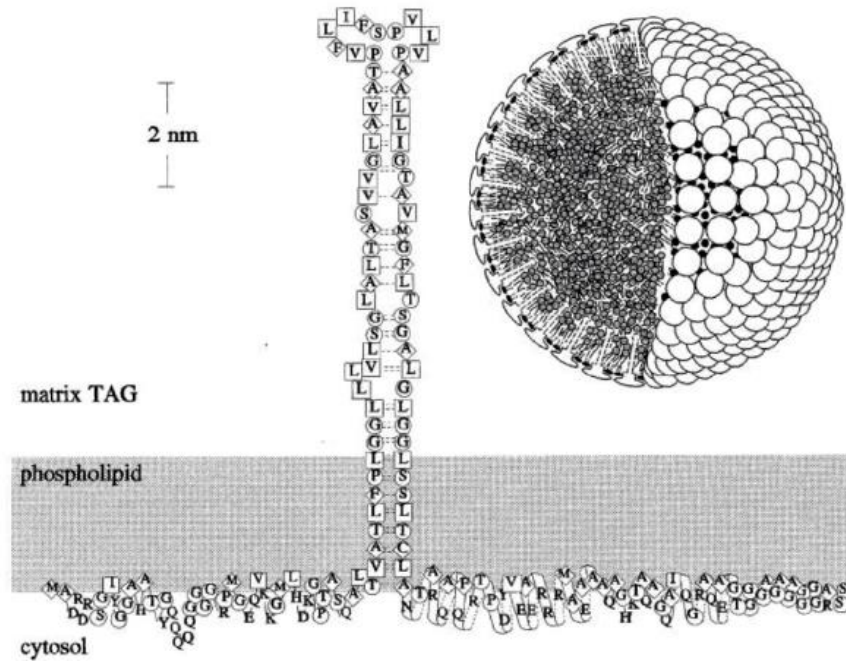


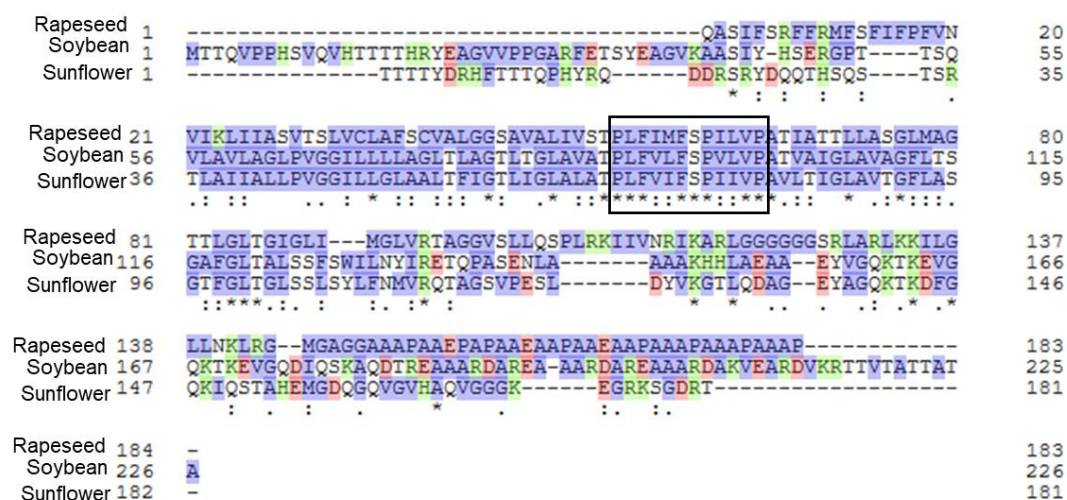
Figure 1.4: Structural model of an oil body. Oil body contains a central triacylglycerol core, surrounded by a monolayer of phospholipids and embedded with integral proteins (mainly oleosins). Adapted from (Huang, 1996).

1.4.1. Oil body proteins

Oleosins are believed to be responsible for the stability *in-vivo* and *ex-vivo* of oil bodies. These proteins prevent coalescence of oil bodies under osmotic and physical stresses, principally during the desiccation-rehydration cycle prior to and after seed dormancy (Murphy, 2001). Moreover, oleosins also maintain an optimal surface area:volume ratio of oil droplets for efficient energy release during germination. Tzen et al. (1993) reported that a decrease in oleosin quantity corresponds to an increase in oil body size. For example, oil bodies from sesame seeds contain 0.6% of proteins with an average diameter of 2 μm , while rapeseed seed oil bodies have about 3.5% of protein with a diameter of 0.65 μm . Two different oleosin isomers have been found to coexist on the surface of seed oil bodies from diverse angiosperms (Tzen et al., 1998), and have been classified as low- or high- molecular oleosin (L- / H-

oleosin) depending on the molecular mass (Tzen et al., 1990). However, the biological significance of the presence of these two isoforms is unclear (Tzen et al., 1998).

The primary structure of oleosin comprises three distinct domains. A long hydrophobic strand of 72 amino acids penetrating the TAG matrix, and amphipathic N- and C- domain flanking the central part covering the surface of the oil body. In Figure 1.5 the amino acid sequence of rapeseed oleosin (*Brassica napus*) is aligned (using Uniprot) to soybean (*Glicine max*) and sunflower (*Helianthus annuus*) oleosin sequences. The hydrophobic portion (highlighted in blue) is highly conserved among different species, which forms a “hairpin type” structure with a central loop usually referred as “proline knot” (in black box, with typical sequence PX₅SPX₃P) (Huang, 1992). The amino acid sequence of the N and C domains are different in composition and length depending on the plant species (Huang, 1992). Positively and negatively charged residues (highlighted, respectively, in green and red in Figure 1.5) are distributed in a non-random pattern. The positively charged residues are oriented towards the interior of the oil body, interacting with PLs, while the negatively charged ones are arranged towards the cytosol (Huang, 1992; Purkrtova et al., 2008; Tzen and Huang, 1992).



Knowledge on the secondary structure of oleosin is more controversial among researchers, mainly due to the low solubility of this protein in aqueous media, and the diversity of analytical strategies. The central hydrophobic domain has been shown to be composed either by two α -helices (Lacey et al., 1998), or made up of two β -sheets structure (Li et al., 2002). However, more recent works (Alexander et al., 2002; Huang and Huang, 2017) support the model of the central polypeptide to be largely formed by α -helices (Figure 1.6).

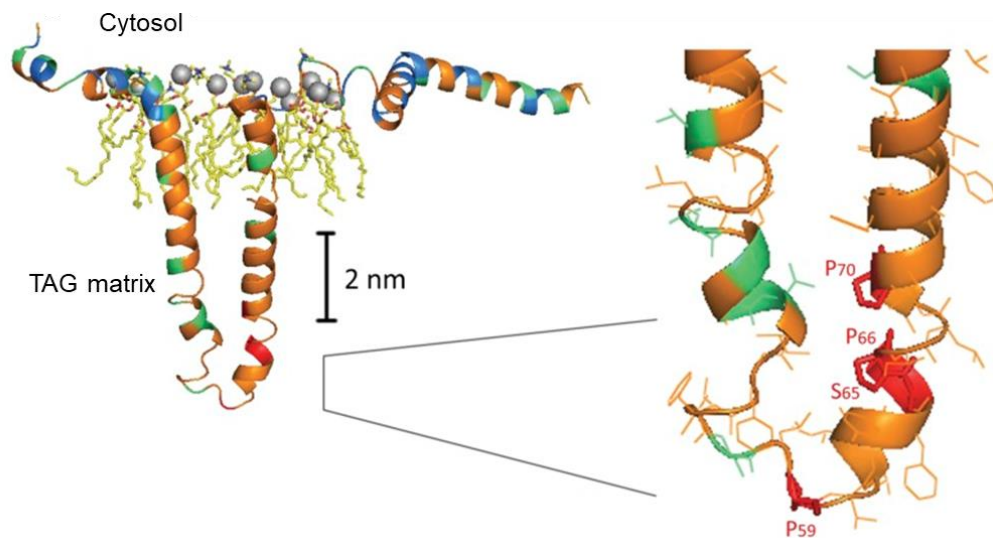


Figure 1.6: Oleosin secondary structures on the surface of an oil body. Residues with nonpolar, polar, and charged side chains are shown in orange, green, and blue, respectively. Locations of the most conserved three Pro and one Ser residues of the hairpin loop PX_5SPX_3P are in red. For the PL molecules, the grey sphere indicates the phosphate head group; yellow zip-zap line represents acyl moiety. Right panel: Enlarged loop region. (Huang and Huang, 2017)

Other proteins known as caleolosin co-exist on the oil body surface; however, they represent only a minor population (Frandsen et al., 2001; Tzen et al., 2003). Caleolosin proteins have a similar three-domain structure to the oleosin, with a much shorter central domain and a calcium-binding site at the N-terminal region. Huang (1996) suggested that the caleolosin may be involved in oil body biogenesis, but the exact functionality is not well known. Seed oil bodies from *Arabidopsis* and sesame also contain enzymes found in minute quantities, called stereolosin (Purkrtova et al., 2008), which may be involved in regulating growth and development in plants (Li et al., 2007).

1.4.2. Oil body phospholipids

Phospholipids are an integral part of the oil body, forming a monolayer around the TAG core, creating an environment where proteins are anchored. It is believed that the tight interaction between oleosin and phospholipids is due to the synergic charge and the polarity distribution within the molecules (Vance and Huang, 1987). As a result of this interaction, oil bodies are protected against coalescence. In fact, artificial oil bodies reconstituted without either oleosin or phospholipids, showed destabilization over time (Tzen et al., 1992).

Oil body phospholipids have been isolated and characterised in *Echium plantagineum* (Payne et al., 2014), rape seed (Katavic et al., 2006; Tzen et al., 1993), and sunflower seed (Furse et al., 2013). Tzen et al. (1993) found that the quantity of phospholipid varied between 0.6 and 2%, but their specific composition is deeply affected by the plant species. Generally, the major phospholipid is phosphatidylcholine (PC) (40-64%), with phosphatidylserine (PS) (18-33%), phosphatidylinositol (PI) (7-21%) and phosphatidylethanolamine (PE) (3-16%) also present (Tzen et al., 1993). The negatively charged PS and PI found in these various plant species interact with the positively charged amino acid residues of the amphipathic portions of the oleosin, making the droplet structure more stable against physical damage (Huang, 1992; Tzen et al., 1992).

1.4.3. Oil body biogenesis

Although the oil body is a relatively simple organelle, the mechanisms by which it is formed are unclear and has been a subject of controversy. Following the initial chemical and structural characterization of seed oil bodies, it was suggested that they arise by vesiculation from the endoplasmic reticulum (ER) (Frey-Wyssling et al., 1963). However, the exact mechanisms by which oil bodies are formed were not clear, and over the decades, different researches led to different theories. What it is clear, is that TAG molecules are synthesised by diacylglycerol acyltransferase and accumulate between the leaflets of the ER membrane (Lacey et al., 1999). Oleosins are synthesised in association with the rough ER and are co-translationally inserted into the ER membrane (Beaudoin et al., 1999; Hills et al., 1993), probably via signal recognition particle pathway (Beaudoin et al., 2000; Thoyts et al., 1995). Sarmiento et al. (1997) suggested that when oleosin bounds the ER (double PL layer), the protein may adopt a different conformation compared to when it is inserted into the oil body surface (single PL layer). Following this theory, the oleosin, once synthesised in the ER, would flow to a more hydrophobic region where the TAG accumulates (Tzen and Huang, 1992), adopting a favourable conformation given by the TAG environment (Sarmiento et al., 1997). Therefore, oleosin targeting into the oil body seems to be driven by the central hydrophobic domain, as also demonstrated by deletion of either N- or C-terminal regions of the proteins which did not drastically affect such targeting (van Rooijen and Moloney, 1995; Huang and Huang, 2017). Once sufficient levels of oleosin are achieved, the vesicle buds off completely (Shimada and Hara-Nishimura, 2010). However, the separation of the oil body from the ER

may not be the final process in its maturation, since Siloto et al. (2006) reported that oil bodies formed at the beginning of seed development were deficient in oleosin compared to oil bodies analysed at the end of seed maturation. Therefore, oil bodies could either coalesce to reach a stable oleosin:TAG ratio, or re-fuse with the ER to undergo further modifications before they reach their mature composition (Figure 1.7) (Murphy, 2001).

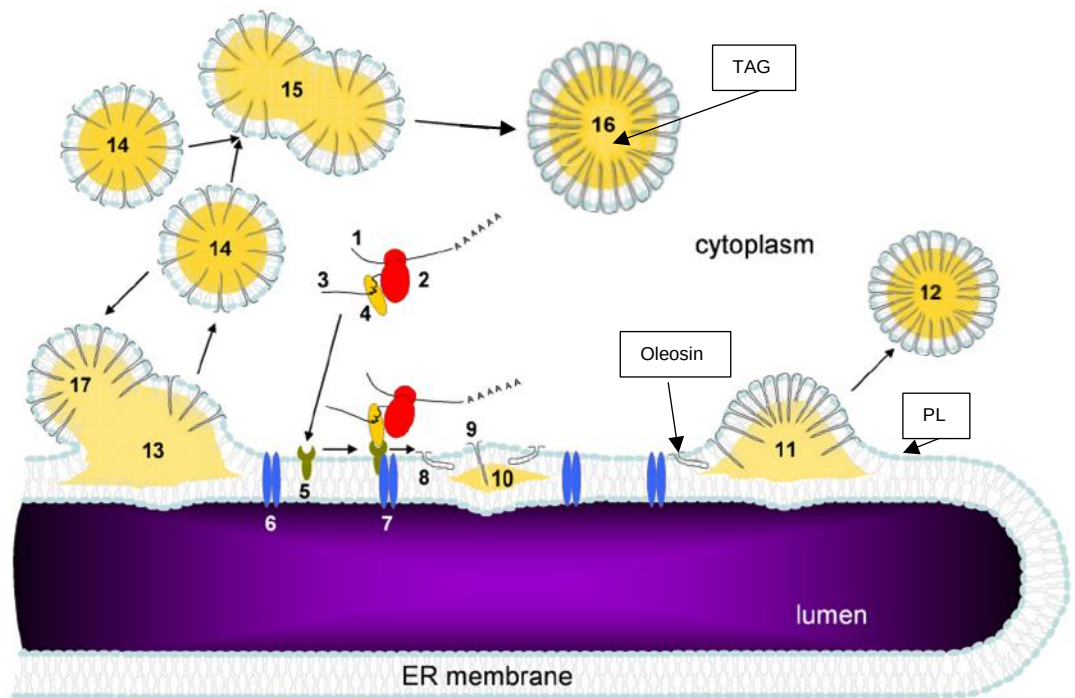


Figure 1.7: Schematic representation of oil body assembly. 1) oleosin mRNA; 2) ribosome; 3) nascent oleosin peptide; 4) signal recognition particle (SRP); 5) SRP receptor; 6) translocation-insertion site; 7) oleosin translation machinery/SRP/translocation - insertion site complex; 8) constrained oleosin in ER double membrane; 9) relaxed oleosin in oil body (OB) phospholipid monolayer-TAG core. TAG molecules are accumulated between the double layer of the ER (10) forming a vesicle stabilised by PL and oleosins (11); when OBs with maximum density of oleosins reaches a critical size, buds off from the ER (12). Maturing OB with low density of oleosins (13) are released from the ER membrane (14) and can coalesce with other OBs (15) with low density of oleosins, until reaches an optimal TAG:oleosin ratio (16) or can re-fuse with the ER (17) for further modifications. Image modified from (Tzen et al., 2008).

1.4.4. Oil body functionality in vivo

The main biological role of oleosin proteins on the surface of oil bodies is to stabilize the neutral lipid in small diameter droplets for storage, in preparation for germination. Oleosin, providing steric hindrance and electrostatic repulsion, helps the oil bodies to maintain their individuality over seed maturation, drying (Tzen and Huang, 1992) and following rehydration. However, the actual role of those integral proteins over germination has remained speculative among researchers (discussed in Section 1.4.5). This process starts with TAGs hydrolysis, which is mediated by lipases at the oil body interface and yields glycerol molecules and free fatty acids (FA, Figure 1.8). FAs are then transferred to specialized peroxisomes to produce acetyl CoA, which is subsequently converted into sugars by the glyoxylate cycle and gluconeogenesis (Graham, 2008).

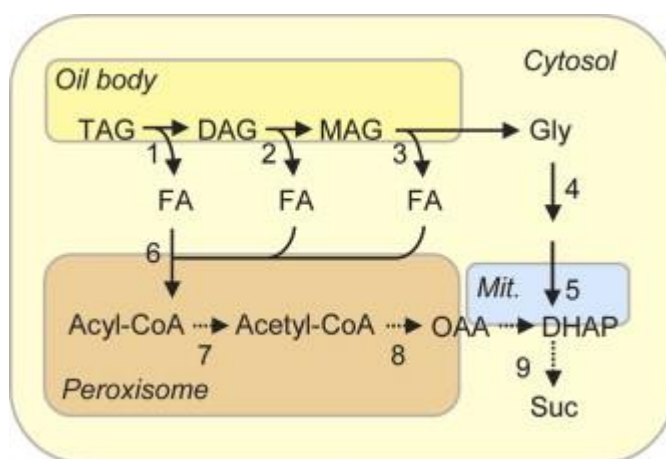


Figure 1.8: Schematic representation of TAG breakdown during germination. Gly: Glycerol; OAA: oxaloacetate; Suc: Sucrose. (Quettier and Eastmond, 2009)

1.4.5. Oil body-associated enzymes

The purity of oil body extracts from seed enzymes is mainly dependent on the type of aqueous solution used, with the alkaline media (pH> 8) being the most adopted in literature (Chen and Ono, 2010; De Chirico et al., 2018; Zaaboul et al., 2018; Zhao et al., 2016). However, the degree of association of these enzymes with the oil bodies is plant specific, and it has been demonstrated that in rapeseed, enzymes are strongly associated to the oil bodies (Jolivet et al., 2009; Katavic et al., 2006; Zhao et al., 2016).

TAG lipases (EC: 3.1.1.3) hydrolyse TAG at the oil/water interphase, to yield to free fatty acids and glycerol (Quettier and Eastmond, 2009). They have been characterized from a large number of organisms, and all true lipases belong to the super-family of α - β fold hydrolases that contain a catalytic triad made of Ser and His in a conserved motif of five amino acids (GX SXG) (Ollis et al., 1992).

According to Villeneuve (2003), plant lipase specificities can be divided into different types:

- a) Substrate specificity: natural substrates of lipases are glycerol esters. These enzymes are able to not only catalyse the hydrolysis of TAGs, but also di- and mono-acylglycerols and even phospholipids, in the case of phospholipases.
- b) Positional specificity or regioselectivity: Lipases may only hydrolyse TAGs at C1 and C3 glycerol bonds, producing fatty acids, 2-monoacylglycerols and 1,2 or 2,3-diacylglycerol (Figure 1.9). Other

lipases can instead hydrolyse TAGs into fatty acids and glycerol in a random way (Figure 1.9).

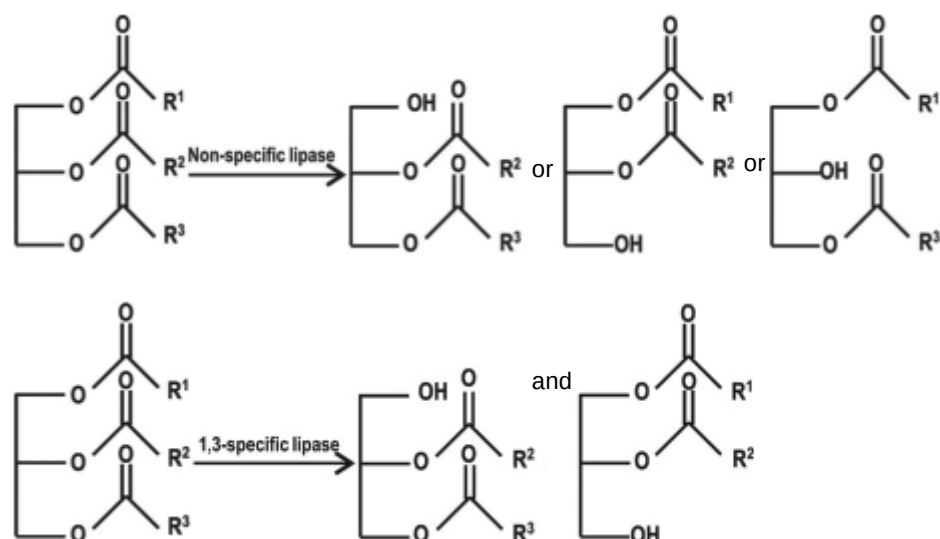


Figure 1.9: Reaction catalysed by non-specific and 1,3 specific lipases.
Modified from (Barros et al., 2010)

- c) Fatty acid specificity or typospecificity: lipases can be selective to a particular fatty acid, or more commonly to a group of fatty acids.

According to Hellyer (1999), oilseed lipases show high selectivity for the dominant fatty acid present in the TAG molecule. For example, castor bean lipases show selectivity for triricinolein, while canola has high specificity for trierucin, and preferably catalyses the hydrolysis of fatty acid moieties from the sn-1 and sn-3 position, to yield 1,2 (2,3)-DAGs (Hills and Mukherjee, 1990).

Depending on the plant species, these enzymes can be located on oil body, peroxisome or microsomal membrane (Mukherjee, 1994). In rapeseed,

it has been suggested that oleosins (together with caleosins, another interfacial protein in oil bodies) may play a role in facilitating the access of lipases to its substrate (Lin et al., 1983; Lin and Huang, 1983), as this enzyme can act only upon binding to a membrane. It is also believed that the presence of oleosin is crucial to have a high surface-to-volume ratio, which provides sufficient area for lipase and/or interaction with organelles such as glyoxysomes during TAG mobilization for ATP production (Murphy, 2001; Siloto et al., 2006). In many lipases, the active site of lipase is sheltered by a lid made of an α -helix stretch that is moved out upon the adsorption of the enzyme to the interface (Allen and Tao, 2007).

However, it is highly controversial among researchers how lipase enzymes can reach the substrate. It was hypothesized that the oil body surface may form a barrier against the lipolytic action, and that, following germination, a phospholipase A2 provides TAG lipases access to the substrate, by degrading the phospholipid monolayer (Gupta and Bhatla, 2007; May et al., 1998a; Noll et al., 2000). In a different work (Sadeghipour and Bhatla, 2002), it was shown that proteolysis of oleosin was essential to allow TAG mobilization in sunflower seeds. However, Beisson et al. (2001) did not support this theory, suggesting that lipolysis may occur without any pre-hydrolysis of the protein coat.

1.4.6. Oil body recovery

In general, the recovery of oil bodies has key points that are similar among the authors, comprising imbibition of the seeds to increase the internal moisture; disruption of the seed matrix in aqueous media; collection of a “crude” oil body cream after centrifugation and washing of the cream (Adams et al., 2012; Chen and Ono, 2010; Iwanaga et al., 2007; Karkani et al., 2013; Payne et al., 2014). Imbibition, the uptake of water by the dry seed, involves the absorption of water by the cell wall and protoplasmic macromolecules (e.g. proteins, polysaccharides), wherein water molecules are “held” by electrostatic forces such as hydrogen bond. The movement of the water into the seed is due to diffusion and capillary action with water moving from a region of higher to lower water potential (Bewley et al., 2013; Woodstock, 1988). Permeability of the seed coat is the major factor controlling the rate of imbibition. The latter stage of oil body recovery is aimed to remove the polysaccharide, seed debris and exogenous proteins that are not covalently bonded to the oil body (Tzen et al., 1997). The degree of purification, which refers to the amount of seed material removed from the oil body extract, is dependent on the number of washing cycles and on the aqueous solution used. The exposure to urea is a proven method to completely remove contaminant material (Millichip et al., 1996). However, the chemical nature of the solution limits its application to food purposes. Alkaline buffers have also been demonstrated to lead at higher extract purity compared to deionised water (Chen and Ono, 2010; De Chirico et al., 2018; Luping Zhao et al., 2016), extending the oil body shelf life, due to the removal of seed hydrolytic enzymes (De Chirico et al., 2018). Thermal treatment of oil bodies can improve oxidative stability (Chen et al., 2012;

Karkani et al., 2013; Zhao et al., 2016b), and change physical properties (Chen et al., 2012; Iwanaga et al., 2007; Wu et al., 2012; Zhao et al., 2016). However, the effect of this process on the physicochemical characteristics over storage has not been characterized.

1.5. Oil body and common emulsions

Emulsions consist of two immiscible liquids (usually oil and water) with one dispersed as small spherical droplets in the other (McClements, 2004). If oil droplets are dispersed in the aqueous phase, emulsions are classified as oil-in-water emulsions, or water-in-oil emulsions when water droplets are dispersed into an oil phase. Emulsions that are produced by homogenization of water and oil are thermodynamically unstable, and tend to separate into a top layer of oil and a bottom layer of water (Dickinson, 1992). To form an emulsion that is kinetically stable, emulsifiers need to be added; these are surface-active molecules that adsorb onto the surface of the droplets during homogenization. By this way, emulsifiers form a protective layer around the droplet (known as interfacial region) that can prevent them to coalesce. Common emulsifiers used (generally amphipathic molecules) include surfactants, phospholipids, and protein (McClements, 2004).

Common emulsions are prepared with compounds that are sometimes extracted using environmentally unfriendly. However, the recovery of oil body occurs from more environmentally friendly resources (Bhatla et al., 2010), and is likely to be more energy efficient compared to the conventional emulsion process (Hetherington, 2014). Moreover, oil bodies can be used as a carrier system for intrinsic and extrinsic vitamins (Fisk et al., 2006), extrinsic aroma (Fisk et al., 2013), and drugs (Acevedo et al., 2018, 2014), for protection during storage or for safe delivery.

1.5.1. Emulsion instability

Colloidal interactions govern whether emulsion droplets aggregate or remain as separate entities. The overall stability depends mainly on the magnitude and sign of droplet charge, which is determined by the interfacial characteristics (type of emulsifier, surface load, thickness, electrical charges), and the properties of the continuous phase (viscosity, chemical composition) (McClements, 2004). For example, in protein-stabilized emulsions, when the pH value of the continuous phase is far from the protein's isoelectric point (pI), the charged droplet will be surrounded by a cloud of counterions, which will give rise to repulsive interactions when two droplets will approach each other. By this way, electrostatic repulsive forces will prevent the droplets to flocculate or coalesce. In proximity of the pI value, the electrostatic repulsion is reduced, leading to droplet aggregation and creaming.

The mechanisms responsible for emulsion can be broken down into two main physical categories: gravitational separation (creaming or sedimentation) and aggregation-based instability (aggregation, coalescence, Ostwald ripening) (Figure 1.10).

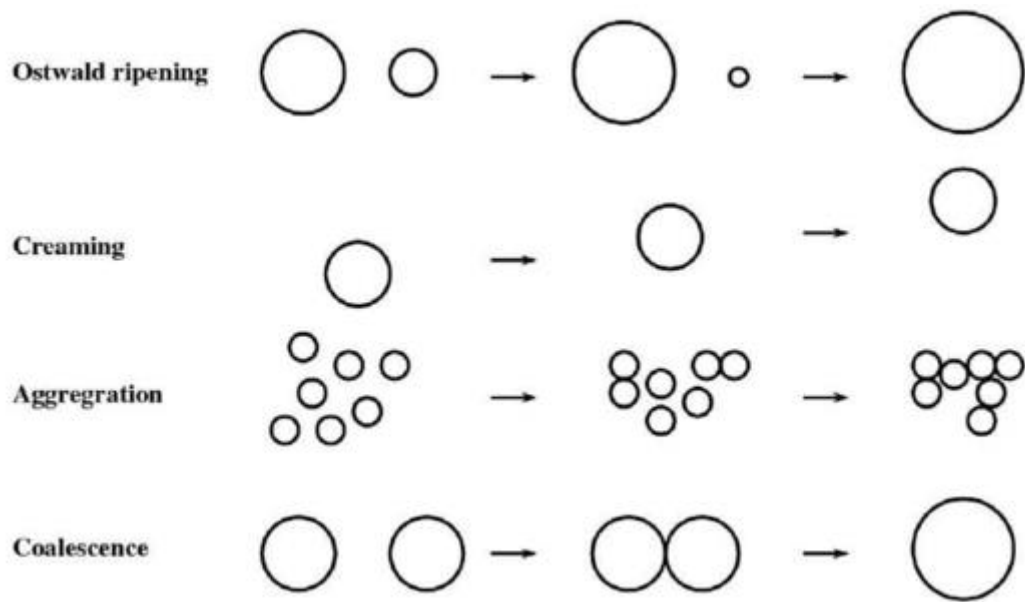


Figure 1.10: Potential instability mechanisms in oil-in-water emulsions (Fredrick et al., 2010).

Gravitational separation is a phenomenon driven by the difference in density between two phases under the gravitational force. Physically described by the Stoke's law, creaming or sedimentation may occur depending on the density of the droplets and the continuous phase (Hunter, 2001). Aggregation is a phenomenon occurring when droplets collide forming flocs. This is driven by a combination of attractive (van der Waals) and repulsive (electrostatic) forces. However, when the interfacial film is disrupted following the collision between two droplets, coalescence occurs, leading to the fusion between them and the formation of a larger one. This is the main mechanism of destabilization in emulsions, as it leads to decrease the contact area between the dispersed and continuous phase, which corresponds to a more thermodynamically favourable state (McClements, 2004).

Various studies have shown that the interfacial composition and thus the physicochemical properties of oil bodies, is dependent on the extraction procedure used (Iwanaga et al., 2007; Karkani et al., 2013; Nikiforidis et al., 2013; Zhao et al., 2016). However, similar oil body recovery methods used in different plant species may yield to different results. For example, while in maize oil bodies exogenous proteins were not detected after a mild washing using sucrose, residual seed proteins are detected in sunflower seed (Nikiforidis et al., 2013).

Long term stability (e.g., no change in size, creaming) can be enhanced by recovering oil bodies with an external layer of exogenous proteins (Nikiforidis and Kiosseoglou, 2009), or by the addition of hydrocolloids, such as xanthan gum (Nikiforidis and Kiosseoglou, 2010). However, the effect of the carried-over enzymes on the chemical stability was not considered. Coatings of pectin (Iwanaga et al., 2008) or carrageenan (Wu et al., 2012, 2011), can be used to improve the stability against freeze-thaw cycles, creaming in different salt concentrations media, and high temperatures.

1.5.2. Characterising emulsions

1.5.2.1. Particle size

Particle sizing is an important tool for the physical characterization of food emulsions. Most laboratories use light scattering techniques as characterization method. The LS 13320 particle sizer (used in this thesis) measures the particle size by measuring the pattern of light scattered by the sample. Each particle's scattering pattern is characteristic of its size. The most

important components of this machine are the Fourier lenses: the scattered light of any particle at a specific angle will be refracted by the lens, to a particular detector, regardless of the particle's position in the beam (Figure 1.11). The individual scattering patterns from the moving particles in the cell are superimposed, creating a single scattering pattern that represents the contribution from all the particles present in the sample. Detectors ($n = 126$) record this composite scattering pattern, which is deconvolved to give a series of individual number (one for each size classification) and their relative amplitude. This mathematical process is based on either the Fraunhofer or Mie theories of light scattering.

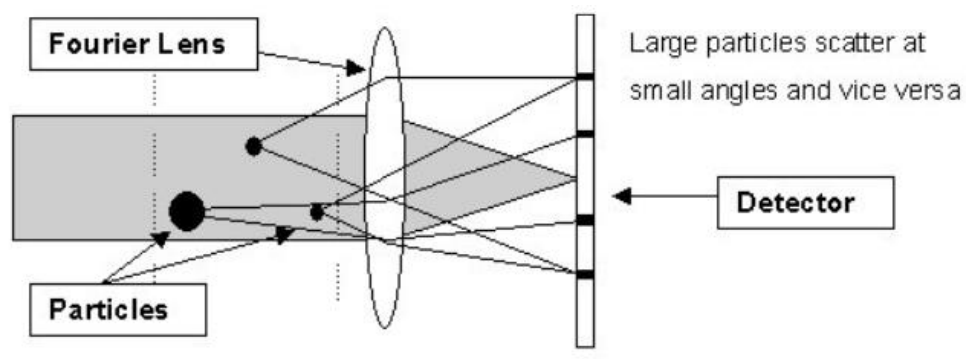


Figure 1.11: Fourier lenses in LS 13320 particle sizer.

Particle size distributions (PSD) are expressed in this thesis by normalised frequency distribution curve, which describes the amount of fractions within a defined interval of particle sizes. However, it can also be expressed by cumulative distribution, which describes the quantity of particles smaller or larger than a certain diameter.

In order to relate the PSD with the product quality, weighted mean diameters are usually used. Both particle property (e.g., size, volume) and weighting (e.g. by number, area), are used in accordance to the information of interest with regard to the application of a particular system, e.g. if the surface area is the determining factor, weighting of diameter by surface area in the Sauter mean diameter is used (Merkus, 2009). Some typical examples are:

$$D_{3,2} = \frac{\sum n_i D_i^3}{\sum n_i D_i^2} \quad (\text{Eq. 1.1})$$

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \quad (\text{Eq. 1.2})$$

$D_{3,2}$ is the surface weighted diameter (also called Sauter mean diameter), and $D_{4,3}$ is the volume weighted diameter, n_i means the (number-based) frequency of occurrence of particles in size class i , having a mean diameter D_i .

1.5.2.2. Zeta potential

In this technique, an emulsion sample is placed into a measurement cell and a static electrical field is applied via a pair of electrodes. The presence of a charge on a particle surface affects the distribution of ions in the surrounding region, resulting in an increase of counter-ions close to the surface. By this way, an electrical double layer exists around each particle (Hunter, 2001). The continuous phase around the particle can be defined in two parts: an inner region (Stern layer) where the ions of opposite charge of the particle are strongly bond, and an outer region (diffuse layer) where they are less firmly

associated. The ions found in the stern layer are immobile and at the extremity of this is the shear plane. Within the diffuse layer, there is a limit inside which the ions and particles form a stable entity. When the particle moves under the electric field, the ions within this limit will move with the particle, while the one beyond will stay freely in the continuous phase. The potential at this limit (shear plane) is the zeta potential (Figure 1.12).

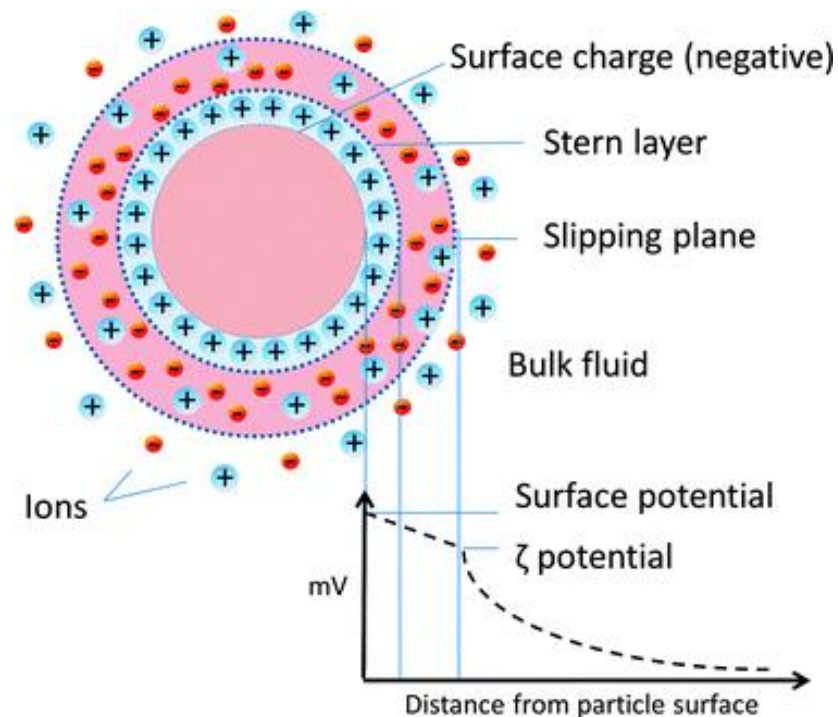


Figure 1.12: Electric double layer of a particle illustrating a reduction in surface charge with increasing distance from the charged core (Beckman coulter, USA).

When the electric field is applied, any charged droplets move towards the oppositely charged electrode, so the direction of travel is determined by the nature of the charge on the emulsion droplets (Hunter, 2001). Under the electrical field, the droplets accelerate until they reach a constant velocity, v ,

while the drag force of the surrounding liquid balances the electrical pulling force. The velocity under the electric field (electrophoretic mobility) is dependent on the strength of the electric field, size and charge of the emulsion droplet, and is related to zeta potential by the Henry equation:

$$U_E = \frac{2 \varepsilon z f(\kappa a)}{3\eta} \quad (\text{Eq. 1.3})$$

Where U_E : electrophoretic mobility, z : zeta potential, ε : dielectric constant, η : viscosity and $f(\kappa a)$: Henry's function (Hunter, 2001; McClements, 2004a).

In Henry's function, the units of κ , termed Debye length, are reciprocal length and κ^{-1} is taken as a measure of the thickness of the double layer. The parameter "a" refers to the radius of the particle; thus, κa measure the radius of the particle to the electrical double layer thickness. In aqueous media, and in the presence of a moderate number of electrolytes, $f(\kappa a)$ is 1.5 and is referred as Smoluchowski approximation.

1.6. Degradation of lipids

The degradation of lipids can occur via enzymatic and non-enzymatic (free radical chain reaction) oxidation processes (Figure 1.13). Non-enzymatic reactions are typically slow at ambient temperature and are mainly related to oxidation and isomerization of C=C bond structure in lipids. In contrast, the enzymatic reactions are reported to be associated with hydrolytic pathways. Lipid oxidation is a serious problem in many sectors of the food industry for being one of the major causes of deterioration in natural processed food (McClements and Decker, 2000). These processes can lead to off-flavours and formation of compounds with adverse health effects.

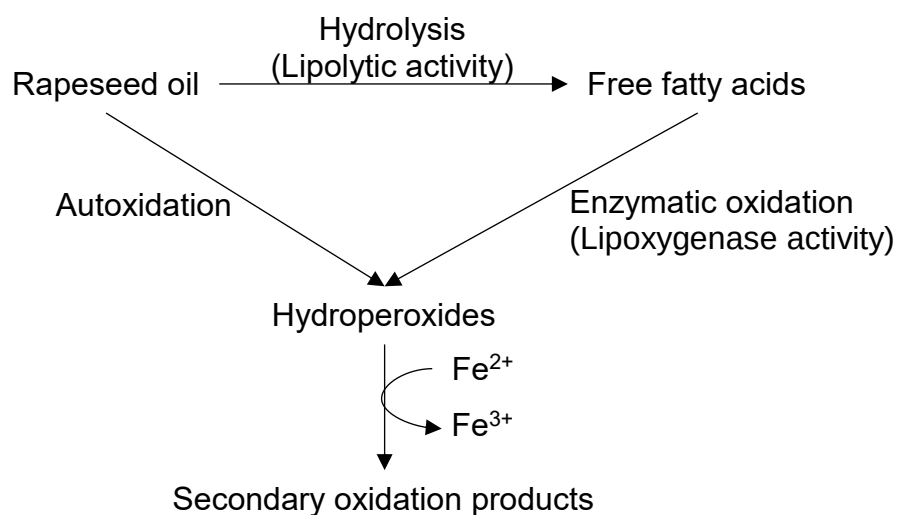
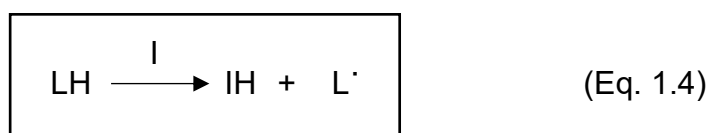


Figure 1.13: Pathways of hydrolytic and oxidative degradation in rapeseed oil

1.6.1. Free Radical chain reaction

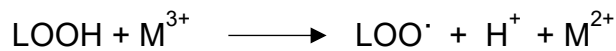
Autoxidation of lipids is a chain reaction occurring through the interaction of unsaturated lipids with oxygen. Lipid hydroperoxides are the initial products of this reaction, which can be further broken down to form volatile products. This is a reaction that occurs spontaneously and it can be initiated by light, heat, metals, peroxides or hydroperoxides (Frankel, 2005). The free radical reaction can be separated into three phases: initiation, propagation and termination.

Lipid radicals are not formed by the direct reaction of unsaturated lipids with atmospheric oxygen, as this reaction is thermodynamically unfavourable (Bradley and Min, 1992). However, in the presence of initiators (I), unsaturated lipids (LH) lose a hydrogen radical (H[•]) to form lipid free radical (L[•]) (Equation 1.4). The energy required to remove hydrogen from a fatty acid is dependent on the hydrogen's position. Allylic hydrogens (bound to carbons next to double bonds) are preferably removed, such as those in polyunsaturated fatty acids (Frankel, 2005). The most likely initiation process, by which free radicals may form, is the metal-catalysed decomposition of preformed lipid hydroperoxides species (Equation 1.5).

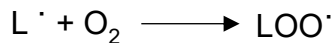




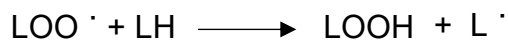
(Eq. 1.5)



During propagation, the alkyl radical of unsaturated lipids ($\text{L}^\cdot$) formed during initiation is able to react with atmospheric oxygen to form peroxy radicals ($\text{LOO}^\cdot$, Equation 1.6). Lipid peroxy radicals abstract hydrogen from another lipid molecule to form hydroperoxide (LOOH) and another alkyl radical ($\text{L}^\cdot$, Equation 1.7).



(Eq. 1.6)



(Eq. 1.7)

The latter reaction (1.7) is the rate-limiting step in the propagation of lipid radicals; thus, hydrogen abstraction from unsaturated lipids becomes selective for the most weakly bound hydrogen. Therefore, the susceptibility of lipids to autoxidation depends on the availability of allylic hydrogens and their relative ease by which they react with peroxy radicals to form hydroperoxides (Frankel, 2005). Each new $\text{L}^\cdot$ radical in turn adds oxygen, forms a peroxy radical, abstracts hydrogen from another lipid, forming another hydroperoxide

and generates a new $L^\bullet$ radical to provide the driving force in the chain reaction (Schaich et al., 2013).

The final phase of the free radical chain reaction is termination, where the peroxy radicals ($LOO^\bullet$) react with one another to form non-radical products and oxygen through the Russel mechanism (Dix and Aikens, 1993). Other reactions that may occur involve the formation of stable alcohols through reaction with unsaturated lipids, or fragmentation of peroxy radicals into unsaturated aldehydes. One of the most important termination reactions involves an antioxidant, which compete with unsaturated lipids and donate hydrogen ions to lipid radicals during propagation, producing stable antioxidant radicals (Frankel, 2005).

1.6.1.1. Breakdown of hydroperoxides

Hydroperoxides are generally stable at room temperature, but in the presence of metal ions or heat, they can be easily broken down to produce secondary oxidation products. The generation of these secondary products is responsible for aroma and flavour properties deterioration that occurs over oxidation. Depending on the parent hydroperoxide, a wide range of secondary products can be produced.

Scission of alkoxyl radicals has probably the greatest consequences in lipid oxidation, because the product generated are responsible for the distinctive volatile off-flavours that are a strong marker of rancidity (Chan et al., 1982). Alkoxyl radicals are formed from hydroperoxides by metal catalysis or thermal decomposition, which will form, alcohols, ketones and hydrocarbons

by β -scission, as well as aldehydes (e.g., malondialdehyde, MDA) which are frequently used as oxidation marker. The most widely employed method for determination of MDA is the spectrophotometric determination of the red fluorescent MDA-thiobarbituric acid (MDA-TBA) complex (Section 2.2.9.3). These products are produced from unsaturated fatty acids but as the degree of unsaturation increases so does the complexity of secondary oxidation products (Frankel, 2005).

1.6.1.2. Oxidation in oil-in-water emulsions

In emulsions, lipid oxidation occurs at the surface of the droplet and thus the properties of the emulsifier such as charge, thickness and antioxidant capacity can have large impacts on the rate of oxidation. For example, Mancuso et al. (1999) revealed that the use of negatively charged surfactants (e.g., sodium dodecyl sulphate) attracted positively charged pro-oxidant metal ions, as these emulsions oxidized at an increased rate compared to surfactants being either neutral or positive. In addition, protein emulsifiers may act as chemical barriers to oxidation, as they may sacrificially oxidise protecting the lipid core (Tong et al., 2000), or repel ions if they are positively charged.

Oil bodies have been reported to be stable against oxidation (Chen et al., 2012; Fisk et al., 2008; Gray et al., 2010; Karkani et al., 2013; Wang et al., 2017). Production of hydroperoxides and secondary products is significantly lower in these natural emulsions compared with technically-prepared emulsions formulated with other surfactants. This higher stability was

dependent on the formation of a stable interface (Fisk et al., 2008), the presence of tocopherols (Fisk et al., 2006), as well as the formation of the coat made out of exogenous seed proteins (Karkani et al., 2013). Furthermore, differently from common emulsions, oxidative stability of oil bodies can be further improved after thermal treatment (Chen et al., 2012). Chen et al. (2012) suggested that this effect was due to inactivation of oxidative enzymes, but this hypothesis is yet to be tested.

1.6.2. Enzymatic hydrolysis

Lipase enzymes catalyse the hydrolysis of acyl glycerol to fatty acids and glycerol. In rapeseed, enzymatic extracts from dry seed contain a low level of lipase activity, which remains nearly constant over the first hours of germination, to then dramatically rise and reach maximum activity at day 4 (Hoppe and Theimer, 1997). The intracellular location of lipase in germinating oilseed is found to vary between species: peanut and soybean do not contain an oil body-lipase, and lipase is found in the glyoxysomal membrane (Hills and Murphy, 1988). In rapeseed, the characteristics of lipase have been discussed controversially (Hills and Murphy, 1988; Hoppe and Theimer, 1997a; Hoppe and Theimer, 1997b; Theimer and Rosnitschek, 1978). However, the work of Hills and Murphy (1988) revealed that the controversy was due to the presence of two type of lipolytic activity, and that these were found on both oil body and microsomal membranes. Oil body-associated rapeseed lipase was found to be most active on trierucin (TAG molecule containing three erucic acids)(Lin et al., 1986), which is the major fatty acid component of storage TAG. Moreover,

its activity is strongly activated by Ca^{2+} and NaCl (Rosnitschek and Theimer, 1980).

Lipoxygenases catalyses the oxidation of free polyunsaturated fatty acids, such as linoleic acid and linolenic acid, into conjugated hydroperoxide fatty acids (Gardner, 1991). The formed hydroperoxides can then be decomposed enzymatically by hydroperoxide lyase, or by autoxidation (as described in Section 1.5.1). Although lipoxygenase catalyses lipid oxidation after lipid hydrolysis, this oxidative process plays important biological roles in plants and animals, such as pathogen attack, or involves other biological activities such as protein induction, program cell death and growth inhibition (Gardner, 1991).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All chemicals were conforming to specifications by the Committee on Analytical Reagents of the American Chemical Society (ACS grade) or higher and sourced from Sigma-Aldrich Co. (St. Louis, MO, USA). Oilseed rape seeds (DK Exalte) were less than 10 months old and provided by a local farm following summers 2015/2016/2017. Seeds were stored in paper bags at 20 °C and rH 60%.

2.2. Methods

2.2.1. Effect of soaking oilseed rape seeds

To monitor the imbibition process, 10 g of seeds were soaked in dH₂O, Tris-HCl (0.1 M, pH 9.5) or NaHCO₃ (0.1 M, pH 9.5) at ratio of 1:4 (w/v) at 4°C. Independent seed samples were soaked for 0, 1, 3, 7, 10, 15, 20, 24, 30 h; thereafter, the seeds were drained, the excess of water was removed with filter paper and the mass of the soaked seed measured using a precision scale. Three independent replicates per time point were performed. Results are expressed as net weight increase expressed in %. To study the effect of soaking time on oil body integrity, dry or soaked seeds were ground in the same soaking solution following the protocol described in Section 2.2.3 (Urea-WOB).

2.2.2. Conductivity measurements

The conductivity of soaking solutions at 4°C was directly measured using a conductivity meter (HI 9033 multirange conductivity meter, HANNA instruments, Romania).

2.2.3. Recovery of oil body

Seeds (200 g) were soaked in deionised water (dH₂O), Tris HCl (0.1 M, pH 8.5) or NaHCO₃ (0.01, 0.1 or 0.3 M pH 9.5) between 0 to 30 hours unless stated otherwise and the soaking solution discarded. The soaked seeds were then ground in the same medium type as used on soaking (4 °C) at ratios (dry seed weight based) of 1:4 or 1:7 (w/v) in a Kenwood blender (BLX52) at 800 W for 90 seconds. Where specified, the ground material was left in agitation overnight (4 °C) in grinding solution. The solution was filtered through three layers of cheesecloth (grade 80, thread count: 40 x 32 threads per square inch). The filtrate was transferred in 400 ml tubes and centrifuged at 10,000 g for 30 minutes at 4 °C using a Beckman J2-21 centrifuge, fixed rotor JA-10. The upper layer was isolated using a spatula, drained on filter paper (Whatman, grade 5) and called crude oil body fraction (COB). Sodium azide (0.02 mM) was added to all soaking/grinding media to avoid microbial spoilage. The COB preparation was dispersed in pre-chilled (4 °C) washing solution (0.1 M NaHCO₃, pH 9.5; 9 M urea, or 0.1 M NaCl) at ratio 1:4 w/v and centrifuged at 10,000 g (4 °C, 30 min.). The recovered oil body cream was designed as washed oil body (WOB I). To remove any residue of the washing solution, the fat pad was isolated using a spatula, drained on filter paper, suspended in pre-

chilled (4 °C) deionised water (1:4 w/v) and centrifuged (10,000 g, 30 min, 4 °C), unless stated otherwise. After centrifugation, the washed cream layer (WOB II) was collected and designated as urea-washed oil body cream (Urea-WOB), sodium bicarbonate washed oil body cream (NaHCO₃-WOB) or salt washed oil body cream (NaCl-WOB). All creams were stored at 4 °C and analyses were completed within 24 hours.

2.2.4. Thermal treatment of oil body emulsions

WOB I cream was resuspended in dH₂O (15 % w/v lipid weight basis), and the thermal treatment consisted in heating this emulsion at 95 °C for up to 6 minutes, in a circulating thermostatic water bath (Grant instruments, Cambridge). Oil body emulsions (5 ml) were pipetted in glass tubes (8 mm inner diameter, 15 cm length), flashed with nitrogen and closed with a cap. A thermocouple, connected to a data logger, was inserted at the centre of the tube to recorder the time-temperature history during the treatment. Prior to the treatment, the samples were equilibrated at 40 °C (3 minutes) to shorten and standardise the come-up time (35-40 seconds). The treated samples at different times were then cooled in ice and centrifuged (10,000 g, 30 minutes, 4 °C). The collected cream was designed as thermally treated WOB II.

2.2.5. Oil body compositional analysis

2.2.5.1. Extraction of neutral lipid

COB or WOB creams (approximately 0.2 g) were dried in a vacuum oven (40 °C, -900 mbar) for at least 48 h until constant mass. The weight was

recorded for moisture content analysis. Dry matter was ground (mini-bead beater, 3450 rpm) for 2 min in isooctane (1 ml) to extract the neutral lipid (Gray et al., 2010). Samples were centrifuged (Thermo Heraeus Fresco 21) at 17,000 g for 5 min at 4 °C and the upper phase was removed with a pipette; this process was repeated three times and the recovered lipids dried under nitrogen and the weight recorded using a precision scale. The resultant pellet after delipidation was used for protein extraction (Section 2.3.2).

2.2.5.2. Protein extraction and quantification

Oil body proteins were extracted from delipidated dry material (as described in Section 2.3.1) using 2% sodium dodecyl sulphate solution (SDS, 1 ml) and incubated for 30 min at 60 °C in a water bath. Protein extracts were vortexed for 1 min and centrifuged at 17,000g (5 min, 4 °C). The supernatant was collected and assayed on the same day for protein content using BCA assay (Smith et al., 1985). On performing the analysis, samples were diluted to obtain absorbance values within the range of the standard curve.

2.2.5.3. Protein analysis by SDS-PAGE

Protein samples were appropriately diluted in 2% SDS solution normalised by the amount of oleosin (as discussed in Section 3.3.2.2), unless specified otherwise. 20 μ L of protein extract was then mixed with an equal volume of sample buffer (Laemmli buffer (Biorad, UK) + 5% β -mercaptoethanol), heated at 95 °C for 5 minutes and then cooled on ice. Proteins were resolved by SDS-PAGE using 4–15% polyacrylamide gels (Mini-

Protean TGX Gels, 10-well, 50 μ L, Bio-Rad, Hercules, USA); gels were positioned within a SE 600 BioRad separation unit and suspended in tank buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was run at 100 V for 40 minutes. After electrophoresis, the gels were fixated and stained (1 h) using Coomassie brilliant blue R-250 (0.1 % Coomassie, 50 % ethanol, 10 % acetic acid) and de-stained with water. Gels were imaged using Bio-Rad Gel Doc XR System.

2.2.6. Oil body Lipid analysis

2.2.6.1. Freeze drying

Samples were flash frozen in liquid nitrogen and stored at -80 °C for 4 hours prior to freeze drying. Samples were freeze dried (Edwards Freeze Dryer Super Modulyo Pirani 1001) at - 40 °C under vacuum (48 hours).

2.2.6.2. Fatty Acid composition

Fresh or aged (stored for 7/ 14/ 21/ 28 days, 20 °C) COB or WOB creams (approximately 0.5 g) were freeze-dried (as detailed in Section 2.4.1) and used for total fatty acid characterization through Gas Chromatography Mass Spectrometry (GC-MS). A mixture of Chloroform/Methanol (2:1 v/v, 1 ml) was added to the samples, shaken in a mini bead-beater (3450 rpm, 2 minutes), centrifuged (Thermo Heraeus Fresco 21) at 17,000g for 5 min at 4 °C, and the upper phase (containing total lipid) was removed with a glass pipette. This process was repeated two more times and the recovered lipids were pooled and dried under nitrogen. Lipid samples were resuspended in

chloroform (final concentration of 100 mg/ml) containing BHT (0.1 %) to avoid oxidation and stored at -80 °C until use. An aliquot of the total fatty acids from TAGs and PLs (200 μ l) was transmethylated by heating the samples at 80°C for 1 hour with 1 mL of a solution containing methanol/ toluene/ dimethoxypropane/ H₂SO₄ (66 :28 : 2 : 1 v/v) (Ruiz-Lopez et al., 2015). Methyl ester derivatives of total fatty acids were extracted with 1 ml of water and 1 ml of hexane and analysed. GC-MS was performed using a CTS Analytics PAL system autosampler (injection operated into split mode), DSQ mass spectrometer and TRACE GC Ultra gas chromatograph (Thermo Electron, Loughborough, UK). One microliter of sample was injected into a capillary column (Phenomenex Zebron ZB- FFAP, California, USA). The oven temperature was initially held at 120 °C for 1 min and increased to 250 °C at a rate of 5 °C/min. The final temperature of 250°C was held for 2 minutes. The carrier gas was helium (30 ml/min). Methyl pentadecanoate (250 μ g/ml in chloroform) was used as an internal standard. Identification of fatty acids was verified by comparing mass spectrum with standard library through Thermo Scientific Xcalibur V1.4 software program.

2.2.6.3. Phospholipid and neutral lipid extraction from oil body creams

Oil body phospholipids were isolated according to the method by Tzen and Huang (1992) with the following modifications. Fresh oil body creams (0.5 g) were placed into a 2 ml sample tube, 1 ml diethyl ether was added, and the sample was vortexed. The sample was centrifuged for 5 min at 15,000 g, 5 °C.

After centrifugation, the upper diethyl ether layer containing neutral lipids was removed, this procedure was repeated twice more. Neutral lipids were pooled, dried under nitrogen and resuspended in 5 ml chloroform ready for thin layer chromatography (TLC) analysis (Section 2.4.5). Residual diethyl ether present in the interfacial layer and the aqueous phase was removed with nitrogen gas for 1 h. Upon removal of the ether, 750 µl chloroform/methanol (2:1 v/v) was added and samples were gently shaken. The lower chloroform phases containing phospholipids were washed three times with 1 ml methanol/water (1:1 v/v) and dried under nitrogen gas. The dried phospholipids were resuspended in 100 µl chloroform ready for TLC analysis (Section 2.2.6.5).

2.2.6.4. Lipid analysis from rapeseed seeds

Seeds (0.5 g) were ground in mortar and pestle and placed in an 8 ml glass tube with 5 ml of chloroform/methanol (2:1 v/v) mixture and BHT (0.1 %). Oil was extracted placing the tubes on a rotatory bed for 24 hours (4 °C). The samples were centrifuged for 5 minutes (2,000 g, 4°C) to induce phase separation and the solvent was collected. Residual oil in the ground material was recovered adding 5 ml of chloroform/methanol (2:1 v/v) mixture and BHT (0.1 %), vortexed and centrifuged (5 minutes, 2,000 g, 4 °C). The solvent was collected, then the samples were pooled and dried under nitrogen and resuspended in chloroform/acetic acid (100:1 v/v, 3 ml). Fractionation of phospholipid and neutral lipid was performed as described by Ruiz-Lopez et al. (2015) with modifications. Lipid extracts (3 ml) were loaded on a Sep-Pak column (www.waters.com) and neutral lipid, glycolipid and polar lipid eluted by

adding chloroform/acetic acid (100:1 v/v, 5 ml), acetone/acetic acid (100:1 v/v, 10 ml) and methanol (5 ml), respectively. Lipid fractions were dried under nitrogen. Polar and neutral lipid were resuspended in 500 μ l and 2.5 ml, respectively, and used for TLC analysis (Section 2.2.6.5).

2.2.6.5. Thin layer chromatography

Oil body phospholipids (50 μ l) and neutral lipids (20 μ l) samples were spotted along with standards (Avanti Polar Lipids, Alabama, USA) onto a BioRad Silica gel 60A TLC plate. Polar lipids were developed in chloroform/methanol/water (65:25:4 v/v/v) and neutral lipids in hexane/diethyl ether/acetic acid (70:30:1 v/v/v). The individual lipid classes were identified under UV light after spraying with primuline (0.05% w/v in acetone/water, 80:20 v/v) and used directly for methylation (as described in Section 2.2.6.2).

2.2.6.6. Phospholipid and Triacylglycerol fatty acid analysis

Phospholipid and neutral lipid spots on TLC plates were scraped off and collected in to a 8 ml glass tube. Samples were directly methylated in 2 ml of a solution containing methanol/toluene/ dimethoxypropare/ H₂SO₄ (66:28:2:1 v/v) for 1 hour at 80 °C and analysed using GC-MS as described in Section 2.2.6.2.

2.2.6.7. Tocopherol analysis

Fresh and aged (stored for 2/ 4/ 6/ 8/ 10 days, 40 °C) oil body emulsions (10 % lipid weight basis) were used for tocopherol analysis. Each sample was centrifuged (10,000 g, 30 minutes, 4 °C) and the cream (0.5 g) freeze dried (as described in Section 2.2.6.1). Methanol (1.5 ml) was added to the mini bead vials, capped, and placed Mini Bead Beater for 1 min at maximum speed. After the extraction, the samples were centrifuged for 5 min at 2600 g, 5 °C and supernatant was pooled. The above steps were repeated twice more, and the solvent was removed by nitrogen gas then resuspended in 1 ml methanol, filtered (0.45 μ m) and analysed by HPLC. The HPLC system consisted of Agilent 1100 separation module equipped with a photodiode array detector and a Jasco intelligent fluorescent detector FP-920. Samples were injected at a volume of 10 μ l through a Security Guard guard-column and separated on a Zorbax RX-C18 5 μ m (250 $\times$ 4.6 mm) column with the column oven set at 20°C. A gradient system of two mobile phases was employed; the first (solvent A) contained 45:45:5:5 (v/v/v/v) Acetonitrile: Methanol: Isopropanol: 1% (v/v) Acetic Acid solution and the second (solvent B) contained 25:70:5 (v/v/v) Acetonitrile: Methanol: Isopropanol. The flow rate of the mobile phase was set at 0.8 ml/min starting with 100% solvent A. After 6 minutes of solvent A running isocratically the mobile phase changed linearly to 100% solvent B over the following 10 minutes. This was held for 12 minutes before being returned to the initial conditions with a total run time of 36 minutes. Detection was performed at an excitation wavelength of 298 nm and emission wavelength of 328 nm. Identification and quantification were made using standards of α -, β -, γ -, and δ -tocopherol and tocotrienol (Sigma Ltd., Gillingham, United Kingdom).

2.2.7. Lipase activity

2.2.7.1. Preparation of enzyme solution from oil body creams

Freshly extracted cream (1 g) was placed in a 50 ml falcon tube and 20 ml of acetone were added. The sample was frozen under liquid nitrogen and the oil bodies broken using an Ultra Turrax (T18, Ika, Oxford) at 20.000 rpm for 1 minute. Samples were centrifuged (5,000 g, 10 minutes, 3 °C) and the supernatant (containing the oil) collected. This step was repeated twice. The residual pellets were completely dried under nitrogen until any trace of acetone was removed. Dry pellet were then suspended in sodium phosphate buffer (50 mM, pH7) with the addition of 0.1% Triton X-100 to avoid precipitations and agitated at 4 °C for 6 hours. The samples were filtered (0.45 µm) and the clarified enzyme solution was used as raw extract for lipase assay on the same day.

2.2.7.2. Preparation of enzyme solution from seeds

Lipase was extracted as described by Ponne et al. (1996) with the following modifications. Dry (internal moisture content of 8 %) or soaked (16 hours, in dH₂O or 0.1 M NaHCO₃, pH 9.5) rapeseed seeds (5 g) were crushed using a cool mortar and added to a falcon tube. 20 ml of cold acetone were added, and the seeds were mixed for 1 minute using an Ultra Turrax (T18) at 20,000 rpm. The samples were centrifuged (5,000 g, 10 minutes, 4 °C) and the oil phase removed. This step was repeated twice, and residues of acetone removed by nitrogen gas. The pellet was resuspended in sodium phosphate buffer (50 mM, pH 7) with 0.1 % Triton X-100 and incubated for 6 hours (4 °C)

on a rotary shaker. The mixture was filtered using cheesecloth, and the crude extract was centrifuged at 21,000 g (4 °C). The water phase was collected and used on the same day for lipase assay.

2.2.7.3. Clarification of serum phases from residual oil bodies

The serum phases were collected after centrifugation of WOB I emulsions and was centrifuged (21,000 g, 4 °C) to remove oil body contamination. The lower phase was carefully removed with a syringe and needle. This step was repeated three times. Finally, the serum was passed through 0.2 µm membrane. All clarified samples were stored at 4 °C and analysed within 24 hours.

2.2.7.4. Lipase assay

Lipase activity was measured using a modified protocol of Ruiz et al. (2004). The non-coloured substrate p-NPL was dissolved in 2-propanol at a final concentration of 20 mM by sonication for 3 minutes. A 1:10 (v/v) dilution in sodium phosphate buffer- Triton X-100 was prepared with gentle agitation until a clear solution was forming. 1 ml of enzyme solution (in 50mM sodium phosphate buffer, pH 7) was mixed with 1 ml of substrate mixture to obtain a 2ml final reaction mixture (1 mM p-NPL, 5% 2-propanol, 0.6% Triton X-100, 50 mM sodium phosphate buffer), which was incubated at 37 °C for 2 hours. At the end of the incubation time, the samples were cooled in ice for 1 minute to stop the reaction and the absorbance at 405 nm was measured. The blanks, corresponding to the absorbance of the reaction mixture without enzyme

solution, were determined and subtracted from the total absorbance. The net absorbance was compared to a standard curve built using commercial lipase. One enzymatic activity unit (U) is expressed in this thesis as the enzymatic activity that released 1 μmol of p-NP under the assay condition. This was normalised either by weight of the cream (dry weight basis), or protein content.

2.2.8. Oil body emulsion stability

2.2.8.1. Oil body emulsion preparation

Oil body emulsions were prepared by mixing the oil body cream with deionised water to achieve a 10% emulsion based on the total lipid content. A uniform dispersion of oil body was achieved by vortex of the emulsion for 1 minute. Absence of aggregates was checked by particle size (Section 2.2.8.2) and light microscopy (Section 2.2.8.4). Analysis of emulsions were performed on fresh samples (within 24 hours) unless stated otherwise. All emulsions contained NaN_3 (0.02 mM) to avoid microbial spoilage (Lichstein and Soule, 1944).

2.2.8.2. Particle size

The particle size of oil body suspensions was measured with an LS 13320 laser diffractometer from Beckman-Coulter (FL, USA) using the Mie theory of the scattering of light by spherical particles. The samples were diluted as appropriate prior to measurement. According to the Mie theory (Mie, 1976), the complex refractive index is a function of the real and imaginary part of the refractive index. The real part of the refractive index, used for the calculation

of oil body size, was 1.462, corresponding to the refractive index of rapeseed oil. The imaginary part, corresponding to the attenuation coefficient that describes the turbidity of a sample, was set to 0.01 according to the laser diffractometer guidelines for lightly coloured translucent materials. The percentage of intact droplets ($\leq 2.5 \mu\text{m}$ in diameter), was found using the LS 13320 software from the cumulative volume frequency distribution. The surface protein load (Γ_s) was found according to the formula $\Gamma_s = C_a D_{3,2} / 6\Phi$, where C_a and Φ is, respectively, protein and oil content, and $D_{3,2}$ is the surface mean diameter (McClements, 2004).

2.2.8.3. Zeta potential

A series of oil body emulsions were prepared in ultrapure filtered (0.2 μm) water at a concentration of 0.008 % (lipid weight basis) and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH. After stabilization of the pH, the emulsions were injected into the measurement chamber of the particle electrophoresis instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA). The instrument settings used were: temperature = 25 °C; refractive index of dispersant=1.333; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm; voltage = 35 V. The ζ -potential was then determined by measuring the direction and velocity of the oil bodies moving under the applied electrical field. The ζ -potential value was reported as the average and standard deviation of three independent samples from each emulsion replicate, with one reading taken per sample.

2.2.8.4. Light microscopy

The microstructure of oil body suspensions was investigated using light microscopy (EVOS, UK). A small drop of oil body emulsion was placed on a glass slide, covered with a cover slide and imaged at a magnification of 40 $\times$.

2.2.8.5. Confocal microscopy

Oil body emulsions were stained to visualise the protein and oil fractions. Nile Red (Sigma Aldrich, 0.01%), was used to label lipids, and Fast Green FCF (Merck 0.01%) was used to bind to structural proteins. Microstructural Imaging Images of the emulsion sample was acquired using CLSM. Samples were mounted on a glass slide and examined using a Zeiss LSM880 Laser Scanning Microscope. A band pass filter between 550-625 nm was selected for the detection of Nile Red; when excited at 488nm, and 640-700 nm for the detection of Fast Green FCF; when excited at 633nm. Images were acquired using an x63 oil objective, with argon, and HeNe laser respectively. An overlay of the two channels, to the same area, gave the final image of the network seen in the samples, showing in red the lipid, and in green the protein.

2.2.9. Oil body oxidation

2.2.9.1. Emulsion preparation

Oil body creams were resuspended in sodium phosphate buffer (10 mM, pH 7.5) at a concentration of 10% (lipid weight basis), with NaN₃ (0.02 mM) to

avoid microbial spoilage. Emulsions were placed into 20 ml tubes, stored at 40 °C protected from light. Oxygen headspace was renewed every day and aliquots were removed at each time point.

2.2.9.2. Hydroperoxide detection assay

Hydroperoxides were detected according to the method by Nuchi et al. (2001). An aliquot of stored emulsion (200 μ l) was mixed with isooctane/2-propanol (3:1 v/v) (1.5 ml), vortexed for 10 seconds every 2 min for 10 min, followed by centrifugation at 2,000 g for 2 minutes. The organic phase (200 μ l) was then removed and added to methanol/1-butanol (2:1 v/v) (2.8 ml); this was followed by the addition of ammonium thiocyanate (3.94 M) (15 μ l) and iron (II) solution (0.072 M) (15 μ l) (formed by mixing equal volume of 0.132 M BaCl₂ (in 0.4 M HCl) and 0.144 M FeSO₄·7H₂O). After 20 min, the solution absorbance was measured at 510 nm against a blank which contained everything but the sample emulsion. The concentration of hydroperoxide was calculated from a standard curve produced using cumene hydroperoxide. The weight of lipid was determined gravimetrically by taking a further 200 μ L of the above organic phase and evaporating the solvent on a hot plate (200°C).

2.2.9.3. Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were measured according to the method by McDonald et al. (1980). TBA reagent was prepared by the addition of trichloroacetic acid (TCA) (15% w/v) and thiobarbituric acid (TBA) (0.375% w/v) in 0.25 M HCl with the addition of 3% butylated

hydroxytoluene (BHT) (2% in absolute ethanol). The TBARS assay was performed by adding TBA reagent (2 ml) to emulsion solution (1 ml) and vortexing followed by heating for 15 min at 100°C. The samples were cooled for 10 min and centrifuged at 1000 g for 15 min. Absorbance was measured at 532 nm against a blank containing everything but the sample. Concentrations of TBARS were determined from a standard curve produced using 1,1,3,3-tetraethoxypropane.

2.2.10. Statistical analysis

All experiments were performed in triplicate. Data and graphs presented were compiled using Microsoft Excel (2016) including averages and standard deviations. Statistical analysis was performed by one-way ANOVA (ANalysis Of Variance) using SPSS version 13.0 software (IBM, Chicago, USA).

CHAPTER 3.

ENHANCING THE RECOVERY OF OIL BODIES FROM OILSEED RAPE SEEDS

3.1. Background

Seeds are the most common site for the accumulation of oil bodies in plants, mainly functioning as energy storage, with oil content ranging from 20% in soybean, to 50% in rapeseed and more than 75% in larger nuts (Murphy, 1996). The recovery of oil bodies requires the rupture of cotyledon cell wall and the release of these droplets from the cytoplasmic network. Despite an increasing interest has been expressed in the use of enzymes such as cellulases, hemicellulases and pectinases to enhance the recovery of oil bodies (Towa et al., 2011, 2010), the use of mechanical methods in aqueous media is frequently reported in literature. During the aqueous extraction, together with the oil bodies, other water-soluble seed material is solubilised (Rosenthal et al., 1998). Given a sufficient amount of solution, the protein diffuse first, followed by the release of oil bodies (Nikiforidis et al., 2014).

Oil bodies have been recovered from a large number of oil crops and cereals, including pumpkin (Adams et al., 2012), soybean (Chen et al., 2012; Chen and Ono, 2010; Iwanaga et al., 2007; Tzen and Huang, 1992), maize (Nikiforidis and Kiosseoglou, 2009; Tzen et al., 1993), sunflower (Fisk et al., 2006; Makkhun et al., 2015; Nikiforidis et al., 2013) and rapeseed (Katavic et al., 2006; Tzen et al., 1993; Tzen and Huang, 1992). Despite the differences between the authors, the key points for the extraction of oil bodies are similar, comprising: soaking of the seed, disruption of the matrix in aqueous media; collection of a crude oil body cream after centrifugation and washing of the cream. The latter stage is aimed to remove the polysaccharide, seed debris

and exogenous proteins that are loosely associated to the oil body (Tzen et al., 1997) using buffers, detergents or chaotropic agents.

The aim of this chapter was to investigate which are the driving factors in each step of the recovery process that affect the final physicochemical characteristics of oil bodies. By this way, it could be possible to recover oil bodies free from exogenous material, and with similar composition, structure and properties to the ones found in seeds. The results thereof have been already published in: “Enhancing the recovery of oilseed rape oil bodies (oleosomes) using bicarbonate-based soaking and grinding media”, 2018, *Food Chem*, 241, 419-426, by De Chirico et al. The first section of this chapter focuses on the effect of soaking variables (as shown in Figure 3.1) on oil body integrity. Subsequently, an overview on the composition and physical properties of oil bodies, with emphasis on protein content, particle size, and surface charge is given upon changing the grinding solution. In the last section, the effect of washing protocol is reported.

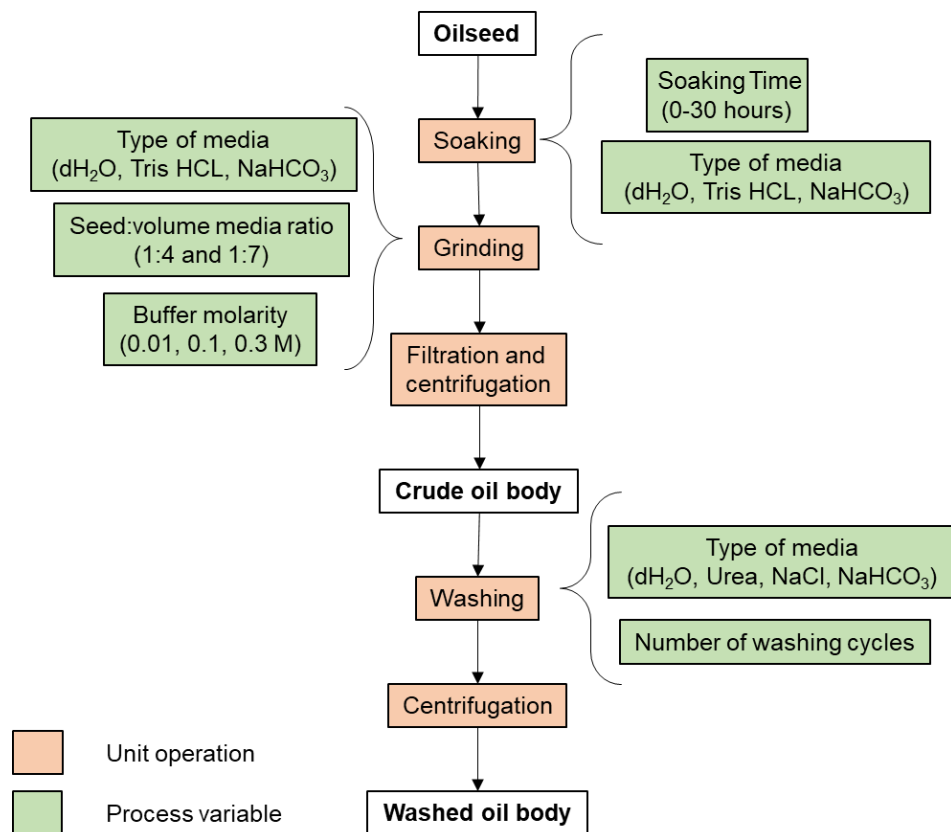


Figure 3.1: Variables considered over the recovery process of oil bodies. The unit operations (pink boxes) for oil body recovery are reported. The process variables over soaking, grinding and washing are shown in the green boxes.

3.2. Effect of soaking time and media type on oil body size

An effective cell disruption is essential to release the oil bodies in the recovery solution. It was initially reported by Gupta and Das (2000), that the energy required to initiate seed rupture decrease with an increase in moisture content, and that the entry of the water in the seed, a process called 'imbibition', is greatly influenced by the permeability of the seed coat. Previous works have attempted to recover oil bodies from a wide range of oilseed species, but the soaking time varied among authors: Tzen et al. (1993) did not soak rapeseed seeds prior grinding, Chen et al. (2014) soaked soybeans for 18 hours and Iwanaga et al. (2007) used a soaking overnight. The use of different soaking times or media could potentially influence the characteristics of recovered oil bodies. The aim of this section was to determine the correlation between length of imbibition and type of media over soaking on oil body integrity, in order to determine the optimum soaking protocol to be used in this thesis (Figure 3.2).

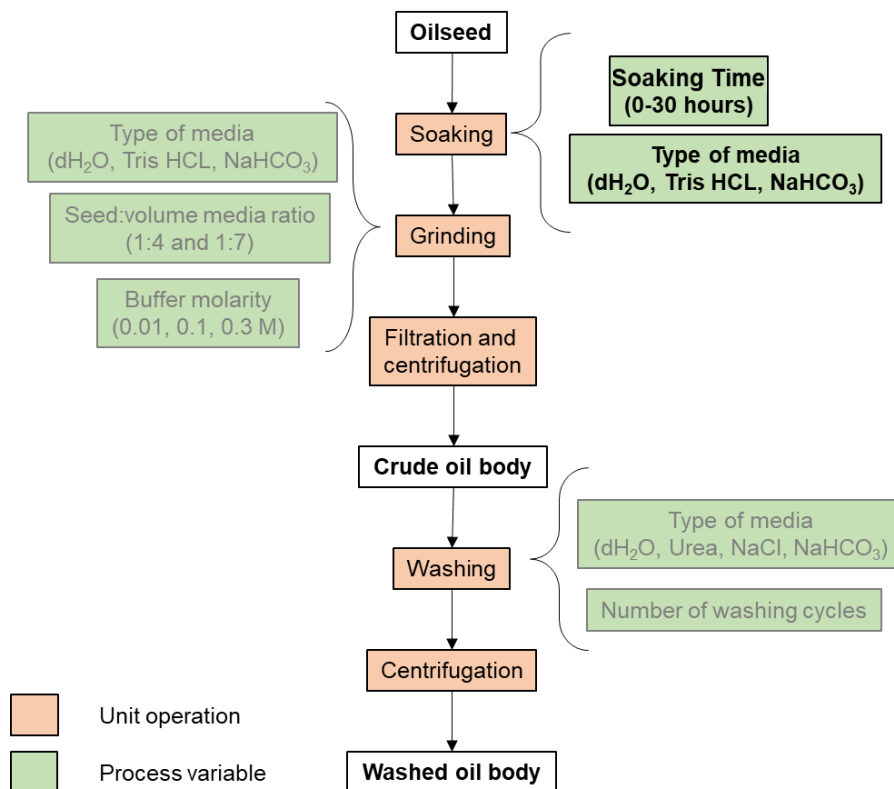


Figure 3.2: Variables considered over soaking step for the recovery of oil body. The process variables considered in this section are: soaking time; type of soaking media. Variables considered are highlighted in bold.

In this set of experiments oilseed rape seeds were soaked at different times (up to 30 hours), at a ratio 1:4 seed-to-volume of media using either deionised water (dH₂O), Tris-HCl (0.1 M, pH 8.5) or NaHCO₃ (0.1 M, pH 9.5) solution and the change in weight was recorded. Figure 3.3 shows that oilseed rape seeds had a rapid uptake of the medium in the first 5 hours of imbibition, reaching a plateau between 15 and 20 hours. The weight increase was not greatly affected by the type of media.

Bradford (1990) described the imbibition process in three different phases, with a rapid initial water uptake (phase I), followed by a plateau phase with little change in water content (phase II), and a final increase in water

content coincident with radical growth (phase III). Imbibition is essential for seed germination, where a precise transition from increased hydration, through enzyme activation to storage products breakdown occur (Bradford, 1990). Because the enzymes start to be active between phase I and II, the length of imbibition needs to be carefully chosen, as the oil body integrity may be compromised by prolonged soaking times.

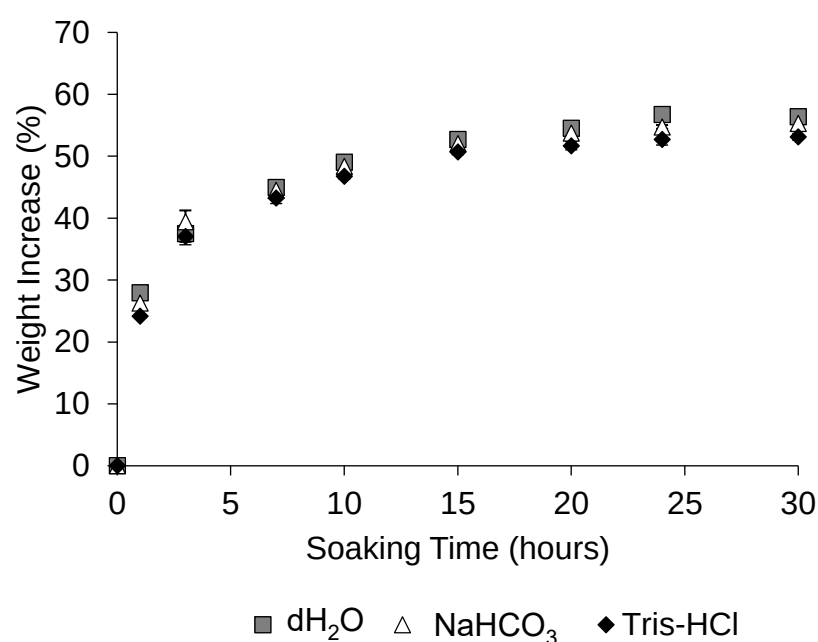


Figure 3.3: Net weight increase (%) of recovered oil bodies as affected by imbibition time. Oil bodies were soaked for up to 30 hours at 4 °C. The ratio seed:volume of media was 1:4 using dH₂O, NaHCO₃ (0.1 M, pH 9.5) or Tris-HCl (0.1 M, pH 8.5).

To study the correlation between seed softening at different moisture content and oil body integrity, rapeseed seeds were milled at a ratio seed-to-volume of media of 1:4, using the same soaking solution (dH₂O, Tris-HCl - pH 8.5, NaHCO₃ - pH 9.5). The mean volume diameter (D_{4,3} value) of recovered emulsions was recorded. It was hypothesised that if significant enzyme activity

was present, the mean diameter would show an increase at prolonged times of soaking. As it can be seen in Figure 3.4, it was found that the softening of seeds had a positive effect on the size of recovered oil bodies: upon increasing the soaking time, the mean volume diameter showed a decrease using all solutions considered, which was nearly constant after 10-16 hours of soaking. This effect may be explained by the decrease in the force needed for seed rupture, as well as bulk and true density of the seed (Izli et al., 2009), which reduced the physical stress on oil bodies. In this experiment, it was also found that the $D_{4,3}$ of oil bodies was dependent on the type of solution used, where the NaHCO_3 solution recovered oil bodies with a diameter significantly smaller ($P < 0.05$) than the ones recovered either in dH_2O or Tris-HCl (Figure 3.4).

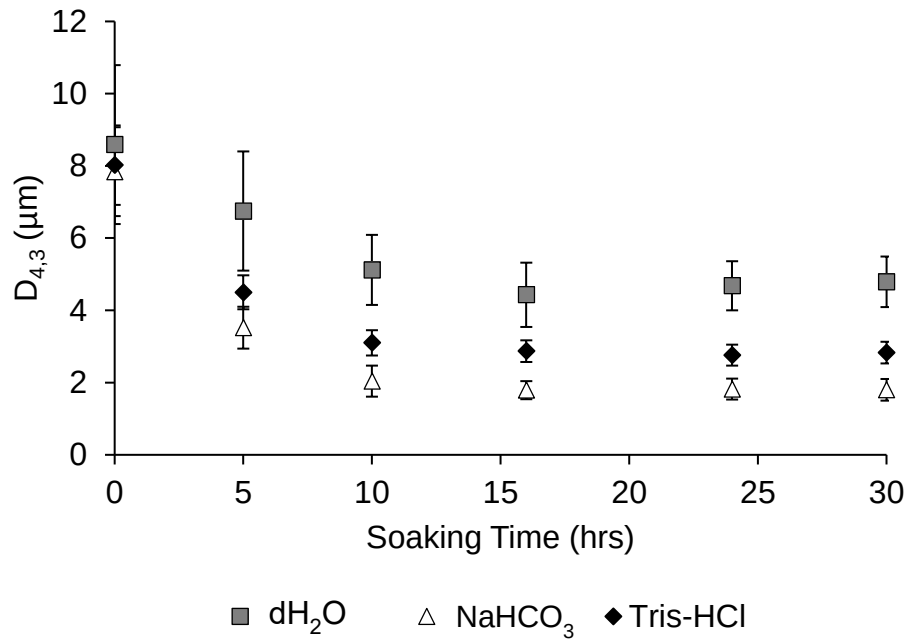


Figure 3.4: Mean volume diameter ($D_{4,3}$) of oil bodies recovered from seeds at different soaking time and with different soaking/grinding media. Seeds were soaked for up to 30 hours, and ground at 4 °C. The ratio seed:volume of media in both soaking and grinding was of 1:4, using dH₂O, NaHCO₃ (0.1 M, pH 9.5) or Tris-HCl (0.1 M, pH 8.5).

Another approach to evaluate the evolution of seed over soaking is by measuring the electrolytes solubilised from the seed tissue using conductivity (Zhang et al., 2017). Despite no soaking-medium specific difference in the amount of solution absorbed by the seed was found, the electrical conductivity was dependent on the solution used. Taking into consideration the conductivity value at the start of the soaking process, it was registered an increase after 16 hours (corresponding at the start of plateau region in Figure 3.3) of 350 ± 50 , 660 ± 40 and 600 ± 70 $\mu\text{S}/\text{cm}$ for dH₂O, Tris-HCL and NaHCO₃, respectively. These values suggest that the use of alkaline media had a higher capacity to solubilise seed electrolytes, probably deriving from the seed coat. The results shown in this thesis are in line with a recent work on pecan nut (Zhang et al., 2017), where it was suggested that the soaking of the seed causes a decrease

in damage to the oil bodies, due to the plasticisation of the tissue. However, the differences in droplet diameter as seen in Figure 3.4 may be due to a synergic effect of different factors over each step of the recovery process, such as the type of media over soaking and grinding, pH value and buffer molarity. The effect of these parameters on the quality of oil bodies (e.g., integrity, purity) will be addressed in this chapter.

Based on the findings shown in this section, a soaking time of 16 hours (corresponding to the initial plateau phase) was used for all subsequent experiments in this thesis.

3.3. Effect of grinding solution on oil body characteristics

Although oil bodies have been extensively studied in literature, the use of a wide range of grinding solutions usually leads to different results for composition and functionality (Karkani et al., 2013; Wang et al., 2017). A recent trend involves the use of deionised water during grinding, followed by the addition of NaOH to alkalis the produced oil body solution above pH 8 (Cao et al., 2015; Chen and Ono, 2010; Zhao et al., 2016). However, due to the slow addition of alkali, this methodology is usually time consuming; thus, endogenous enzymes may have higher chances to cause oil body breakdown.

In this section, the grinding of soaked seeds in buffer solutions at a specific pH (8.5 and 9.5), has been considered as an alternative to dH₂O (pH 6.5). Initially, the effect of pH and ratio seed:volume media on droplet integrity and protein content of recovered oil bodies were studied. Subsequently, the effect of buffer concentration (at the optimum pH) on physicochemical characteristics of oil bodies was assessed. Over this chapter, oil bodies recovered in deionised water were considered as reference material, and its particle size data are repeated over the next sections for ease of data comparison.

3.3.1. Effect of seed:grinding buffer ratio and pH value during grinding

3.3.1.1. Droplet size

When the seeds are disrupted in aqueous solution, the overall viscosity of the system is reduced on increasing the volume of the medium (Austin and Trass, 1997), which may lead to a reduction in the forces experienced by oil bodies and to a reduction in the concentration of seed particulates that might collide with and damage oil bodies. At seed:grinding media ratio too low or too high, an effective rupture of the seed is not achieved, as there may be low circulation of the media (high ratio) or not enough contact with the blades (low ratio). The optimised solids:media ratio allows an effective circulation of particulates and a sufficient contact with the blades leading to a satisfactory particle reduction.

To study whether a specific seed:grinding medium ratio would cause droplet breakdown, crude oil bodies were recovered at two ratios (1:4 and 1:7 seed:media ratio). Moreover, the effect of the type of media (pH) was studied in this section (Figure 3.5). Soaked seeds (16 hours) were ground in either deionised water (dH₂O, pH 6.5, used as reference), Tris-HCl (0.1 M, pH 8.5) or NaHCO₃ (0.1 M, pH 9.5) solution, collecting the cream layer (crude oil bodies) after centrifugation. The recovered cream (COB) was then washed with urea (9 M) (WOB) as described in method section, and the particle size distributions (PSD) of crude and urea-washed emulsions were measured (Figure 3.6).

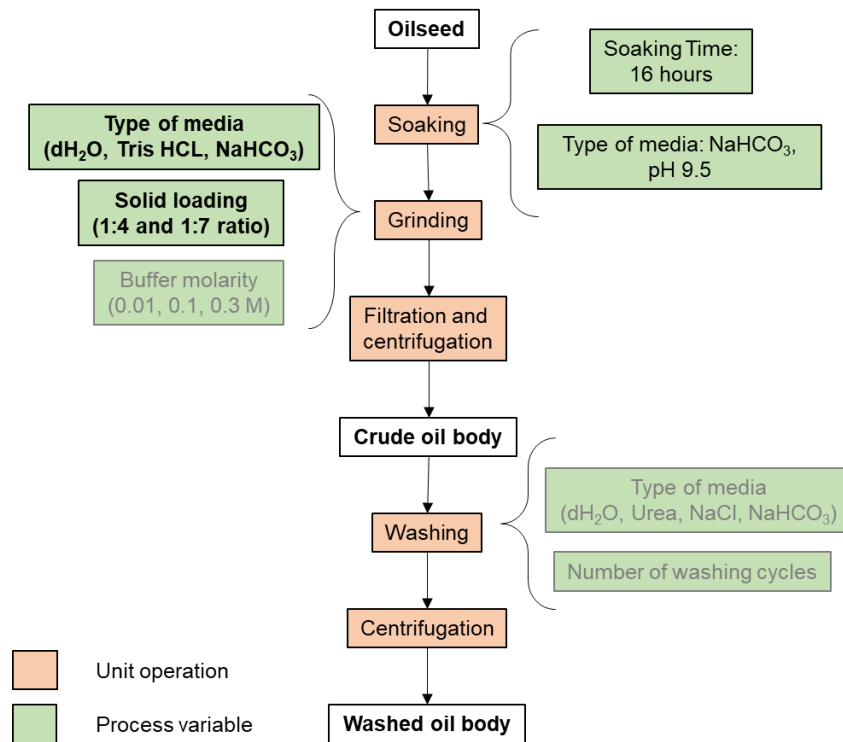


Figure 3.5: Variables considered for the recovery of oil body. The process variables considered (highlighted in bold) in this section are: type of media (dH₂O; Tris-HCL – pH 8.5; NaHCO₃ – pH 9.5); seed:volume grinding media ratio (1:4; 1:7). Variables in grey are yet to be developed.

Urea is a chaotropic agent able to remove the exogenous proteins that are strongly associated with the oil body and the seed proteins that could be adsorbed at the interface of damaged droplets, temporarily stabilising them. Lacey et al. (1998) suggested that the oleosin is not affected by exposure to urea, so the secondary structure would be expected to reform once the agent is removed from the solution. Millichip et al. (1996) also claimed that the physical integrity of oil body is not affected by urea treatment. In this thesis, urea wash (9 M) was used as a methodology to measure the degree of droplet integrity. It is proposed that after exposing the cream to urea, intact oil bodies (presenting only oleosin at the surface) will remain as small discrete entities,

whereas hybrids (damaged oil bodies with altered surface chemistry) will rapidly destabilise and coalesce forming larger droplets.

As it can be seen in Figure 3.6, in both low and high seed:grinding media ratios the COB emulsions recovered at pH 8.5 and 9.5 showed a very similar droplet size profile, with a bimodal distribution ranging between around 0.1 and 6.0 μm . However, using dH₂O, the PSD showed the presence of higher diameters, probably due to the presence of aggregates (indicated by arrows). After exposing the crude material recovered at ratio 1:4 using either Tris-HCl or NaHCO₃ to urea, the PSD of washed oil bodies (WOB) showed a similar shift towards higher diameters, with a major peak at 4 μm . Oil bodies recovered in deionised water showed a larger shift, with a much broader distribution ranging between 0.1 and 120 μm , and a major peak at 12 μm . Droplets having a diameter over 2.5 μm (Tzen et al., 1993) may be the result of coalesced oil bodies, as suggested by optical images in Figure 3.6. However, when a more diluted system is used, such as 1:7 seed:grinding media ratio, the PSD showed a more limited shift towards higher diameters for dH₂O and Tris-HCl, suggesting that this low ratio improved the oil body integrity. Moreover, the quality of oil bodies was significantly improved using NaHCO₃ (pH 9.5). In this case, compared to COB, a slight size decrease was registered, which may be due to the reduction of aggregates after solubilising exogenous protein over the urea wash. The $D_{4,3}$ of the recovered urea WOBs were 3.6 ± 0.5 , 2.0 ± 0.2 and 1.3 ± 0.2 μm for deionised water, Tris-HCl and NaHCO₃, respectively, suggesting that the latter is the most appropriate solution to recover intact oil bodies.

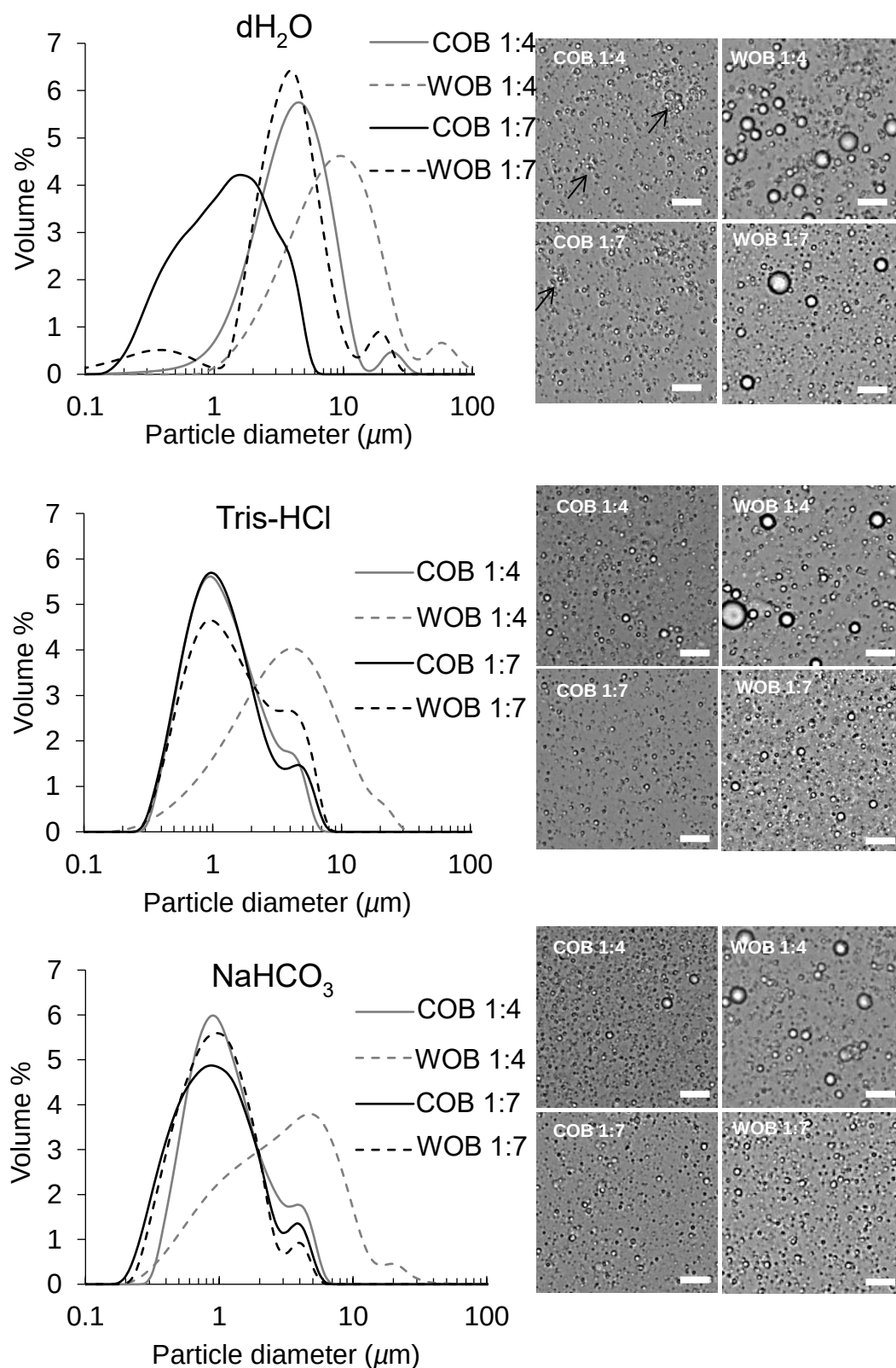


Figure 3.6: Effect of seed-to-volume ratios (1:4 and 1:7) on oil body droplet size using dH₂O, Tris-HCl and NaHCO₃. Seeds were soaked and ground using the same media type at a ratio seed:volume of media of 1:4 or 1:7. The media used was either dH₂O, Tris-HCl (pH 8.5) or NaHCO₃ (pH 9.5). Scale bar represent 10 μm. Aggregates are indicated by arrows.

These results suggest that the ratio seed:media is not the only driving factor preserving droplet integrity, with the pH of grinding solution also playing an important role. The surface charge of oil bodies shows a dependency on solution pH, with increasing repulsion forces at alkaline values (where the oil body surface is negatively charged) that promotes the maintenance of droplets as discrete entities, leading to an increase of the emulsion stability against aggregation (Iwanaga et al., 2007). At pH values close to the isoelectric point of oleosins (4.5-5.5) (Chen et al., 2012; Iwanaga et al., 2007; Nikiforidis and Kiosseoglou, 2009), the overall charge is zero and in the absence of repulsion forces aggregation occurs (Tzen et al., 1993; Tzen and Huang, 1992; White et al., 2008). The results shown in Figure 3.6 clearly indicate that in a highly viscous system, such as in 1:4 seed:grinding media ratio, the presence of aggregates may promote coalescence, making the pH value of grinding solution a critical variable that needs to be taken into consideration. Those results suggest that the use of low ratios (e.g., 1:7), together with a highly alkaline solution (NaHCO_3 , pH 9.5) are the optimal condition to maintain droplet integrity.

3.3.1.2. *Protein Content*

During the conventional extraction of oil using organic solvents, the cell wall is disrupted, and the oil is solubilised in the medium. In this case, fibres, carbohydrates and protein remain in the meal (Nikiforidis et al., 2014). However, using an aqueous extraction, in addition to the integral protein of oil bodies (e.g., oleosins), many seed exogenous proteins (located elsewhere in

the cell) may bind to the oil body surface during their recovery (Tang et al., 1997) and be present in the final extract. The amount and/or the type of proteins that remain associated to the surface of oil bodies could potentially change their physicochemical characteristic (Chen and Ono, 2010; Zhao et al., 2016). Crude oil body material was recovered after soaking the seeds (16 hours) in dH₂O, Tris-HCl and NaHCO₃, and then ground in the same type of media (freshly made) at both seed:grinding buffer ratios under test. The protein content of the COB material was measured (Figure 3.7).

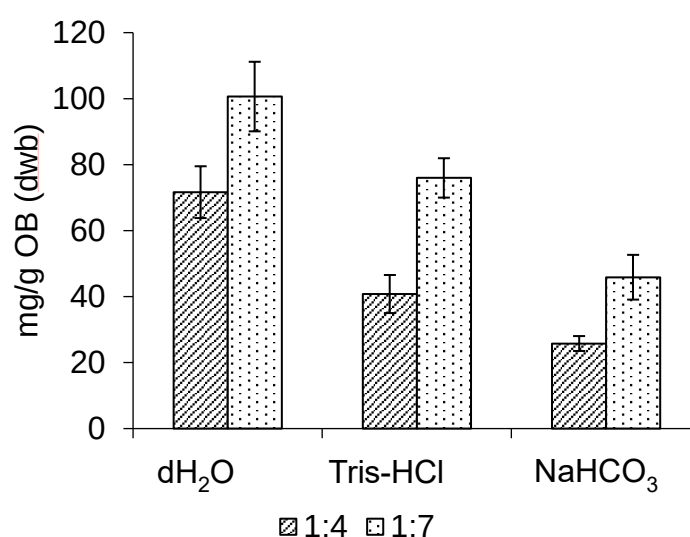


Figure 3.7: Effect of grinding media and seed:media ratio on protein content of recovered crude oil bodies. Oil bodies were recovered soaking and grinding rapeseed seeds in dH₂O, Tris-HCl (0.1M, pH 8.5), NaHCO₃ (0.1M, pH 9.5). Recovered oil body solutions pH values after grinding (prior centrifugation) were 6.3 ± 0.2 , 8.5 ± 0.1 and 8.8 ± 0.1 for dH₂O, Tris-HCl, NaHCO₃, respectively at both 1:4 and 1:7 ratios.

The degree of purity, as measured by the amount of proteins removed, increased in the order dH₂O (pH 6.5) < Tris-HCl (pH 8.5) < NaHCO₃ (pH 9.5); and was higher using seed:grinding media ratio of 1:4. The pH values of the

resultant natural emulsions after grinding the seeds and removal of ground material, were 6.3 ± 0.2 , 8.5 ± 0.1 and 8.8 ± 0.1 for dH₂O, Tris-HCl, NaHCO₃, respectively, in both seed:volume of media ratios considered. The carry-over of proteins (as shown in Figure 3.7) in the extract is affected by type of grinding solution and seed:grinding media ratio. When type of grinding solution is compared, the increase in pH value seems to be the driving force for the higher purity of oil body extracts. This hypothesis was confirmed when the pH of NaHCO₃ solution was reduced to 8.5 (in both soaking and grinding step), recovering oil bodies that had a protein content similar to the ones recovered in Tris-HCl (pH 8.5) (result not shown). Chen and Ono (2010) described a similar effect of alkaline pH on the purity of oil bodies recovered from soybean, where a higher removal of exogenous proteins was achieved alkalisating the COB emulsion to pH 11.

However, when the seed:grinding media ratios are compared (see Figure 3.7), the difference in protein content may not be related to pH values, as they were similar for both ratios. As discussed in Section 3.3.1.1, the use of lower ratios (e.g., 1:7) with any of the solution considered, results in oil bodies with a smaller diameter (Figure 3.6). By recovering smaller oil bodies, the total surface area increases, which may lead to an increase of the passively associated exogenous protein to the oil body surface (Figure 3.7).

The results shown in Figure 3.7 suggest that the pH value in which the seeds are ground does affect the carry-over of proteins, which is further discussed over the next sections. Considering the data shown here, the use of alkaline pH (9.5) and low seed:grinding media (1:7) allow the recovery of oil bodies with characteristics that are more in line with the scope of this thesis.

Among the solution considered, only NaHCO_3 was carried over for the optimization of oil body recovery for its satisfactory results and for being a food grade material.

3.3.2. Effect of buffer molarity on oil body physicochemical characteristics.

The effect of buffer molarity on droplet size and integrity, carry-over of exogenous proteins (seed proteins that are not integral to the oil body) and surface charge of crude and urea washed oil bodies were measured. Figure 3.8 resumes the unit operations that have been studied so far, with the extraction parameters that will be used for this section, and the ones that will be tested (highlighted in bold). Crude oil bodies were recovered soaking and grinding rapeseed seeds using NaHCO_3 (pH 9.5, 4 °C) at molarities of 0.01, 0.1 and 0.3 M, and at a ratio seed:grinding media of 1:7. Urea-WOB were recovered exposing the COB to urea (9 M), then rinsed in dH_2O as specified in method section.

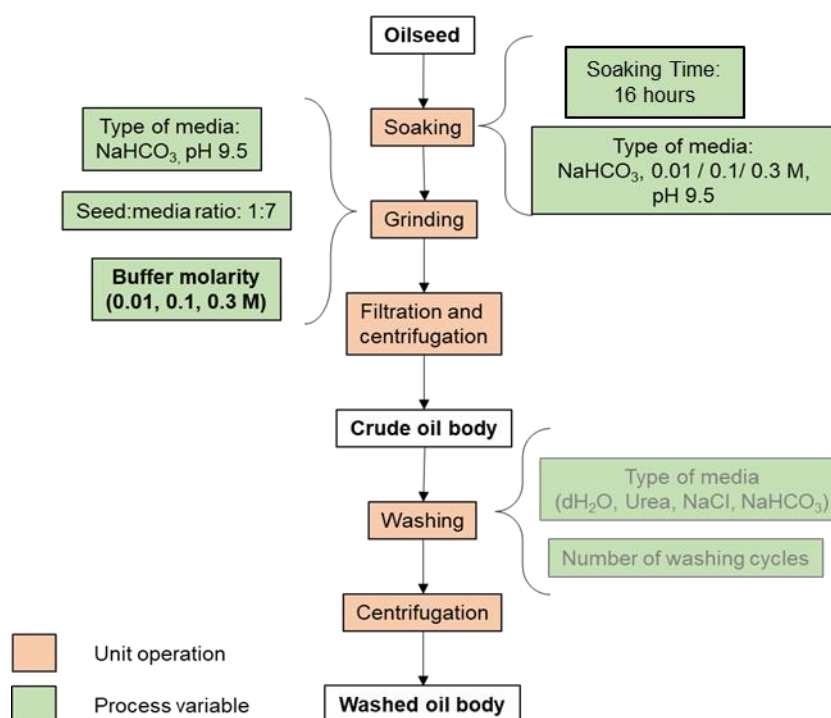


Figure 3.8: Variables considered for the recovery of oil body. The process variable considered (highlighted in bold) in this section is the molarity of NaHCO₃ solution (0.01, 0.1, 0.3 M). Variables in grey (over washing) are yet to be developed.

3.3.2.1. Particle size

Based on the findings shown in the previous section, it was hypothesised that keeping the seed:grinding media ratio constant (1:7), the maintenance of droplet integrity is a function of pH of the grinding solution. If this value is far from the isoelectric point (pI), the repulsion forces between oil bodies should increase, limiting the coalescence at the high shear stress experienced by the droplets during grinding. The ability of the buffers to hold the pH at the desired value against the solubilisation of seed material during grinding, was tested and correlated to the degree of oil body damage. For this reason, NaHCO₃ solution (pH 9.5) at increasing molarity (0.01, 0.1, 0.3 M) was used to recover crude oil bodies and the particle size was measured. Subsequently, all crude emulsions were exposed to urea (9 M) and the shift in size taken as a marker

of droplet integrity and compared with dH₂O. Higher molarities of the media were not considered as the fat layer after centrifugation became too runny and impossible to collect.

The buffering capacity of the solution is dependent on its molarity, reaching a pH value after grinding the seed of 8.8, 7.0 and 6.0 for 0.1 M and 0.01 M NaHCO₃ solution and dH₂O, respectively. At higher molarities (0.3 M), the buffering capacity was able to keep the pH value unchanged (9.5). Similarly to what shown previously (Section 3.3.1.1), crude emulsions recovered using NaHCO₃ showed comparable size distribution for all molarities considered (Figure 3.9). The crude material is characterised by the presence of a high concentration of exogenous proteins, which may facilitate the droplets to form aggregates as confirmed by light microscopy images (indicated by an arrow). On the other hand, the washing with urea removes the proteins that are associated to the oil bodies (Millichip et al., 1996), and the presence of droplets with diameters above 2.5 μm (Tzen et al., 1993) are only due to coalesced droplets. After exposure to urea, the quality of oil bodies (in terms of size) recovered from soaked seeds using NaHCO₃, and ground in 0.01 and 0.3 M NaHCO₃, was reduced compared with COB, but less than when dH₂O-prepared COB material was washed with urea. When seeds were ground in water, the pH value of the media is within the pI of oil bodies (5.5–6.5), neutralising the surface charge of the droplet (Tzen et al., 1993; White et al., 2008). As a result, the oil bodies will form aggregates, experiencing extended coalescence during the grinding process. Among the different molarities of sodium bicarbonate used, only the 0.1 M did not show any shift to particles of

higher diameter after urea exposure (Urea-WOB), and the recovered droplets maintained their singularity.

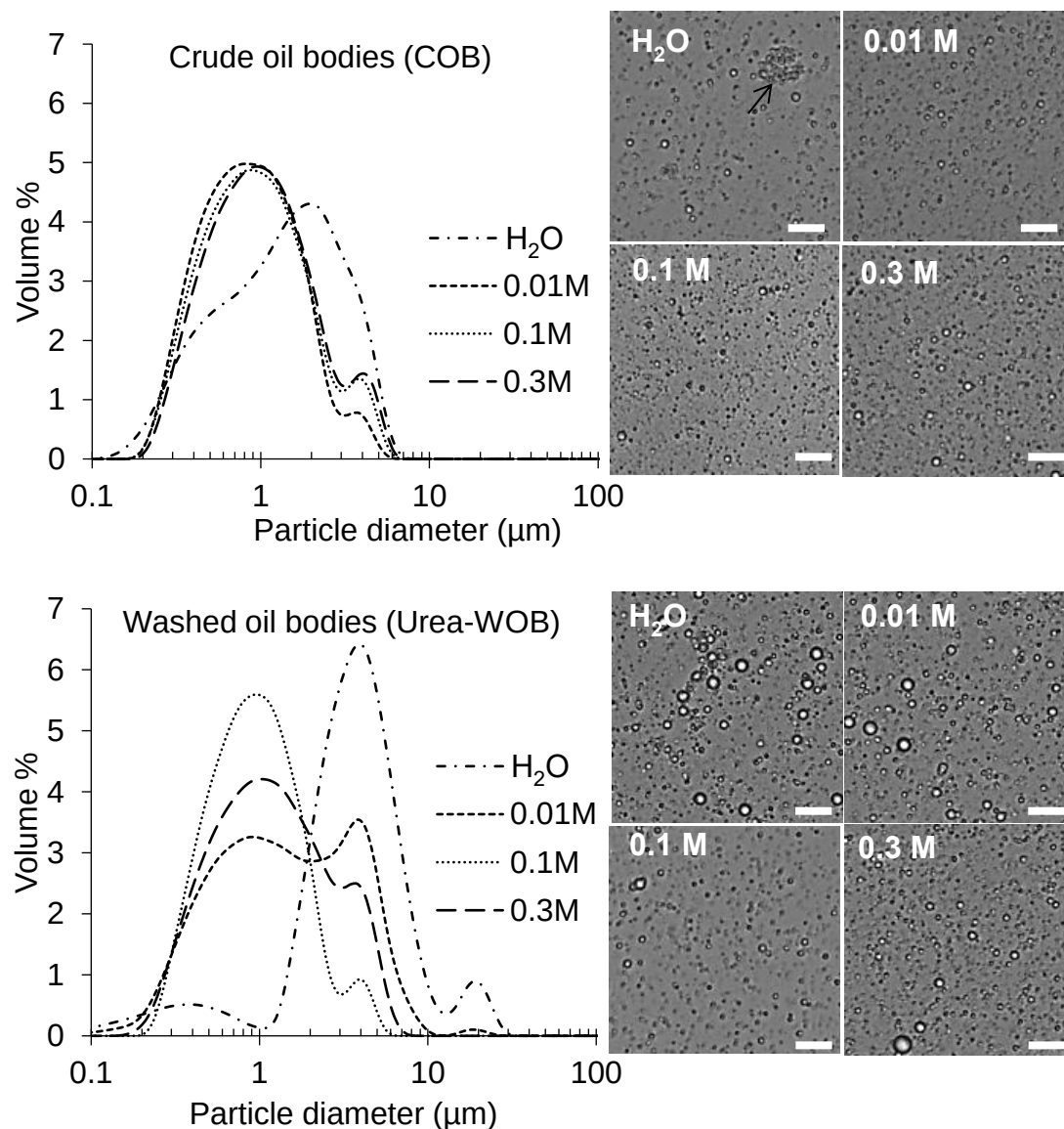


Figure 3.9: Effect of NaHCO_3 solution molarity on oil body size in crude and washed oil bodies emulsions. Oil bodies were recovered using NaHCO_3 over soaking and grinding step. The seeds were ground using different molarities of the buffer: 0.01, 0.1 and 0.3 M. dH_2O extracted oil bodies are reported as reference. Particle size and microscopy images were taken for crude and urea washed oil bodies. Arrow indicates aggregates. Scale bar represent 10 μm

According to these results, holding the pH value above 8.8, the repulsion forces of oil bodies may be strong enough to avoid the formation of aggregates during the grinding step, thus limiting coalescence. However, despite the pH being far from the pI using higher molarities of the sodium bicarbonate solution (0.3 M), the size distribution of urea washed oil bodies showed the presence of coalesced droplets. Oil bodies are organelles that retain their singularity in dry seed (Tzen and Huang, 1992), due to the presence of surface active proteins (oleosins) that are intrinsically imbedded on the surface and are usually retained during the recovery process. However, this work has proved that the exposure to high alkaline pH (>8.8) and high salt concentration (0.3 M), facilitated the coalescence of oil body droplets during grinding.

According to the oil body model from Huang (1992), oleosin is composed by three parts: a central hydrophobic domain inserted into the triacylglycerol matrix (TAGs), and an N- and C-terminus exposed to the continuous phase (Figure 3.10). The positively charged amino acids are bound at the interior to the negatively charged phospholipids by salt bridges (Huang, 1992), which, together with the hydrophobic interactions with TAGs, should make the desorption of oleosin difficult (Frandsen et al., 2001; Huang, 1996, 1992; Tzen et al., 1993, 1992). However, upon exposure to highly alkaline pHs, the positively charged residues of N- and C portions of oleosins were deprotonated, reducing the number of salt bridges between oleosins and phospholipids, which may compromise droplets integrity (Cao et al., 2015).

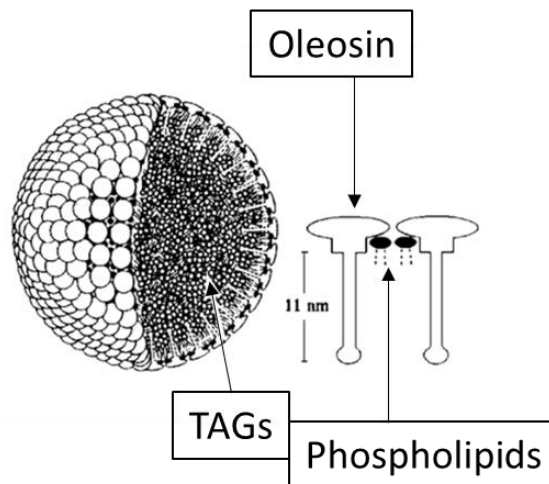


Figure 3.10: Proposed model of an oil body and of oleosin. Central hydrophobic domain of oleosin is inserted into the triacylglycerol (TAG) matrix, while the hydrophilic (N- and C-terminus) portions reside on phospholipid heads and face the continuous phase. Image adapted from Huang (Huang, 1996, 1992).

Using less concentrated solution (0.1 M), over 90% of oil bodies were within the range of intact droplets ($<2.5 \mu\text{m}$, Tzen et al., 1993), while the rest of the population at higher diameters can be attributed to limited coalescence. Those results suggest that the optimum salt concentration to extract intact oil bodies is the 0.1 M, as the buffering capacity of the medium is able to hold the pH value far above the pI, without destabilising the integral proteins.

3.3.2.2. Oil body composition

In this set of experiments, the effect of solution molarity on composition of oil bodies in terms of oil and protein content, was studied. Crude oil bodies were recovered soaking and grinding seeds at increasing molarities (0.01, 0.1, 0.3 M) of NaHCO_3 solution (pH 9.5, 4 °C). As a reference, a set of oil body samples were recovered using dH_2O in both soaking and grinding step. Crude

and urea-WOB were analysed for protein and oil content as detailed in method section and shown in Figure 3.11.

Considering that the exposure to urea leads to the removal of strongly bonded proteins from the oil body surface, leaving only integral ones (e.g., oleosins) (Millichip et al., 1996), in this section urea WOBs are considered to be the purest preparation. Among the condition studied (Figure 3.11), the urea-washed oil body cream showed the highest content of oil ($94.30 \pm 2.32\%$) and the lowest protein content ($2.60 \pm 0.05\%$), which should correspond only to the total amount of integral proteins for that sample (Furse et al., 2013). It was suggested by Tzen et al. (1993) that the composition of rapeseed oil bodies was 95% of total lipids, and up to 3.46% of proteins, depending on the oilseed crop tested. The slight lower protein content than the one presented by Tzen et al. (1993) may be due to the harsh conditions of the washing method used in this thesis.

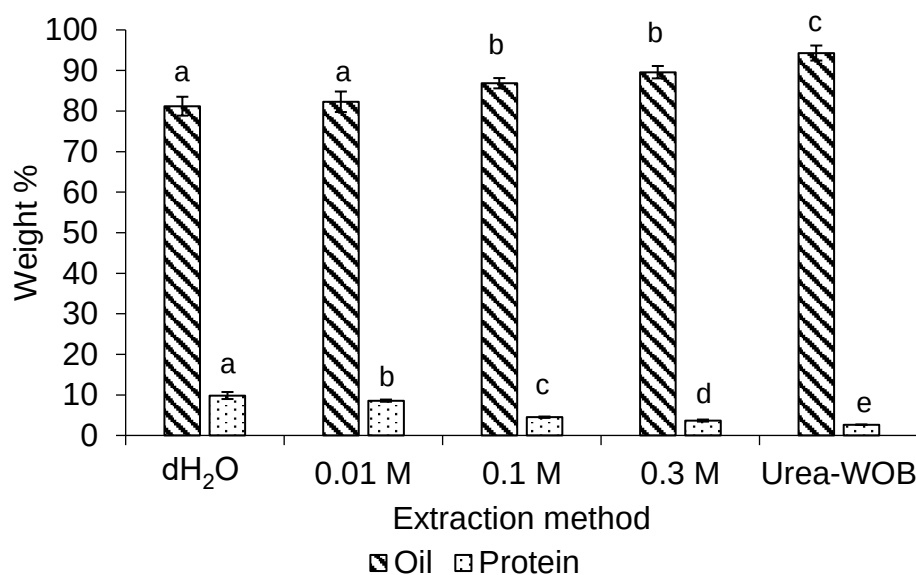


Figure 3.11: Oil and protein content of COB (compared with Urea-WOB) expressed as percentage on a dry weight basis (*dwb). Crude oil bodies were recovered using NaHCO₃ (pH 9.5) in soaking and grinding step. During these steps, the solution had a molarity of either 0.01, 0.1 or 0.3 M. As a reference, oil bodies recovered soaking and grinding seeds in dH₂O are reported. The cream of COB and urea-WOB was analysed for oil and protein content.

The use of alkaline pHs influenced the oil content in recovered creams. On a dry weight basis, it was found that the oil content was similar using dH₂O and 0.01 M NaHCO₃ (around 82%) but increased using 0.1 and 0.3 M reaching about 88% (Figure 3.11).

The COB recovered in dH₂O had the highest fraction of total protein ($9.8 \pm 0.74\%$), which decreased to $8.5 \pm 0.27\%$, $4.5 \pm 0.17\%$ and $3.8 \pm 0.25\%$ when using 0.01, 0.1 and 0.3 M sodium bicarbonate, respectively. The protein extracts were further analysed by SDS page under reducing conditions to analyse the quantity in exogenous protein as affected by extraction media (Figure 3.12). Considering that each sample had different protein content (Figure 3.11), the amount of oleosin in each well had to be similar, so to be

able to make a comparison of the exogenous proteins on SDS-PAGE. This was possible knowing the oil content in each oil body extract, and assuming the ratio between oil and oleosin in oil bodies to be constant. This was confirmed considering the findings of Hu et al. (2009), where a direct correlation between the accumulation of oleosin, the *oleosin1* gene transcript, and oil content in *Brassica napus* cultivars is demonstrated. In the same work, it was also shown that the size of oil bodies in low oil content cultivars was much larger than that previously described by Tzen et al. (1993). Moreover, Siloto et al. (2006) reported that the suppression of oleosin gene expression in *Arabidopsis* affected the formation of the storage organelles and the accumulation of lipids. Clearly, oleosin plays an important role in oil body formation and lipid accumulation. In this thesis, the proteins of urea washed oil bodies are mainly composed by oleosin isomers (Figure 3.12, indicated by arrows), and it was found a ratio oil/protein of 32.88 mg oil/mg oleosin. Considering the composition in oil and protein for the other samples tested, in this experiment it was possible to dilute the protein extracts and standardise the amount of oleosin (based on the ratio in urea-WOB) in each well of the gel. By this way, the amount of exogenous seed proteins (Figure 3.12) can be compared. Lane 2, corresponding to the protein extract of oil bodies recovered in dH₂O, showed several bands which represent the presence of exogenous proteins (at a higher and lower molecular weight than the oleosin isoforms, 20 and 18 kDa) carried over in the extract.

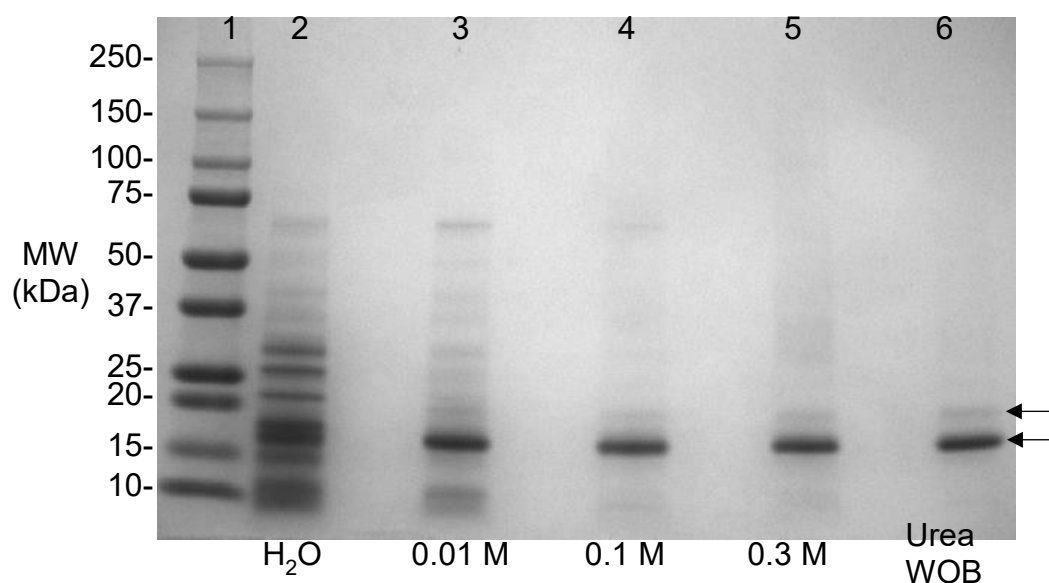


Figure 3.12: SDS-PAGE in reducing conditions of protein extracts from COB and urea-WOB. Oil bodies were recovered soaking and grinding rapeseed seeds in either NaHCO_3 (pH 9.5) at increasing molarities (0.01, 0.1, 0.3 M), or dH_2O over soaking and grinding step. Urea washed oil bodies were produced exposing COB (recovered with 0.1 M NaHCO_3) to urea (9 M). Arrows indicate oleosin isomers.

During seed maturation, proteins are deposited in membrane-bound compartments (Protein Bodies), generated from the protein storage vacuoles (Krishnan and Coe, 2001). In rapeseed, two classes of seed storage proteins are predominant: 11S globulin (legumin-type) cruciferin and napin-type albumins (2S or napins), which compose between 85 to 90 % of the total proteins (Campbell et al., 2016). The solubilisation of those proteins from rapeseed meal is believed to be pH dependent, with cruciferin polypeptides being solubilised between pH values 6.5-10, and napin polypeptides at pH 1.5 and 4.5 (Perera et al., 2016). Wanasundara and McIntosh (2013) described a similar behaviour for the solubilisation of Brassicaceae oilseed proteins from defatted rapeseed meals, which was its highest degree (more than 90% of the total proteins) at $\text{pH} > 11$, and lowest at pH between 3 and 5. The close pH

value to this range using dH₂O (pH 6.0) during oil body recovery, may explain the low solubilisation of exogenous proteins from the oil body surface into the grinding media. Therefore, a higher concentration of these proteins is found in the extract (Figures 3.11 and 3.12, lane 2). By increasing the molarity of the NaHCO₃ solution (and so the pH), the majority of bands at high and low molecular weight lost intensity (Figure 3.12, lanes 3, 4, 5), suggesting that they had been removed from the oil body emulsion. Lane 6 confirms the presence of only integral proteins in urea-washed oil bodies (Furse et al., 2013).

Similar results were reported by Chen and Ono (2010) where, by using neutral pH extraction, the surface of oil bodies from soybean was characterised by the presence of several proteins and enzymes like lipoxygenase, β -amylase, γ -conglycinin, and some not well known proteins; while at alkaline values (up to pH 11) a washing effect (removal of exogenous proteins) occurred. In addition to this, the increase in salt concentration, causes an increase in protein solubility (Arakawa and Timasheff, 1985). pH and salt concentration in our system may be working in synergy to remove exogenous proteins without bringing the system to high pH values.

3.3.2.3. *Surface charge of oil bodies*

The ζ -potential is a measure of the surface potential of a colloidal system, measured when exposed to an oscillating voltage across a measurement cell. When a charged particle is in suspension, the charged ions present in the solution will surround the particle to keep the neutrality, which depends on the charged components present at the surface of the particle. In this study, ζ -potential was used to predict how the protein carry-over affects the surface charge at different pH values. The crude oil bodies were recovered using deionised water or NaHCO_3 at molarities of 0.01, 0.1 and 0.3 over soaking and grinding. An aliquot of the cream was suspended in ultra-pure water at a final concentration of 0.008 % (lipid weight basis). As a reference for the purest preparation (presenting only oleosin isomers at the surface), crude oil bodies recovered in 0.1 M NaHCO_3 were exposed to urea (9 M) and compared to the crude emulsions. Once the pH of the emulsion was stabilised at a value between 3 and 10 using either 0.1 M NaOH or 0.1 M HCl, the oil body suspension was loaded into the measuring chamber and the zeta potential measured. As shown in Figure 3.13, urea WOB showed to have a ζ -potential of approximately +55 mV at pH 3, which decreased upon alkalisation of the solution, arriving at -60 mV at pH 10, while the surface zero charge was achieved at pH 6.5.

This behaviour is typical for protein covered oil droplets (Guzey and McClements, 2006), confirming that the oleosin remained on the surface of the oil bodies despite the harsh washing with urea. The charged residues of the oleosin are organised in a “non-random” pattern, where most of the positively charged residues face the interior towards the negatively charged

phospholipids, while the majority of the negatively charged oleosin residues are exposed to the exterior (Tzen et al., 1992; Tzen and Huang, 1992). Changing the pH of the system from alkaline towards acidic values, causes protonation of the negatively charged oleosin residues until the net charge equals to zero (Tzen et al., 1992).

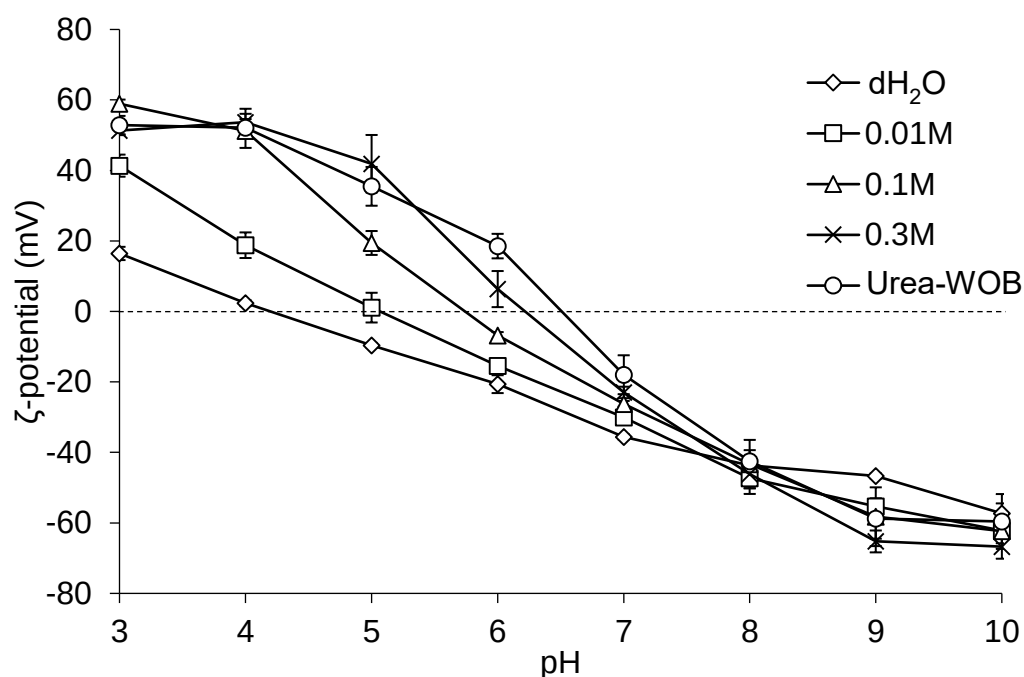


Figure 3.13: Surface charge of crude and urea washed oil body emulsions between pH 3 and 10. Oil bodies were recovered soaking and grinding rapeseed seeds using either dH₂O, or NaHCO₃ at increasing molarities (0.01, 0.1, 0.3 M). Urea washed oil bodies were produced exposing 0.1 M NaHCO₃ COB to urea (9 M). Crude oil body creams were re-suspended in water and the pH adjusted between 3 and 10 for ζ -potential analysis.

The surface charge of the crude oil bodies also moved from positive to negative as the pH increased from 3 to 10, but the magnitude of the charge and the pI of the emulsion was affected by the recovery solution (Figure 3.13). The charge was negative (<-40 mV) with a similar zeta potential for all emulsions at pH from 8 to 10, but differences were observed at pH's 3 to 8,

probably due to the different protein composition at the interface of the extracted oil bodies (Figure 3.11 and 3.12). Increasing the purity of the crude extracts in the order $\text{dH}_2\text{O} < 0.01 \text{ M} < 0.1 \text{ M} < 0.3 \text{ M}$ (as detailed in Section 3.3.2.2), the surface charge showed closer trends to the urea WOB. Among the samples tested, only the crude oil bodies recovered in 0.3 M NaHCO_3 had a similar surface charge to the washed sample, suggesting that the two emulsions had similar interfacial composition. A similar correlation was described by Payne et al. (2014), where the differences in ζ -potential were also attributed to proteins that become associated with the surface of the oil bodies, also altering the pI of the organelle *ex-vivo*. In fact, this is in line with the findings in Figure 3.13, where the pI of crude oil bodies depended on the extraction medium used, ranging from 4.2 using deionised water to 5.0, 5.7 and 6.2 for crude emulsions extracted with, respectively, NaHCO_3 at molarities of 0.01, 0.1 and 0.3. Most of the results in literature show that oil bodies have a pI between 4.5-5.5 (Chen et al., 2012; Iwanaga et al., 2007; Nikiforidis and Kiosseoglou, 2009; Zaaboul et al., 2018). The discrepancy in the results may not only be species and variety dependent, but also due to the purification protocol used.

One of the factors determining the pI of oil bodies is the nature of oleosin residues exposed to the exterior environment (Tzen et al., 1992), but it is unlikely that proteins at the interface are the sole factor contributing to the overall charge of the droplet. The presence of negatively charged surface active molecules, such as the free fatty acids generated from lipolytic activity, may negate the surface, thus requiring a greater degree of protonation to neutralise the total charge. Moreover, the presence of endogenous lipolytic

enzymes (Hoppe and Theimer, 1997; Lin and Huang, 1983; May et al., 1998; Theimer and Rosnitschek, 1978), especially in less purified extracts, can act on free oil, liberating fatty acids which will further negate the surface.

Lastly, the difference in sizes in crude oil bodies (Figure 3.9, top panel) recovered in dH₂O or NaHCO₃ (0.01 M, 0.1 M, 0.3 M), may have affected the ζ -potential values in Figure 3.13. In ζ -potential measurements, when an electrical field is applied across an emulsion, the electrical pulling force will cause droplets to accelerate until they reach a constant velocity, which is balanced by the drag (Stokes) force from the surrounding liquid (McClements, 2004). The ζ -potential is a measurement of the movement of these droplets related to their electrical charge, which depends on several factors such as the viscosity of the medium, strength of the applied electric field, thickness of the Debye layer, charge and size (Hunter, 2001; McClements, 2004). It could be hypothesised that the increase in volume of oil bodies may have affected the velocity of the particle, thus decreasing its electrophoretic mobility.

3.3.3. Physical stability of oil body emulsions during storage

It was hypothesised that a reduction in protein content extracting the oil bodies at increasing molarities of NaHCO_3 solution, would also correspond to the removal of endogenous enzymes from the extract, and so to an extension of the emulsion shelf life. In this set of experiments, crude oil body emulsions (10% lipid weight basis) with a different content in protein (recovered either in dH_2O , or NaHCO_3 , as discussed in Section 3.3.2.2), were kept at ambient temperature (20 °C) measuring the particle size during a period of 28 days using light scattering and assessing droplet structure by microscopy (Figure 3.14, top panel). To study the influence of exogenous proteins during storage, a series of urea washed samples were recovered from the corresponding crude oil bodies and considered for comparison (Figure 3.14, bottom panel). Moreover, NaN_3 (0.02 mM) was added in all preparations to avoid microbial growth (Lichstein and Soule, 1944). By this way, only the active endogenous enzymes in crude emulsions would be responsible for the loss in droplet structure.

During the time course of the study, the crude oil bodies recovered in deionised water were the least stable, with extended coalescence and clear structure disruption after 4 weeks (indicated by arrows). Some improvements in the physical stability of crude oil bodies on storage were achieved by grinding seed in 0.01 M NaHCO_3 , and even more, using higher molarities. However, despite no difference was found in the physical stability of COB recovered using 0.1 or 0.3 M NaHCO_3 , some oil bodies with altered morphology (Figure 3.14, indicated by an arrow) were found using 0.1 M. The highest stability was achieved by removing most of the exogenous proteins

using urea. Those washed preparations showed only a slight size increase during the storage time, which may be due to limited coalescence. The sample recovered using dH₂O had bigger mean diameter at the start of the trial due to the presence of an high number of damaged droplets during grinding (as discussed in Section 3.3.2.1), but the droplets retained their singularity during storage.

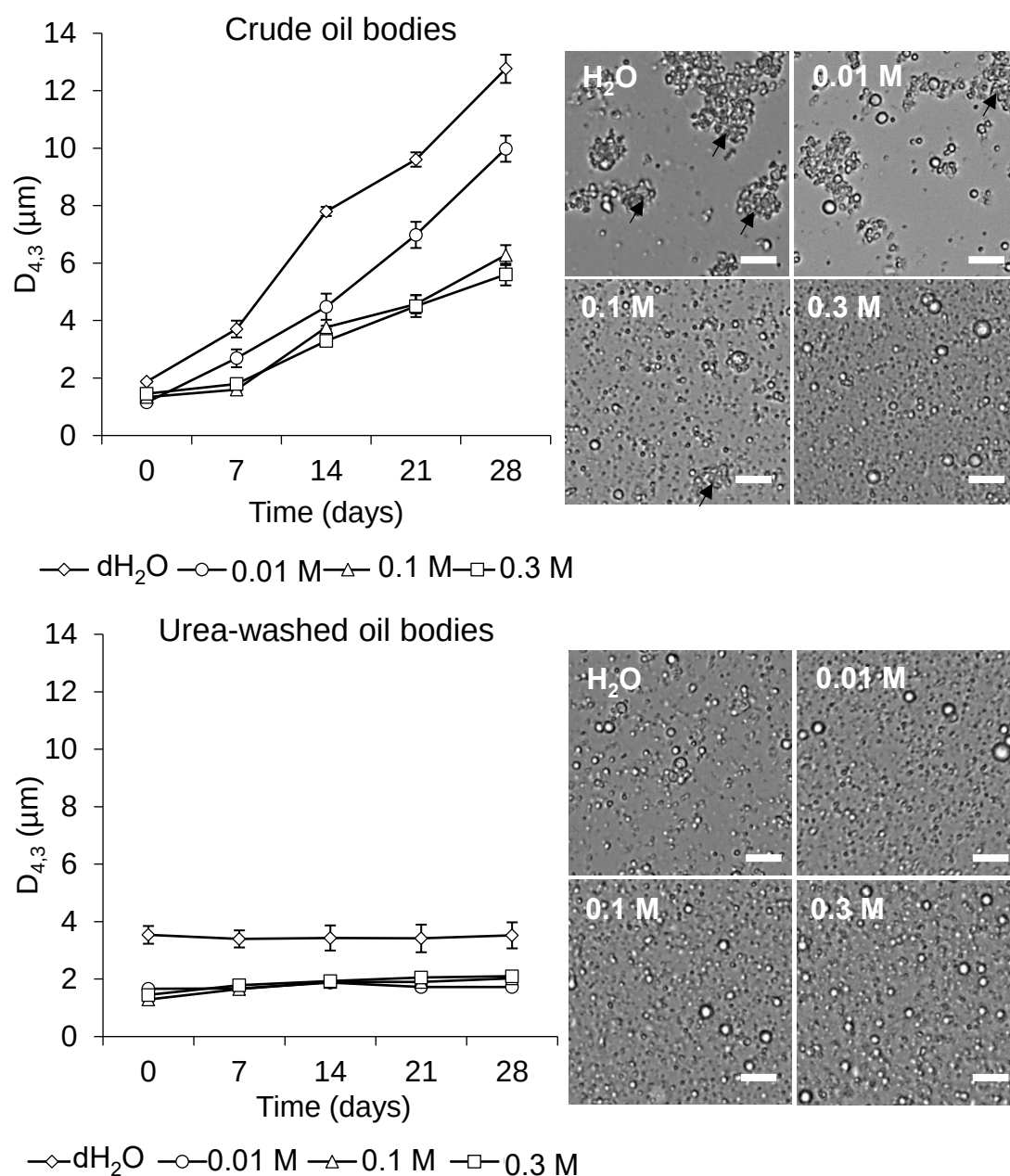


Figure 3.14: $D_{4,3}$ of COB and urea WOB emulsions over 28 days at 20 °C. Crude oil bodies (top panels) were recovered soaking and grinding rapeseed seeds in either dH₂O or NaHCO₃ at increasing molarities (0.01, 0.1, 0.3 M). Each COB sample was then exposed to urea (9 M) and then rinsed in dH₂O to produce urea WOBs (bottom panels). Micrographs represent droplets at the end of storage trial (28 days, 20 °C). Micrographs at day 0 of each sample can be seen in Figure 3.9. Scale bar represent 10 μm.

As described in literature, different enzymes such as lipase (Lin et al., 1983, 1982; Lin and Huang, 1983) and protease (Sadeghipour and Bhatla, 2002), are localised in the proximity of the oil body during the germination process. The size increase in crude emulsions presented in this section may be due to the action of these enzymes. Moreover, the hydrolysis of the surface material of oil bodies releases the oil, with the subsequent formation of structures with altered morphology (probably majorly composed by protein and phospholipids). This is supported by a clear layer of oil formed on top of the emulsions during storage (results not shown) and by the absence of such structures in urea washed samples. The results shown in this thesis suggest, not surprisingly, that higher protein carry-over with oil bodies corresponds to higher enzyme activity, which is present in the extract despite using higher concentrations of the NaHCO_3 solution. Therefore, further processes in purifying the oil body cream are needed, which is discussed in the next section.

3.4. Effect of washing crude emulsions of oil bodies

From the previous section we can see that crude oil body material contains exogenous proteins, including evidence of active enzymes. This activity appears to be reduced, but not completely lost, by exposing oil bodies to an alkaline pH. The aim of this section is to study the effect of washing protocols on protein content, surface charge and droplet size, with the purpose to reach a comparable composition to the urea washed preparations. The variables considered were focusing on the number of washing cycles, and type of washing media (Figure 3.15). Based on the characteristics of the recovered crude emulsions on droplet integrity, in this study only the one recovered in 0.1 M NaHCO₃ was considered.

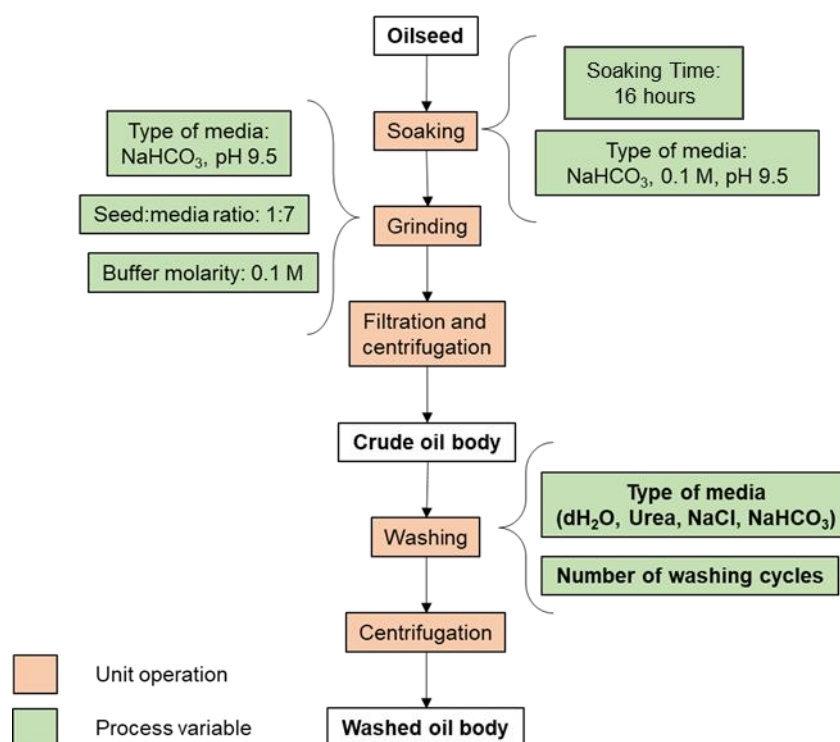


Figure 3.15: Variables considered for the recovery of oil body. The process variables considered (highlighted in bold) in this section are the type of solution used during washing (dH₂O, NaCl, NaHCO₃) and number of cycles.

In literature, different solutions are used for washing the crude material, with the most common being salt and alkali wash (Chen and Ono, 2010; Frandsen et al., 2001; Tzen et al., 1993; Zaaboul et al., 2018). The crude material was recovered soaking and grinding rapeseed seeds in 0.1 M NaHCO₃ at a ratio seed:grinding media of 1:7 (Figure 3.15). To test which washing solution would be the most suitable, COBs were re-suspended in either dH₂O (used as reference), 0.1 M NaCl, or 0.1 M NaHCO₃. After centrifugation and a further rinse in dH₂O to reduce the amount of chemicals in the final product, the washed oil body preparation was analysed.

Upon the washing using 0.1 M NaCl, the oil bodies showed the tendency to form aggregates (Figure 3.16, micrograph A indicated by arrows), which

may be due to the effect of NaCl, reducing the repulsive interactions between droplets due to electrostatic screening (Hunter, 2001; Iwanaga et al., 2007). As a consequence, coalescence was enhanced, as it is shown by the particle size distribution (Figure 3.16), with the presence of higher population of droplets at 4 μm .

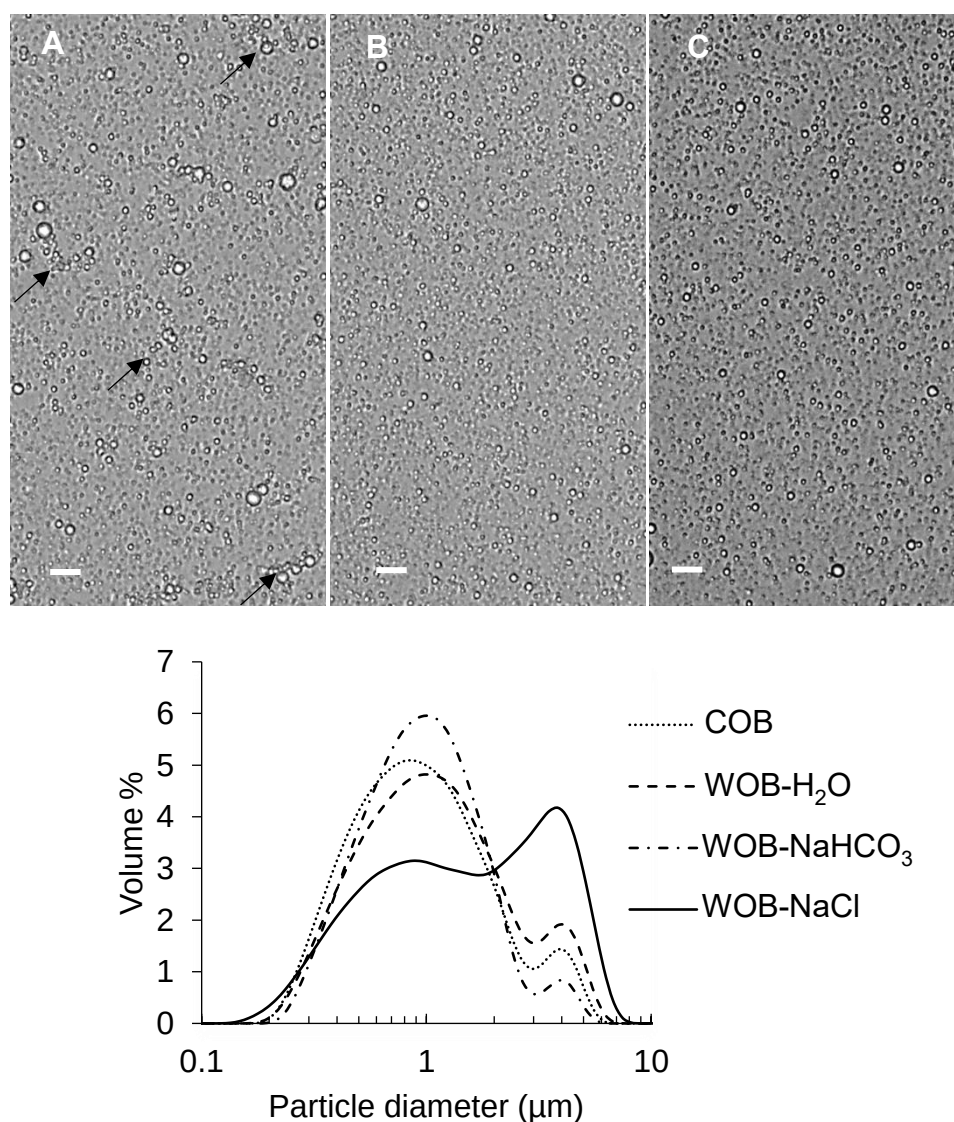


Figure 3.16: Effect of type of media during washing step on oil body integrity. Microscopy images (top panel) of washed oil bodies using NaCl (A), dH₂O (B) and NaHCO₃ (C). Arrows indicate aggregates. Bottom panel: Particle size distribution of washed oil bodies. COBs are shown as reference. Aggregates are indicated by arrows. Scale bar represent 10 μm .

Micrographs of crude emulsions washed with dH₂O or NaHCO₃, did not reveal significant presence of aggregates. Despite the similarity of both washed material in the particle distribution to the crude emulsion, the one washed in NaHCO₃ showed an increase in the population of droplets around 1 μ m, and a decrease in the second peak at around 4 μ m.

In this work, the washing protocol was set to be composed by only two cycles of suspension-centrifugation of the cream, in order to allow comparison with the methodology using urea which was adapted from Tzen et al. (1997). To evaluate which combinations of aqueous solution would remove more exogenous proteins, compositional analyses were performed on oil bodies after being washed using one of the following procedure: one wash in dH₂O (W) or NaHCO₃ (B), two wash cycles in dH₂O (WW), two wash cycles in 0.1 M NaHCO₃ (BB), one in NaHCO₃ and one in dH₂O (BW). The purity of oil bodies is affected by the type of media and number of washes (Figure 3.17). The result suggests that upon washing, more proteins are removed from the oil body cream. This purification process was solvent specific on the first wash cycle, where exogenous proteins are removed to a higher extend using 0.1 M NaHCO₃ (B) compared to dH₂O (W), reaching a protein content of 3.09 ± 0.1 and 3.9 ± 0.13 %, respectively. Considering the total protein content in the product washed using only dH₂O (WW), it was found that proteins remained associated to the oil body, despite the sequential washing cycles. The purest preparations were observed only including NaHCO₃ in the protocol (BB), but no differences were recorded in the use of water over the second wash (BW).

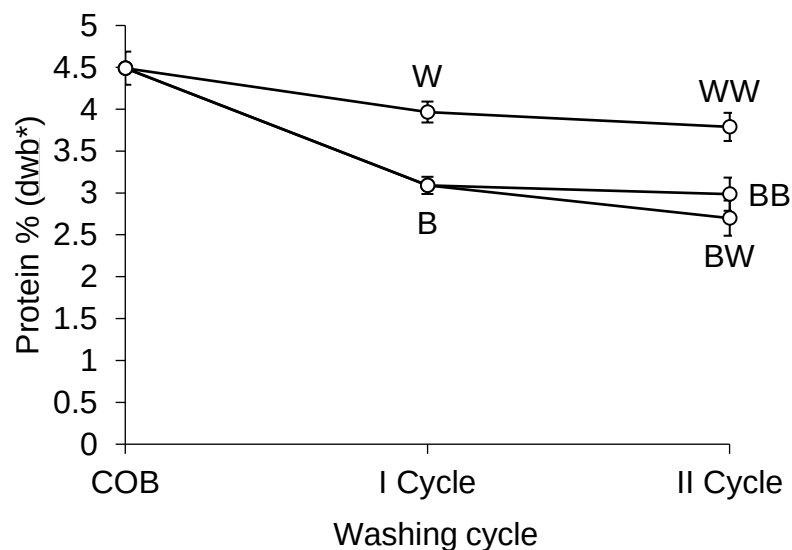


Figure 3.17: Effect number of washing cycles and type of media on the protein content (*dw b, dry weight basis) of recovered oil bodies. W/ WW: one or two washes in dH₂O, respectively; B/ BB: one or two washed in NaHCO₃, respectively; BW: one wash in NaHCO₃ and one in dH₂O.

Considering that if sodium bicarbonate was to be used in an industrial process, a final water wash would be ideal to remove the salt and meet recommended levels of sodium in food and ingredients, making the BW procedure the recommended one. In this case, the protein content was only $2.69 \pm 0.21\%$, which is comparable to the urea washed preparations (as discussed in Section 3.3.2.2).

Analysis of this washed material revealed that the further removal of exogenous proteins influenced both surface charge and storage stability (28 days, 20 °C). The effect of washing the crude emulsion (taken as reference from Section 3.4) using 0.1 M NaHCO₃ and then rinsed in dH₂O is shown in Figure 3.18. Due to the increased purity, the recovered washed oil bodies displayed a ζ -potential trend similar ($P < 0.05$) to the urea washed preparation (Figure 3.18 A), increasing the pI from 5.7 to 6.5. Compared to the crude

emulsion, there is also a clear improvement on the stability of oil bodies during storage. However, despite the similarity in protein content between urea and NaHCO_3 washed material, some destabilisation occurred during storage (Figure 3.18 B), which suggests the presence of enzymatic activity during the storage trial.

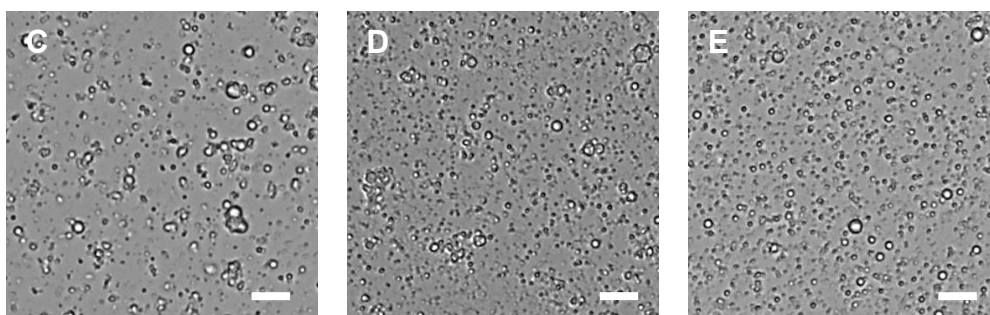
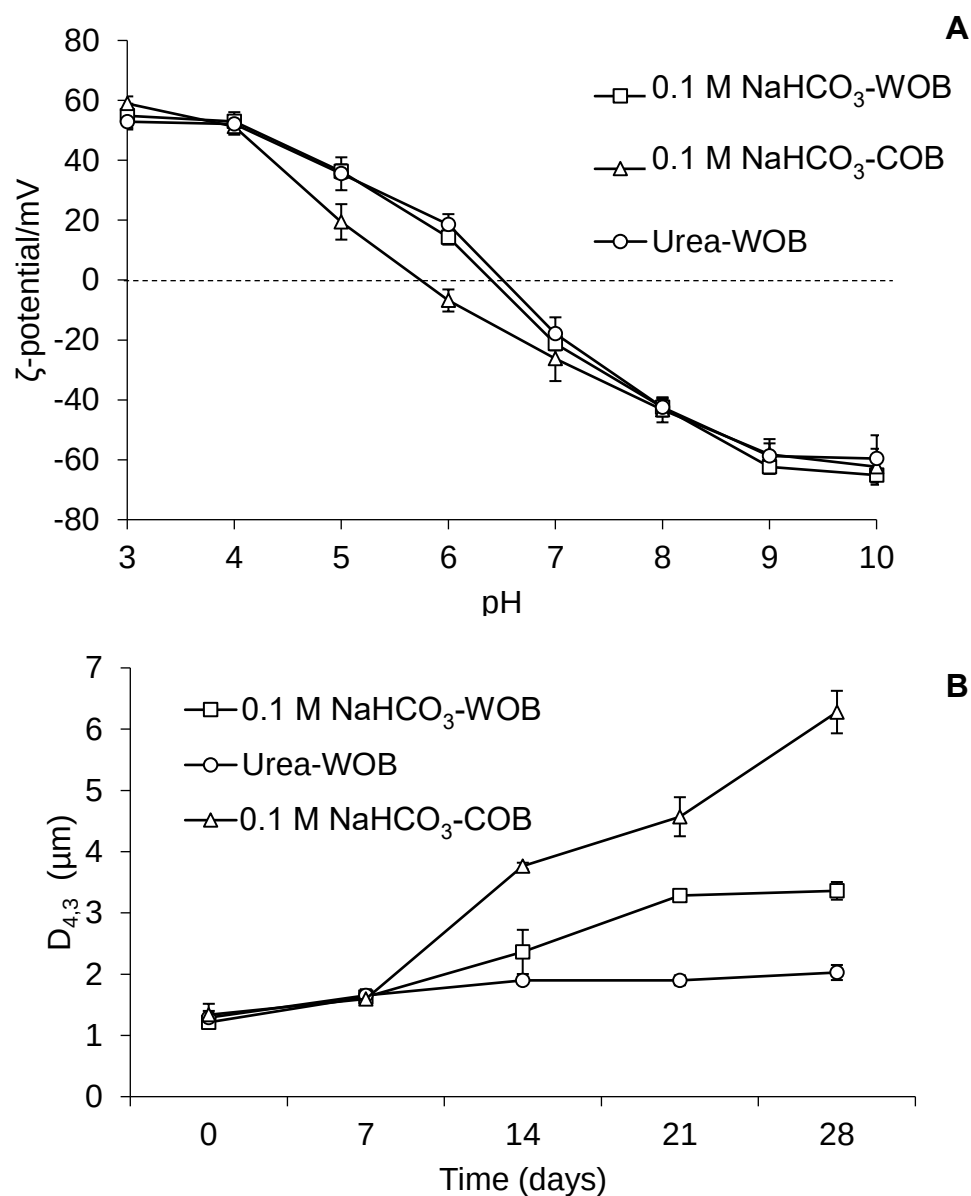


Figure 3.18: Effect of washing crude oil bodies using NaHCO₃ on surface charge and storage stability. Panel A: Surface charge of crude and washed emulsions recovered soaking and grinding rapeseed seeds in NaHCO₃ (0.1 M), then washed in the same media; Panel B: Mean volume diameter trend during storage at 20 °C. Microscopy images (C-D-E) at 28 days of crude, washed and urea washed emulsions, respectively. Images at day 0 can be seen in Figure 3.9. Scale bar represent 10 μm.

3.5. Summary of results

The effect of process parameters on the quality of oil bodies recovered from oilseed rape seed was determined. The soaking of seeds increased their internal moisture, leading to a softening of the tissue thus reducing the physical stress experienced by the oil bodies.

The ratio of seed:grinding buffer during grinding, as well as the pH and molarity of the buffer were all factors contributing to the droplet integrity and purity. This study shows that the use of an alkaline media overcomes the buffering capacity of the seed material in suspension. Using a low solid-to-media ratio (1:7 w/w), and a concentration of 0.1 M of NaHCO₃ solution, the system appears to maintain the integrity of oil body droplets by creating enough repulsion forces and limit coalescence.

The differences in the purity of the oil body preparations is a result of the ability of the alkaline medium to interfere with the bonds between exogenous proteins and the surface of oil bodies. However, the exposure of oil bodies to high pH values and salt concentration, may promote coalescence due to deprotonation of N- and C-terminus of oleosin, decreasing the number of salt bridges with the phospholipid. The increase in oil body purity had a significant effect on surface charge and storage stability, mainly due to the removal of exogenous seed proteins and enzymes.

The protein content in washed preparation is dependent on the type of media and on the number of washing cycles. The 0.1 M NaHCO₃ washed material had similar composition and surface potential to the urea washed

preparations. However, a slight change in oil body size was observed during storage, which was attributed to the catalytic activity of endogenous enzymes.

CHAPTER 4.

EFFECT OF THERMAL TREATMENT ON THE QUALITY OF OIL BODY EMULSIONS

4.1. Background

During germination, triacyclglycerols (TAGs) are hydrolysed to fatty acids (FA) and glycerol by lipases (Huang, 1987; Huang and Wang, 1992). Some researchers suggest that the effective initiation of TAG hydrolysis from oil bodies may require an initial degradation of oleosins (Matsui et al., 1999; Sadeghipour and Bhatla, 2002). However, others believe that lipases can have access to the substrate without hydrolysis of the protein coat (Beisson et al., 2001). Moreover, in this process, the catalytic activity of phospholipases and lipoxygenases may play a role (Gupta and Bhatla, 2007; Matsui et al., 1999; May et al., 1998). In Chapter 3 it was demonstrated that, when preparing oil bodies from oilseed rape, the use of 0.1 M NaHCO₃ over grinding and washing removes exogenous proteins to a similar extent as 9 M urea. What was not established was the magnitude of the passively-associated enzymes removed during washing, so to predict the enzyme-induced deterioration of oil bodies during storage. If the enzyme carry-over could be reduced during the initial process of oil body recovery, then this will lower the thermal treatment required to stabilise the cream.

At the moment, there is a gap in literature about which is the magnitude of enzymatic activity in purified oil body extract, and which are the minimal parameters required to stabilise the recovered material from the present enzymes. The aim of this chapter is to measure the lipolytic activity as a new approach for the assessment of oil body purity from endogenous enzymes, and as a marker for thermal process efficiency. Among the enzymes listed

above, the lipolytic ones were chosen for (i) the proven physical association in recovered oil bodies from oilseed rape seeds, (ii) their resistance at high temperatures, (iii) the wide research available in literature.

In the first section of this chapter, the correlation between protein content and enzymatic activity was tested, looking at which are the driving factors (e.g., type of media or washing steps) that may affect the presence of enzymes in the final product. Afterwards, the remaining activity was monitored over a thermal treatment to determine which would be the minimal conditions to assure enzymatic stability (>90% inactivation). Finally, the effect of thermal treatment on the droplet integrity and antioxidants degradation was assessed.

4.2. Lipolytic activity is affected by the recovery protocol.

Recent works have focused on the physicochemical characterization of oil bodies from oleaginous seeds as affected by extraction media, demonstrating the alkaline solutions (pH>8) to be particularly effective against the carry-over of exogenous seed material (Chen and Ono, 2010; De Chirico et al., 2018; Zaaboul et al., 2018; Zhao et al., 2016). However, despite the efforts made, some enzymes were still found in purified oil body creams (Katavic et al., 2006; Zhao et al., 2016). Moreover, as previously discussed in Section 3.4, despite oil bodies recovered at pH 9.5 (using a NaHCO₃ solution) had a similar protein content to the urea washed preparations ($P<0.05$), loss of droplet structure was seen over storage, probably due to the presence of hydrolytic enzymes. These findings suggest that the quantification of proteins is not a reliable predictor of enzyme removal or of the stability of oil bodies over a period of storage. Therefore, a different approach should be applied.

Among the enzymes that may be involved in the degradation of oil bodies over germination, lipases are one of the most characterised in literature (Allen and Tao, 2007; Beisson et al., 2001; Eastmond, 2006; A Hoppe and Theimer, 1997; Lin et al., 1982; Theimer and Rosnitschek, 1978). In rapeseed, they appear to have some kind of physical association with the oil body, presumably through binding with the integral proteins (Hills and Murphy, 1988; Lin et al., 1983; Rosnitschek and Theimer, 1980). Bearing this in mind, it was hypothesised that the lipolytic activity in the recovered material, instead of the total protein content, could be used as a good indicator of the purity of the

extracted oil bodies and physical storage stability. For this reason, the aim of this set of experiments was to establish the correlation between protein content of oil body preparations and lipolytic activity. Among the methodologies for the lipolytic activity assay, the colorimetric method using p-nitrophenyl laurate (p-NPL) measuring the amount of liberated yellow product (p-nitrophenol, p-NP) from the hydrolysis of the substrate was adopted (Ruiz et al., 2004; Tan et al., 2011). The utilization of titrimetric method using sunflower oil as substrate (Theimer and Rosnitschek, 1978), was considered as well. However, the spectrophotometry revealed to be more sensitive to low level of activity, and easier to use with large numbers of samples.

Oil bodies with low amounts of exogenous proteins were recovered soaking (16 hours) and grinding rapeseed seeds in NaHCO_3 (0.1 M, pH 9.5, 4°C), then washed in the same solution and rinsed in deionised water (dH_2O , 4°C), measuring the lipolytic activity in each step of the extraction (Table 4.1). Moreover, a set of oil bodies with high level of exogenous proteins were recovered using only dH_2O (as discussed in Section 3.3). Lipolytic activity results for a range of oil body preparations, expressed on a dry weight basis, are shown in Table 4.1.

Table 4.1: Effect of oil body recovery and washing process on lipolytic activity.

		Lipolytic activity (U/g dwb)			
	Dry Seeds	Soaked Seeds (16 hrs)	COB	WOB I	WOB II
dH ₂ O	13.8±1.0 ^a	19.1±1.2 ^{b,x}	17.4±3.1 ^{b,x}	12.2±1.3 ^{cd,x}	10.1±0.4 ^{d,x}
NaHCO ₃		19.0±1.5 ^{b,x}	3.9±0.2 ^{c,y}	2.7±0.1 ^{d,y}	2.1±0.2 ^{e,y}

The activity was assayed from protein extracts of recovered oil bodies or dry/soaked seeds. Low protein content oil bodies were recovered soaking and grinding seeds in NaHCO₃ (COB), then washed in the same media (WOB I) and rinsed in dH₂O (WOB II). High protein content samples were recovered using only dH₂O. Different (a, b, c, d, e) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the effect of COB recovery and washing was evaluated. Different (x,y) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the effect of recovery/ washing solution (NaHCO₃ vs dH₂O) was evaluated.

Surprisingly, some lipase activity was already present in mature seeds, which showed an increase of about 38% after soaking (16 hours) using either dH₂O or NaHCO₃ (0.1 M, pH 9.5). However, the type of media did not have any influence ($P < 0.05$). After grinding of seeds in the corresponding grinding solution, the activity in the crude material (COB) showed a dependency on the type of solution used. When soaked seeds were ground in dH₂O, the activity in COB was about four-fold higher than those recovered in NaHCO₃. Washing these COB samples with the corresponding solution (same as soaking and grinding), then rinsed in dH₂O (to produce WOB II) caused a similar reduction in lipolytic activity of 42 and 51 % for oil bodies recovered using dH₂O and NaHCO₃, respectively.

Some reports showed that lipolytic activity is generally absent in dry seeds of different crops, rising only on the onset of germination (Huang, 1992).

However, some other crops (e.g., castor bean) have shown active acid lipase in the dormant seed (Ory et al., 1969, 1960). The presence of active enzymes at this stage of maturity has been a subject of controversy among researchers. Indeed, in contrast with Huang (1992) and Lin and Huang (1983), Hoppe and Theimer (1997) were able to detect low lipase activity in mature seeds of rape, which is in line with the findings in Table 4.1. In the same work, it was shown that despite the presence of activity in mature dry seed, the degradation rate of TAG molecules *in vitro*, exceeded the one found *in vivo* over the early stages of germination. Therefore, the activity measures in Table 4.1 may be an overestimation of real lipolytic activity in seed: differences in enzymatic activity may depend on its attachment to the substrate (oil body, or the artificial emulsion used in the assay) and on the surface composition of the substrate emulsion (Brockman, 2013).

As suggested in Table 4.1, the lipolytic activity is deeply affected by the recovery solution used. There was a significant positive correlation ($R^2= 0.88$, $P< 0.05$) between the activity and the protein content in the oil body samples, suggesting that the removal of exogenous material could lead to better stability against enzymes. However, to further investigate this phenomenon, the data were expressed as specific activity (U/g protein) and shown in Table 4.2. This parameter describes the amount of product formed by an enzyme in a given amount of time under given conditions, normalised by the amount of total protein. For ease of data interpretation, the protein content of each sample (g/g dry weight basis) are reported. As a reference, oil bodies recovered soaking and grinding seeds in NaHCO_3 were washed in urea (9 M) and lipolytic activity assessed in the final cream (WOB II).

Table 4.2: Specific activity and protein content in seed (dry/soaked) and recovered oil bodies.

Specific Activity (U/g protein)	Dry Seeds	Soaked Seeds (16 hrs)	COB	WOB I	WOB II
dH ₂ O	68.99±5.07 ^a	95.2±4.8 ^{b,x}	177.5±4.9 ^{c,x}	154.5±7.9 ^{d,x}	153.2±8.2 ^{d,x}
NaHCO ₃		99.1±3.3 ^{b,x}	87.1±2.7 ^{c,y}	83.6±1.7 ^{c,y}	76.1±2.3 ^{d,y}
Urea-WOB	-	-	-	-	13.5±0.5 ^z

Protein content (g/g dwb)	Dry Seeds	Soaked Seeds (16 hrs)	COB	WOB I	WOB II
dH ₂ O	0.2±0.02 ^a	0.19±0.03 ^{a,x}	0.098±0.010 ^{b,x}	0.078±0.008 ^{bc,x}	0.065±0.005 ^{c,x}
NaHCO ₃		0.2±0.05 ^{a,x}	0.045±0.003 ^{b,y}	0.033±0.003 ^{c,y}	0.027±0.002 ^{d,y}
Urea-WOB	-	-	-	-	0.025±0.001 ^y

Low protein content oil bodies were recovered soaking and grinding seeds in NaHCO₃ (COB), then washed in the same media (WOB I) and rinsed in dH₂O (WOB II). High protein content samples were recovered using only dH₂O. Urea-WOB were produced washing COBs (recovered with NaHCO₃) with urea solution (9 M) for the first wash (WOB I), then rinsed in dH₂O to produce WOB II. Different (a, b, c, d) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the effect of COB recovery and washing was evaluated. Different (x, y, z) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the recovery/ washing solution was evaluated.

When the data are expressed by the amount of protein (Table 4.2), the specific activity in COBs is still dependent on the grinding solution used (as seen in Table 4.1) but compared with expressing the activity relative to the total dry weight, the differences are not as dramatic. Moreover, when the protein and activity data are compared over washing, a reduction by only 13% in WOB II was seen for NaHCO₃ extraction, revealing that this trend did not follow the same decay as seen for the protein content of the corresponding oil body sample (-40%).

The different trends during washing between lipolytic activity and exogenous proteins removed from the NaHCO₃-recovered oil body preparation (Table 4.2, $R^2 = 0.39$, $P < 0.05$), suggests that different factors may play an important role, such as the presence of more than one isoform of lipase, each with a different strength of association with the oil body, or by the presence of other molecules (e.g., cofactors). Lipase isoforms may be located in different compartments of the seed, depending on the species (Hills and Murphy, 1988; Lin and Huang, 1983; Theimer and Rosnitschek, 1978). For example, in rapeseed, lipase activity was found in both oil bodies and the soluble cell fraction after density gradient separation of the homogenised seed, while soy seeds do not have any lipolytic activity associated to the oil body (Hills and Murphy, 1988). In rapeseed, the two isoforms were revealed due to differences in pH optimum and response to substrate type and concentration (Hills and Murphy, 1988). Although both lipases show a neutral to alkaline pH optima, the microsomal lipase showed maximum activity at pH 7.5 and the oil body lipase at pH 9 (Hills and Murphy, 1988). However, other works reported contradictory results for the optimum pH value: Theimer and Rosnitschek showed maximum activity of the microsomal lipase fraction at pH 9, while Lin and Huang (1983) reported a pH optimum of 6.5 for the oil body-associated lipase.

The results in Table 4.2 suggest that these enzymes may have a different degree of association with the oil body; and upon exposure to NaHCO₃, only the one more tightly associated to the oil body is carried over in the cream. On the other hand, cofactors are non-protein molecules that can increase (or initiate) the catalyst activity of a particular enzyme (Nelson et al.,

2008), which may have affected the results in Table 4.2. The action of lipase cofactors has been thoroughly investigated in monolayer systems (Bläckberg et al., 1979; Brzozowski et al., 1991; Crandall and Lowe, 2001; Larsson and Erlanson-Albertsson, 1991; Van Tilbeurgh et al., 1993). Beisson et al. (2001) tested oil bodies as substrate of lipolytic activity, showing that the addition of colipase increased the turnover of lipase on intact almond oil bodies, reducing at the same time its lag time. It is commonly thought that the activation of many lipases is a result of conformational changes, which opens a lid made of α -helix stretch that is moved out upon the adsorption of the enzyme at the interface, allowing the access to the active site (Derewenda et al., 1994; Van Tilbeurgh et al., 1993). The presence of a cofactor could potentially explain why a decrease of 40% in the protein content of washed oil bodies recovered with NaHCO_3 , does not correspond to a similar decay in the specific activity.

Lastly, small amounts of lipolytic activity were found in urea-washed samples. Despite the similarity in protein content between urea and NaHCO_3 -WOB (as discussed in Section 3.3.2.2), the lipolytic activity as shown in Table 4.2 was significantly higher in the latter, which could suggest that either cofactors or lipase enzymes are more effectively removed with urea (9M) than with sodium bicarbonate. Katavic et al. (2006) analysed by mass spectroscopy the protein extracts from rapeseed oil bodies after exposure to urea (8 M), showing the presence of proteins that were sharing homology (81 % similarity) to an *A. thaliana* enzyme that belongs to the lipase/acylhydrolase family. In fact, the catalytic properties of oilseed rape lipases are largely consistent with those reported for the oil body-associated TAG lipases in *Arabidopsis* (Hills and Murphy, 1988; Lin and Huang, 1983; Quettier and Eastmond, 2009). An

endogenous, oil body-associated lipase enzyme, not easily removed in the presence of chaotropic agents, such as urea, may explain the residual lipase activity in our rigorously washed oil body samples.

The results shown in this section suggest that protein analysis may not be a good indicator of oil body purity from endogenous enzymes. Given the presence of residual activity in the NaHCO_3 washed product, a thermal treatment seems to be a necessary step to assure oil body stability over time. This aspect is discussed in the next section.

4.3. Effect of thermal treatment on oil body extracts

The manufacturing process of food emulsions involves thermal treatment at some stage of the process, either during production, or utilization (Heldman and Lund, 2006). Thermal treatment is one of the most common techniques used in food processing to assure product quality, to avoid spoilage and to prevent food-borne illnesses. Nevertheless, it is essential that the emulsion, in this case the oil body, is capable to withstand the thermal process without breaking.

Despite some information on the effect of thermal treatment on the physical properties of oil bodies can be found in literature (Chen et al., 2012; Iwanaga et al., 2007; Karkani et al., 2013; Naziri et al., 2017; Wu et al., 2012), the minimal parameters required for the inactivation of enzymes is yet to be established. In this regard, the lipolytic activity could potentially be used as marker of thermal process efficiency, as it is a fairly thermoresistant enzyme (Ponne et al., 1996). In the last section, it was shown that lipolytic enzymes are present in the oil body preparations to varying degrees depending on the recovery protocol. It is worth considering that despite the use of optimised conditions for the removal of exogenous material (NaHCO_3 , 0.1 M, pH 9.5), further residual lipolytic enzymes could be removed by exposure to urea (9 M). However, urea is not an acceptable agent for preparing material for food applications. For this reason, the thermal treatment (95 °C) to inactivate residual lipolytic activity was tested (Figure 4.1).

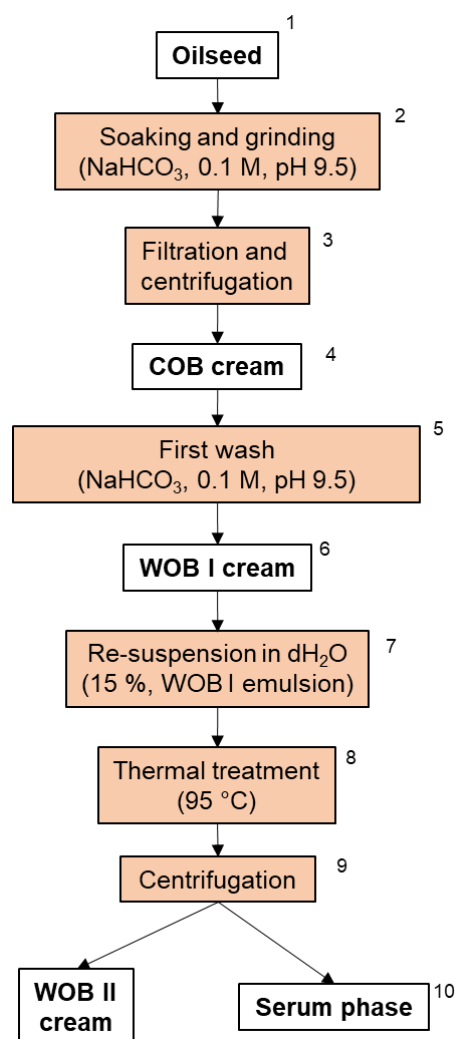


Figure 4.1: Inclusion of thermal treatment in the protocol for the recovery of oil bodies from rapeseeds. After the first washing cycle, WOB I creams were re-suspended in dH₂O (producing WOB I emulsions, 15 % oil weight basis), which were thermally treated (95 °C). After cooling and a centrifugation step, WOB II cream and a serum phase were collected.

The possible synergic effect of thermal treatment and centrifugation, not only on lipolytic activity, but also on the protein composition, compared with centrifugation only (discussed in Chapter 3) was studied. For this reason, after centrifugation (step 9 in Figure 4.1), the cream and serum phases were separately collected and analysed (step 10). The aim of these experiments was to study not only the trends of lipolytic activity post thermal treatment and

centrifugation, but also if those changes were caused by physical removal (solubilisation in the serum phase) or heat-inactivation.

Initially, heat treatments at 75 °C and 85 °C were tested, trying to find the correlation between temperature and enzyme inactivation. The preliminary data showed that at short-time thermal treatment (1.5 - 2 minutes), the activity increased, reaching values even higher of the initial one ($t=0$ s). This was not expected and finding what was causing this increase would be beyond the scope of this thesis. For this reason, 75 °C and 85 °C will be not considered in this work.

4.3.1. Oil body cream phase: lipase activity and protein content

Oil bodies were recovered after soaking and grinding rapeseed seeds and further washed using NaHCO_3 (0.1 M, pH 9.5, 4°C) (WOB I, step 5 in Figure 4.1). After re-suspension of 1g WOB I cream in dH_2O (15% WOB I emulsion, lipid weight basis), the emulsion was carefully poured into glass tubes (8 mm inner diameter) and thermally treated in a water bath at 95°C. After cooling in ice and centrifugation (10,000 g, 30 minutes, 4°C) the cream was collected, analysed for lipolytic activity and the results shown in Figure 4.2. The data were normalised by the protein content in 1g of WOB I cream, assuming that over thermal treatment and subsequent centrifugation the protein mass balance between WOB I (recovered from step 5, Figure 4.1) and WOB II + serum phase (recovered from step 9, Figure 4.1) was maintained.

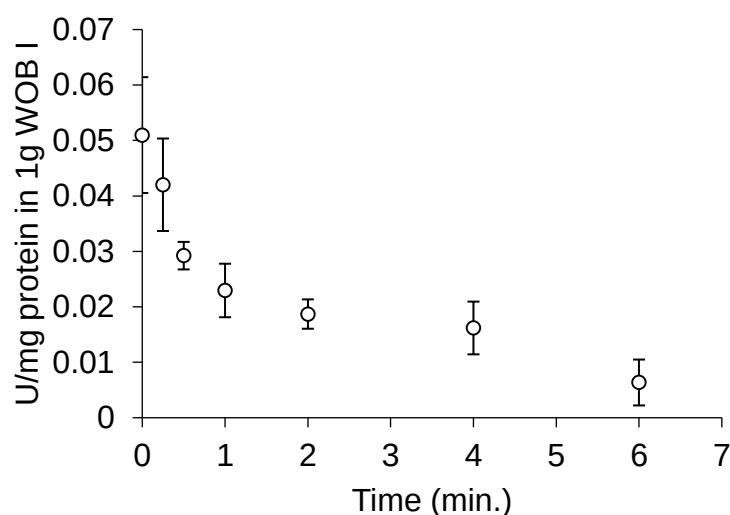


Figure 4.2: Effect of thermal treatment (95 °C) on lipase activity in cream phase of WOB II. Oil bodies were recovered and washed once in NaHCO_3 (0.1 M, pH 9.5). The cream (WOB I) was then suspended in dH_2O , thermally treated (95 °C) and centrifuged.

It can be seen from Figure 4.2 that lipolytic activity in the cream phase decreased significantly from 0.050 ± 0.01 U to 0.023 ± 0.005 U over the first minute held at 95 °C. Afterwards, the decay was slower, reaching 0.006 ± 0.004 U after 6 minutes. These results suggest that applying the sufficient amount of heat, enzyme inactivation takes place. The catalytic activity is dependent on the integrity of the native protein conformation: if the original tertiary and quaternary structures are altered (e.g. due to heating), the catalytic activity is usually lost (Nelson et al., 2008).

In literature, the thermal process variables for oil bodies are not standardised among researchers, applying temperatures between 30 and 120 °C, for up to 30 minutes (Farah et al., 2018; Iwanaga et al., 2007; Karkani et al., 2013; Wu et al., 2012). The results shown in Figure 4.2 suggest that the holding time for thermal treatment could remarkably be reduced. The effect of

thermal treatment on other enzymes present in oil body preparations (e.g., proteases), looking at the hydrolysis of oleosin, will be discussed in Chapter 5.

As already discussed in Section 4.2, one possible explanation for the reduction of lipase activity in oil body preparations could be the loss of the enzyme into the serum phase during washing and centrifugation steps. However, this phenomenon may be promoted by heating the WOB I emulsion. For this reason, the protein content of WOB II samples was measured and compared with the amount of protein derived from the parent WOB I material (1 g). The recovered protein extracts were quantified and studied by SDS-PAGE (Figure 4.3). Heating the oil body emulsions (95 °C), followed by centrifugation, had a significant effect on the protein content already over the first 30 seconds (Panel A), decreasing from 22.29 ± 1.29 mg in the untreated, to 18.47 ± 0.82 mg. Extending the thermal treatment for up to 6 minutes, the protein content was furtherly reduced to 15.44 ± 0.61 mg.

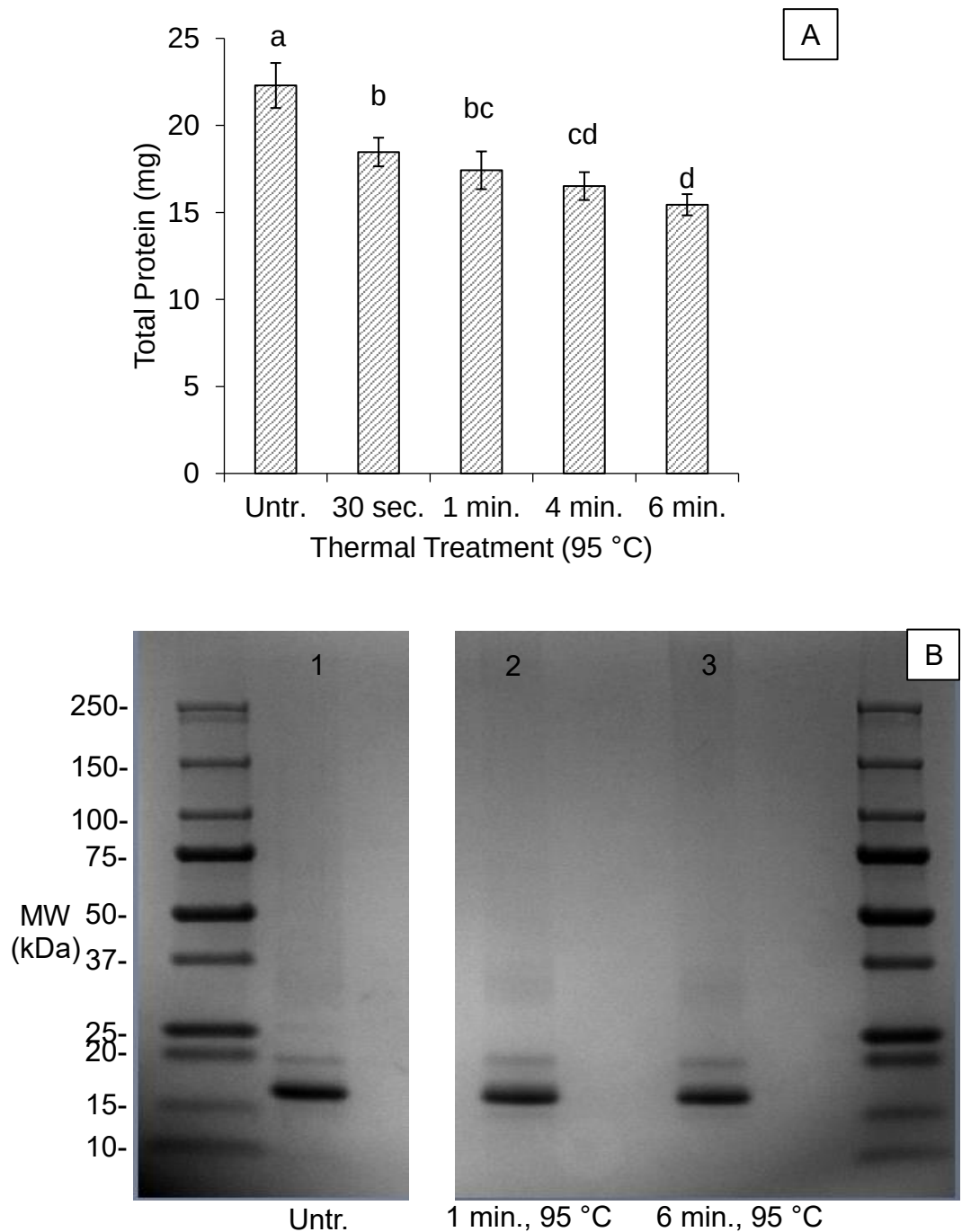


Figure 4.3: Effect of thermal treatment on oil body cream protein content and composition. A) Total protein content of WOB II extracts recovered from 1g of WOB I. The untreated sample (Untr.) was recovered from 1g of WOB I after suspension of the cream and centrifugation. The other samples were thermally treated (0-6 minutes, 95 °C), and the creams recovered after centrifugation (10,000 g, 30 minutes). B) SDS-PAGE of protein extracts (3.5 μ g loaded in each well) from untreated (lane 1), and thermally treated (1-6 minutes, 95 °C, lane 2 and 3) oil bodies.

In principle, when the denaturation energy provided by heating is sufficiently high, the association between exogenous proteins, and the surface of oil bodies, can be disrupted. Due to the thermal treatment and subsequent centrifugation step, the dissociated protein molecules from the oil body surface, are probably removed and solubilised in the serum. To confirm this hypothesis, the protein content in serum phases recovered after centrifugation of thermally treated emulsions, will be discussed in Section 4.3.2.

Panel B in Figure 4.3 shows the protein composition of the different oil body preparations, with equivalent protein loadings ($3.5\ \mu\text{g}$) per sample lane. Despite the decrease in protein content after heating, there is no difference in the composition between untreated (lane 1) and thermally treated (lane 2 and 3, corresponding to 1 and 6 minutes at $95\ ^\circ\text{C}$) oil body emulsions. Moreover, no change in oleosin band intensity was found, suggesting that the integral proteins are not be affected by the process (as discussed in Section 4.3.3).

4.3.2. Serum phases: lipase activity and protein content

After thermal treatment (95 °C, 0-6 minutes) and centrifugation of WOB I oil body emulsions, the serum phases were collected and clarified from residual oil bodies to avoid cross contamination. This process involved three centrifugations at high speed (21,000 g, 1 hour), removing the top fat layer in each step. After a final filtration (0.22 µm membrane), the lipolytic activity was assayed on an aliquot of clarified serum (1 ml) and the result shown in Figure 4.4.

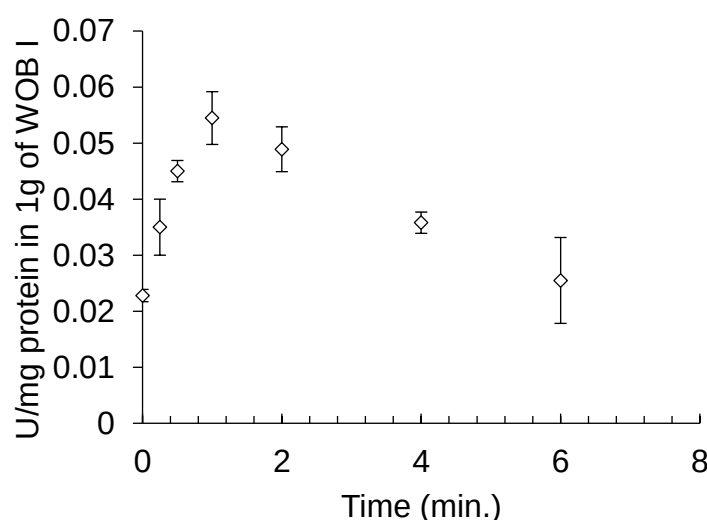


Figure 4.4: Effect of thermal treatment (95 °C) on lipolytic activity from serum phases. Samples were recovered post centrifugation of WOB I emulsions (produced upon re-suspension of WOB I cream in dH₂O). Serum phases were clarified to minimise cross contaminations from oil bodies.

During thermal treatment, the activity in the serum phase increased from 0.023 ± 0.001 U in the untreated, to 0.055 ± 0.005 U after 1 minute of heat treatment. Afterwards, the activity decreased linearly reaching 0.026 ± 0.007 U after 6 minutes. Combining these results with the ones shown for the cream phases (Figure 4.2), it could be concluded that the reduction of lipolytic activity

in the thermally treated WOB II cream samples is due to a combination of different phenomenon. The decrease in lipolytic activity in the oil body cream over the first minute (Figure 4.2), which amounts to 65% of the total, may not only be due to thermal inactivation, but also to the solubilisation of enzyme molecules into the serum phase due to heating. Therefore, an increase in activity in the serum phase can be observed. Over longer treatment times (1 to 6 minutes), thermal inactivation of the lipolytic enzymes takes place, showing a slow decay in both serum and cream phases (Figure 4.2 and 4.4), probably due to their thermic resistance (Ponne et al., 1996). However, this trend may be due also by the presence of distinct enzymes with lipolytic activity and different tendencies to remain associated with oil bodies (as anticipated in Section 4.2) (Hills and Murphy, 1988).

The total protein content in the serum phase (Figure 4.5) increased from 1.24 ± 0.26 mg to 5.98 ± 0.3 mg over the first minute of treatment time, to achieve 7.35 ± 0.29 mg at the end of thermal treatment (6 minutes).

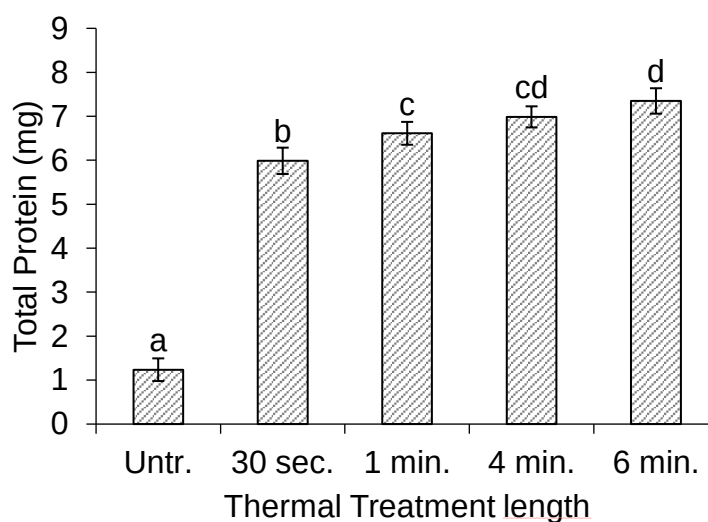


Figure 4.5: Protein content of serum phases. Samples were recovered from the lower phase after centrifuging untreated or thermally treated (0-6 minutes, 95 °C) WOB I emulsions (from 1 g of WOB I cream).

This trend can be correlated to the one already seen in the cream (Figure 4.2, Panel B), where the dissociated protein molecules from the oil body surface, are probably removed and solubilised in the serum, keeping constant the protein mass balance between WOB I and WOB II + serum.

To assess whether the heating would lead to destabilization of the emulsion, the physical stability (particle size and zeta potential) was measured (Section 4.3.3).

4.3.3. Effect of thermal treatment on physical stability of oil body emulsions

If thermal treatment is employed to inactivate enzymes that could cause the deterioration of oil body quality, it is vital that any heating does not lead to the physical destabilisation of the oil body dispersion. The purpose of these experiments was to examine the effect of high temperature on oil bodies extracted from rapeseed. For this reason, particle size (Figure 4.6 A) and ζ -potential (Figure 4.6 B) were measured to analyse the impact of heating (95 °C, 1-6 minutes) WOB I emulsions (produced from the re-suspension of WOB I cream in dH₂O) on the integrity of the final product.

Untreated emulsions recovered in 0.1 M NaHCO₃ (pH 9.5) have a bimodal distribution ranging between 0.2 and 6 μ m, with major peak at 1 μ m, as already shown in Section 3.3. The ζ -potential changes from +55 $\pm$ 4 mV to -65 $\pm$ 2.5 mV as the pH increases from 3 to 10 (pI 6.5). Upon heat treatment, no significant change either in particle size (Figure 4.6 A) nor ζ -potential (Figure 4.6 B) was observed. Moreover, there was no evidence of aggregation or phase separation in the heated samples. These results suggest that the process did not significantly affect the surface charge and integrity of oil bodies.

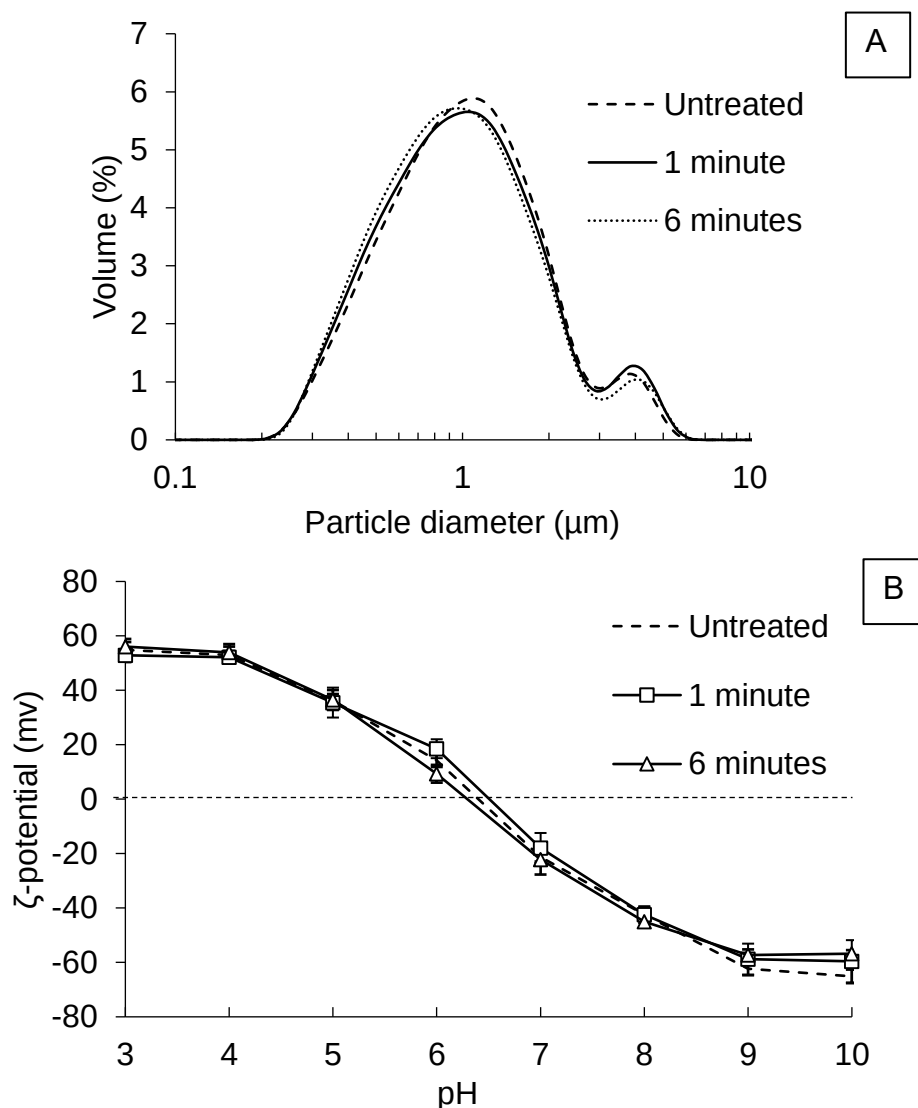


Figure 4.6: Particle size (A) and ζ -potential (B) of untreated and thermally treated (1 and 6 minutes, 95°C) oil body emulsions. WOB I cream was re-suspended in dH₂O (15% lipid basis emulsion) and thermally treated. After cooling in ice and centrifugation, the WOB II cream was recovered and analysed.

In general, emulsions stabilised by globular proteins are particularly sensitive to thermal treatments (David Julian McClements, 2004). Upon heating, the adsorbed proteins will unfold, losing their structure and exposing reactive groups (e.g., hydrophobic) that were originally located in the interior. As a consequence, the interaction between those groups (in the same or different droplets) can lead to flocculation and coalescence (Kim et al., 2002).

The occurrence of protein denaturation and the effect on the protein integrity and functionality is dependent on the intensity of heat exposure and on the environmental condition, such as pH and ionic strength (deMan et al., 2018; McClements, 2004).

Particle size and ζ -potential analyses in Figure 4.6 suggest that oleosins have a good stability against high temperatures, which is in line with other studies (Farah et al., 2018; Iwanaga et al., 2007). The good thermal stability is presumably due to the T-like structure of oleosin molecule: the central portion, which penetrates through the phospholipid layer into the lipid core (Huang, 1992; Tzen and Huang, 1992) remains tightly associated to the non-polar matrix during heating, while the salt bridges between phospholipid and the N and C terminus of the oleosin are weakened, releasing them from the oil body surface. However, after cooling they return to their intrinsic locations (Chen and Ono, 2010).

4.3.4. Effect of heating on tocopherol content

Food processing carried out in industry is expected to have an effect on bioactive compounds present in the product, namely antioxidants, such as tocochromanols (commonly known as vitamin E). In the development of a process, it is important that the effects of each step are taken into consideration when assessing vitamin stability in food, since they can be subjected to different adverse factors, being heat the most common deleterious one (Braunstein, 2006).

One class of tocochromanol showing a fully saturated side chain are called tocopherols, and are distinguished into four natural sub-form: α - β - γ - δ -tocopherols (Munné-Bosch and Alegre, 2002). These vitamers are very important compounds commonly found in oilseeds (Mène-Safrané and DellaPenna, 2010), as they have the ability to react with and quench free radicals in cell membranes and other lipid environments, preventing polyunsaturated fatty acids (PUFA) from being damaged by non-enzymatic lipid oxidation (Bramley et al., 2000). It is likely that the majority of tocopherols in oilseeds are located in oil bodies (Farah et al., 2018; Fisk et al., 2006; Fisk and Gray, 2011; Zaaboul et al., 2018), but they may be affected by chaotropic agents, such as urea (Fisk et al., 2006). Considering that the developed process leads to a composition of oil bodies similar to urea washed preparations, it is important to assess if those molecules will be affected by the exposure to NaHCO_3 . Moreover, the effect of heating of oil body emulsions (95 °C, up to 6 minutes) on tocopherols was investigated.

In the first set of experiments, oil bodies were recovered (COB) and washed using NaHCO_3 (0.1 M, pH 9.5), then rinsed in water (WOB) and the tocopherols extracted to be analysed using HPLC. The quantity found in the recovered cream was then compared to the one found in seeds and the results shown in Table 4.3. On a dry weight basis, it was found that crude oil bodies had a significantly higher level of tocopherols compared to seeds, which increased further after the washing protocol (WOB, Table 4.3 A). These results confirm that the oil bodies are loaded with tocopherols and their concentration increases over the recovery. This is expected, considering their amphipathic nature where the hydrophobic chain would be within the single phospholipid layer. In line with other studies in literature (Fisk et al., 2006; Fisk and Gray, 2011; Wang et al., 2017), the concentration of tocopherol on a lipid basis remains constant in all the samples (Table 4.3 B). Looking at the composition, it was found that the major isoforms were α - and γ -tocopherols, but no δ -tocopherol were found (Table 4.3 B).

Table 4.3: Tocopherol content in rapeseed seeds and oil body cream on a dry weight basis (A) and on lipid weight basis (B).

A				
Tocopherols (mg/kg dwb)				
	TOTAL	Alpha	Delta	Gamma
SEED	484.2 ± 13.1 ^a	219.5 ± 6.6 ^a	4.5 ± 0.6 ^a	260.2 ± 5.9 ^a
COB	1264.8 ± 55.9 ^b	555.3 ± 40.5 ^b	12.7 ± 4.4 ^b	696.8 ± 11.0 ^b
WOB	1442.3 ± 81.7 ^b	610.8 ± 46.3 ^b	12.7 ± 4.9 ^b	818.8 ± 30.5 ^c

B				
	Total (mg/kg oil)	Alpha (%)	Delta (%)	Gamma (%)
SEED	1152.9 ± 31.4 ^a	45.3 ± 1.3 ^a	0.9 ± 0.1 ^a	53.7 ± 1.2 ^a
COB	1201.8 ± 121.9 ^a	49.5 ± 5.3 ^a	1.2 ± 0.4 ^a	53.5 ± 4.7 ^a
WOB	1205.1 ± 88.7 ^a	47.0 ± 3.2 ^a	1.2 ± 0.5 ^a	56.3 ± 4.0 ^a

Different (a, b, c) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the content (mg/kg dwb, mg/kg oil) or composition (%) are compared among samples (Seed, COB, WOB).

According the findings of Dolde et al. (1999) and Abidi et al. (1999) the total content in tocopherols in rapeseed is highly variable, depending on the environmental condition in which the plant was grown, ranging between 80 to 1000 mg/Kg (on a dry weight basis). However, according to Marwede et al. (2004) the major components of tocopherols from rapeseed are represented by γ - (up to 64 %) and α -tocopherols (35 %), while the others (β - and δ - isoform) are present only in trace amounts (around 1 %). In all samples used

for the analysis in this thesis, primarily γ - and α -tocopherols were found, and the δ - did not exceed 1.2 %. Moreover, the analysis between seed and oil body revealed that the tocopherols were not affected by the vigorous recovery protocol (Table 4.3 B).

Previous research on thermal decomposition of tocopherols was carried out on whole seeds during roasting procedures (Marzena et al., 2014; Shrestha and De Meulenaer, 2014; Wijesundera et al., 2008). However, no research was done on the effect of thermal treatment of oil body emulsion on tocopherol content. For this reason, oil body emulsions (WOB I) were thermally treated at different time length and the tocopherols extracted from the WOB II creams. Figure 4.7 shows the degree of vitamin E decomposition.

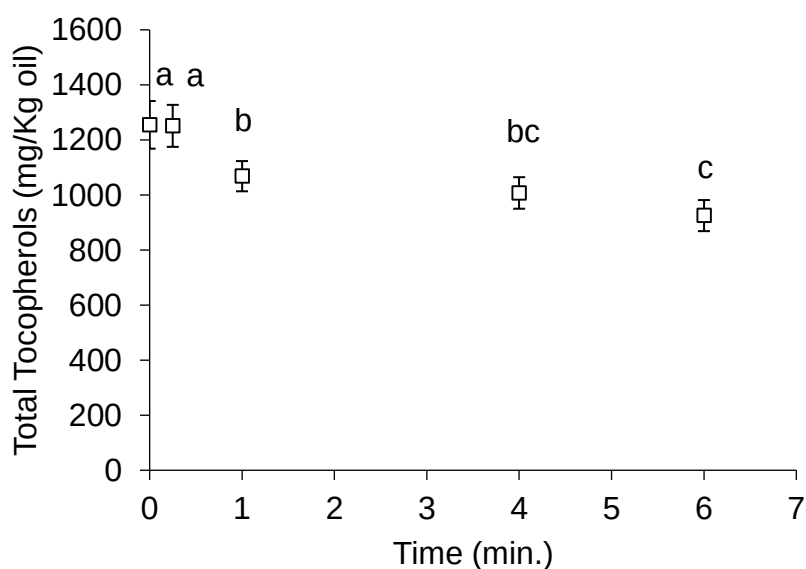


Figure 4.7: Tocopherol decomposition on heating oil body emulsions (95 °C). WOB I emulsions were thermally treated at different time lengths and the cream was recovered after centrifugation.

The decay of tocopherols increased slowly to 14 % over the first minute and reached 26 % at the end of thermal treatment (6 minutes). These results suggest that the heating process considered (as detailed in Section 4.3) is not able to completely decompose tocopherols, and that significant levels of tocopherol remain, at levels known to offer protection of oil dispersed emulsions. The correlation between thermal treatment, tocopherol degradation and lipid oxidation will be discussed in Chapter 5.

4.4. Summary of results

The findings in this work demonstrate that lipase is strongly associated to the surface of oil bodies, despite the efforts in the purification process from the exogenous material. This study shows how this activity is affected by seed soaking time, the chemical nature of the recovery medium, and the nature of the washing protocol applied. A crude oil body preparation recovered after soaking and grinding seed in NaHCO_3 , had a significantly lower lipolytic activity than the one recovered using dH_2O . The washing of this crude oil body material did not lead to a complete loss of lipolytic activity, so thermal treatment is necessary to reduce enzyme activity further.

A short-time thermal treatment (95°C , up to 1 minute) of the milk (re-suspended WOB I cream), and subsequent centrifugation, is an efficient way to further reduce the amount of exogenous proteins, reducing by 65% the total lipase activity in the recovered cream. This may be enough for commercial preparations, as total enzyme inactivation may not be necessary. Longer thermal treatments (>1 minute) enhanced the inactivation of the enzymes ($>90\%$ inactivation for 6 minutes). The use of lipolytic activity as a marker for oil body purity and/or thermal process efficiency, could be a new approach for the assessment of the stability in oil body-based products.

The thermal process applied to the oil body emulsion did not significantly alter droplet integrity or surface charge, presumably attributed to the structure of oleosin. Tocopherol concentration in the oil bodies was high (about 1200 mg/kg oil) and remained high after thermal treatment;

approximately 75% remained even the longest thermal treatment (6 minutes 90°C).

CHAPTER 5.

EFFECT OF THERMAL TREATMENT ON PHYSICAL AND CHEMICAL STABILITY OF OIL BODIES OVER STORAGE

5.1. Background

To the best of our knowledge, the understanding of how enzymes may affect the physicochemical stability of oil bodies over storage, is usually limited to *in-vitro* studies using commercial proteins. Moreover, how and at what rate the residual enzymes associated with the oil body surface affect this stability *ex-vivo*, is poorly understood. Despite some researchers believed in the presence of enzymes (e.g., proteases) integral to the oil body (Herman et al., 1990), later works showed that these results were artefacts due to the extraction procedure (Kalinski et al., 1992; Sadeghipour and Bhatla, 2002). Despite no hydrolytic enzyme has been identified as an integral component of oil bodies, it was shown in Section 4.2 how their association to the surface of oil bodies depends on the recovery process used. Thermal denaturation of seed enzymes in oil body extract has not been the major focus of recent research, and the parameters used in this process are not consistent among authors. In fact, thermal treatment may vary between 10 to 30 minutes, at temperatures ranging between 70 to 100 °C (Chen et al., 2012; Iwanaga et al., 2007; Karkani et al., 2013; Naziri et al., 2017; Wu et al., 2012). Clearly, a guideline for the minimal requirements to enhance oil body stability against enzymes needs to be developed.

In chapter 4, a new approach was presented for this purpose, measuring the lipolytic activity over thermal treatment. The aim of this chapter is to assess the effect of enzyme inactivation (based on 60 and 90% reduction in lipolytic activity at 95 °C), on the chemical and physical stability of oil bodies over

storage. The first section of this chapter focuses on the effect of thermal treatment of oil bodies on proteolytic activity over storage. The integrity (particle size), morphology (microscopy imaging) and surface charge (ζ -potential) of aged oil bodies were studied over a period of 30 days. In the second section, lipid and fatty acid composition of fresh and aged emulsions were studied, focusing on the effect of lipolytic enzymes. In the last section, the effect of thermal treatment on oxidative stability was considered.

5.2. Physical stability of aged oil body emulsions

5.2.1. Oleosin hydrolysis

To confirm whether reducing lipase activity is a good marker for the inactivation of other enzymes over thermal treatment, the catalytic activity of these enzymes needs to be assessed.

Oleosins are hydrolysed rapidly during post-germinative growth in many plant species, including maize, rape, sesame and sunflower seed, with the concomitant conversion of triacylglycerols (TAGs) into fatty acids (FAs) (Fernandez et al., 1988; Murphy et al., 1989; Sadeghipour and Bhatla, 2002). Matsui et al. (1999) initially hypothesised that the proteolysis of the oil body protein coat is the first step required for the oil mobilization; a cytosolic thiol-protease detected in germinating sunflower seed, was found to be associated to the surface of recovered oil bodies, and may be a possible candidate for this catalytic activity (Sadeghipour and Bhatla, 2002; Vandana and Bhatla, 2006). Bearing this in mind, the hydrolysis of oleosins through carried over proteolytic activity was observed with SDS-PAGE analysis of aged emulsions. Oil bodies were recovered from oilseed rape seeds using NaHCO_3 grinding media (0.1 M, pH 9.5, 4°C), thermally treated at 95 °C for 1 and 6 minutes (corresponding to about 60 and 90% inactivation of lipolytic activity), and the WOB II creams were recovered after centrifugation of this emulsion. A series of untreated and thermally treated WOB II emulsions (10%, lipid weight basis, produced after re-suspending WOB II creams in dH_2O), were stored at 20 °C, and the degree of oleosin hydrolysis was studied on SDS-PAGE after 1 month (Figure 5.1). To avoid microbial spoilage, all stored emulsions contained 0.02 mM of NaN_3 (Lichstein and Soule, 1944).

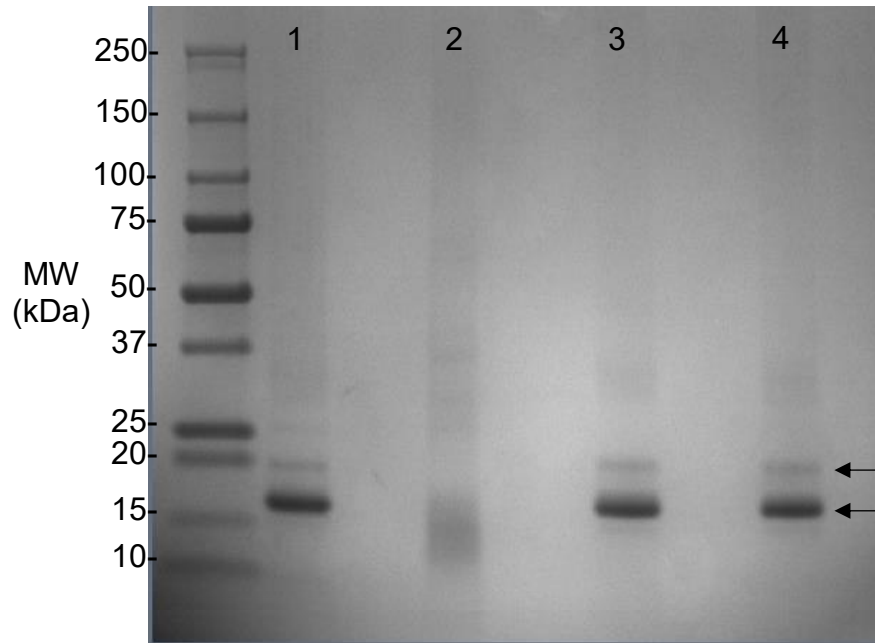


Figure 5.1: SDS-PAGE of protein extracts from fresh (lane 1) and aged (lane 2-4) oil body emulsions. Oil bodies were recovered, and emulsions (10%, lipid weight basis) stored at 20 °C for 1 month. Protein extract from fresh emulsions (lane 1) are included as reference.

At the end of the storage trial, the protein extracts on SDS-PAGE from untreated oil body samples (Figure 5.1, lane 2), showed proteolytic activity on oleosins, resulting in a series of peptides at the low molecular weight range (10-15 kDa). Heated emulsions (lane 3 and 4), retained a protein composition similar to the fresh sample (lane 1), revealing that the oleosin isoforms (indicated by arrows) were not affected by enzymes after either heat treatment.

Tzen and Huang (1992) and Lacey et al. (1998) tested the effect of commercial proteases on oleosin, which lead to the degradation of oleosins to a fragment of 8 kDa. This peptide was thought to be the central hydrophobic domain of oleosin, which was therefore protected by the hydrophobic environment of phospholipids (PLs) and TAGs from the catalytic activity of

proteases. However, in Figure 5.1 no distinctive peptide that could be related to the central domain of oleosin was found, suggesting that the prolonged proteolysis from carried-over enzymes may have completely hydrolysed the protein coat. This hypothesis is supported by the work of Maurer et al. (2013), who found that the addition of commercial trypsin did not produce any distinct peptide. The results shown in Figure 5.1 suggest that there is no proteolytic activity after thermally treating oil bodies. It is therefore confirmed that proteolytic enzymes are more thermolabile than lipolytic ones.

5.2.2. Particle size of aged emulsions

The progressive degradation of oleosin relegates the phospholipid layer to stabilise the oil body, weakening the emulsification system which could promote coalescence. The aim of these experiments was to examine the effect of thermal inactivation of the carried over enzymes (based on lipolytic activity at 95 °C) on storage stability of oil bodies extracted from rape seeds using 0.1M NaHCO₃ (pH 9.5). Particle size measurements over storage, supported by light microscopy, can give important information on the stability of the emulsion and on the effectiveness of the process.

Untreated (Figure 5.2, reported as reference) and thermally treated oil body emulsions (Figure 5.3 and 5.4, for 1 and 6 minutes treatment, respectively), were stored at 20 °C for 1 month. Moreover, confocal imaging (Figure 5.5) of fluorescently stained oil bodies was performed at the start and end of the trial, so structural information could be determined.

At the start of the storage, confocal imaging showed that the oil bodies were small ($D_{4,3} = 1.2 \pm 0.05 \mu\text{m}$), discrete organelles stabilised by a layer of proteins (in green, Figure 5.5 A) surrounding the oil core (in red, Figure 5.5 A). Over the time course, untreated emulsions (Figure 5.2) showed poor storage stability, with the first signs of size increase already over the first seven days of storage (20 °C). Microscopy analysis (Figure 5.2, C-D) showed some aggregated droplets (indicated by arrows), which could have affected the particle size distribution over the first days. However, between 7 and 30 days, the effects of enzymatic hydrolysis are visible to a greater extent, with enhanced size increase and presence of free oil (Figure 5.2, E). Moreover, confocal images (Figure 5.5 B) clearly show droplets with altered morphology and disordered protein aggregates (in green, indicated by an arrow), which are probably the result of the proteolytic activity.

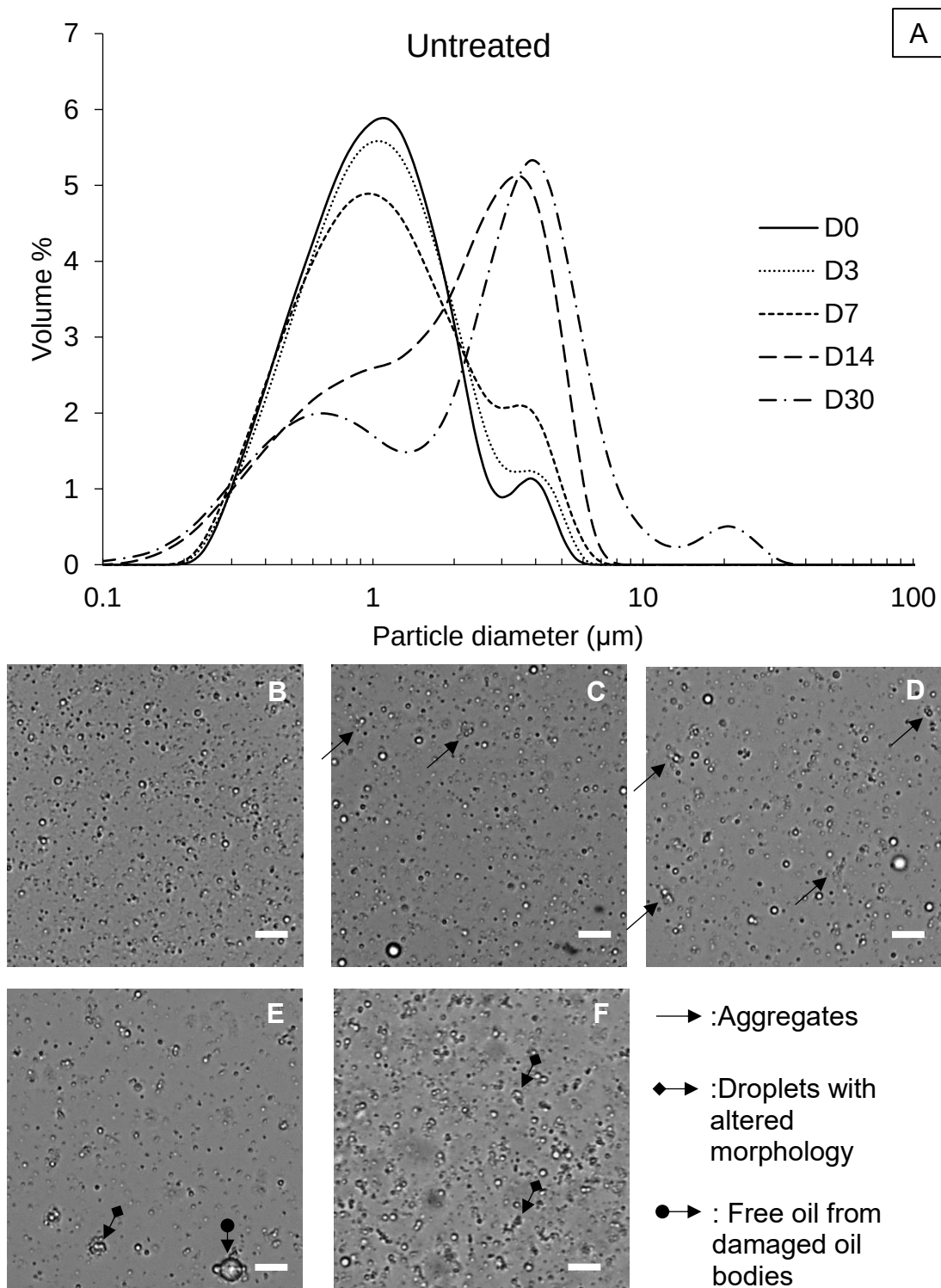


Figure 5.2: Particle size distributions (Panel A) and microscopy images (Panel B-F) of aged untreated oil body emulsions recovered in NaHCO_3 (0.1 M, pH 9.5). Emulsions (10%, lipid weight basis) were stored at 20 °C with the addition of NaN_3 (0.02 mM). Measurements were taken at day (D) 0/ 3/ 7/ 14/ 30 (corresponding to micrographs B/ C/ D/ E/ F, respectively). Scalebar represent 5 μm .

Thermal treatment (95 °C) had a significant impact on the storage stability of oil bodies (Figure 5.3 and 5.4). Particle size measurements showed a size increase over the first 7 days of storage for both thermally treated emulsions, but to a much lesser degree than the untreated samples. The oil bodies treated for 6 minutes remained stable and had a $D_{4,3}$ of $1.4 \pm 0.1 \mu\text{m}$ after 30 days (Figure 5.3, A); the samples treated only for 1 minute showed a further increase in size between 14 and 30 days of storage ($D_{4,3} = 1.7 \pm 0.15 \mu\text{m}$, Figure 5.4 A). Despite the thermally treated emulsions were creaming after 3 weeks (data not shown), the thermally treated oil bodies remained as discrete droplets, with no sign of aggregation or altered morphology (Figure 5.5 C-D), suggesting that the structure of the oil body was preserved.

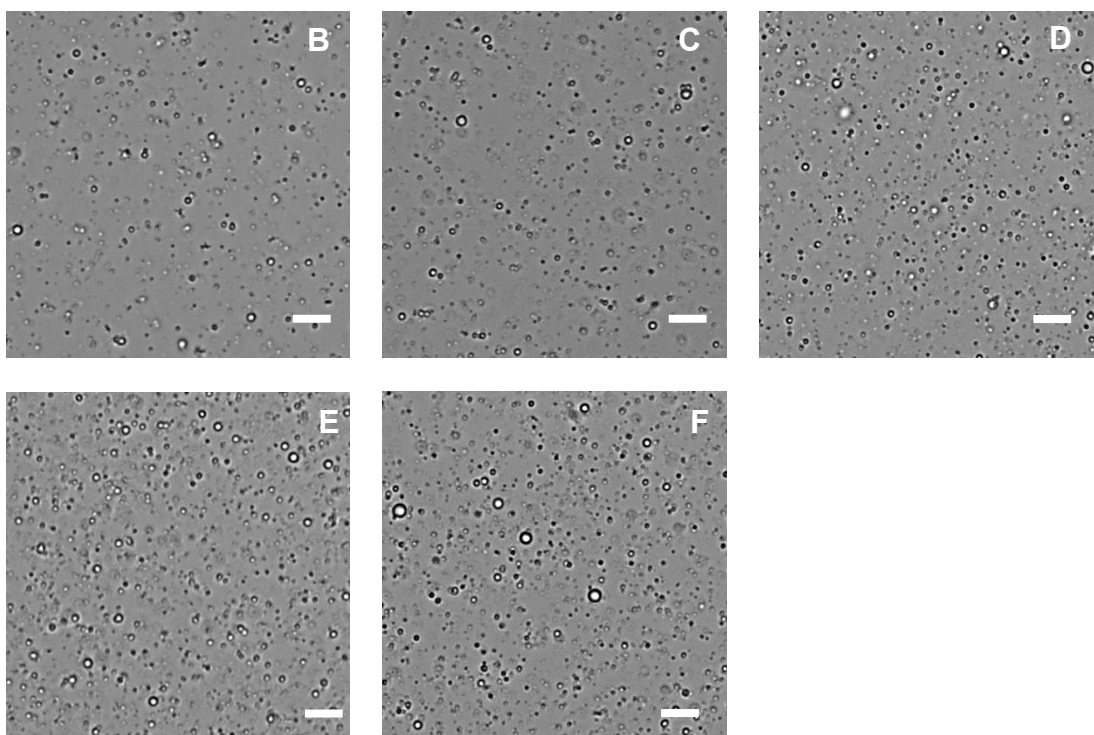
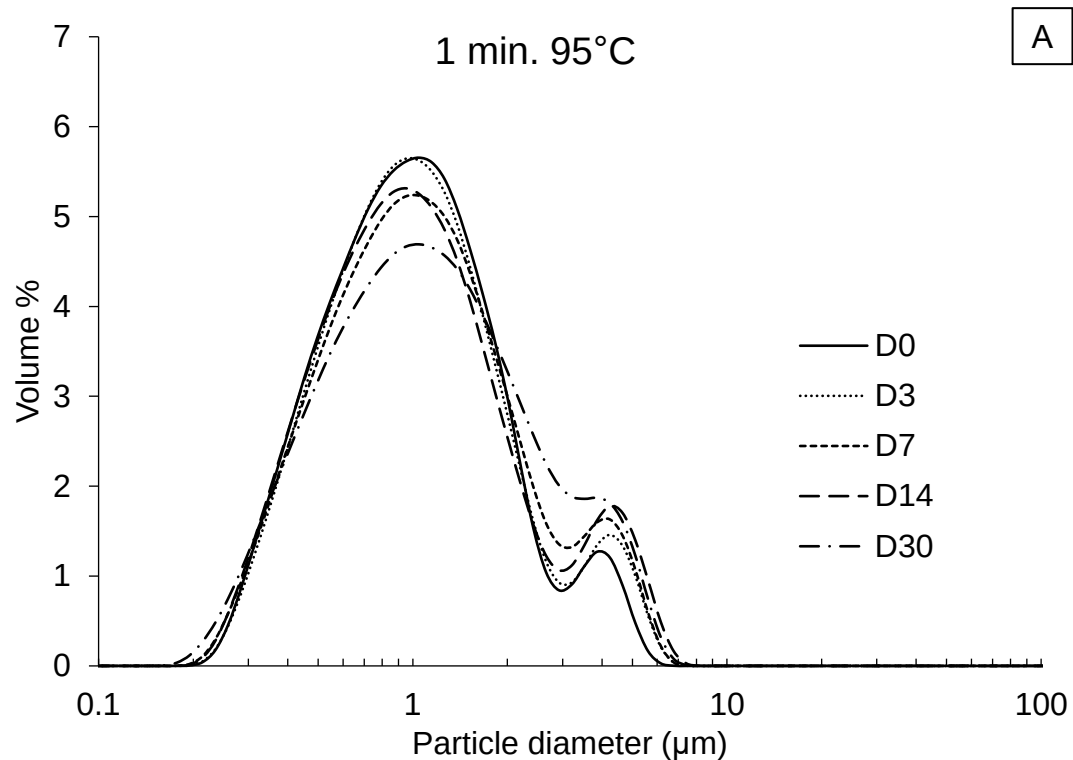


Figure 5.3: Particle size distributions (Panel A) and microscopy images (Panel B-F) of aged oil body emulsions recovered in NaHCO_3 (0.1 M, pH 9.5) and thermally treated (95 °C, 1 minute). Emulsions (10%, lipid weight basis) were stored at 20 °C with the addition of NaN_3 (0.02 mM). Measurements were taken at day (D) 0/ 3/ 7/ 14/ 30 (corresponding to micrographs B/ C/ D/ E/ F, respectively). Scalebar represent 5 μm .

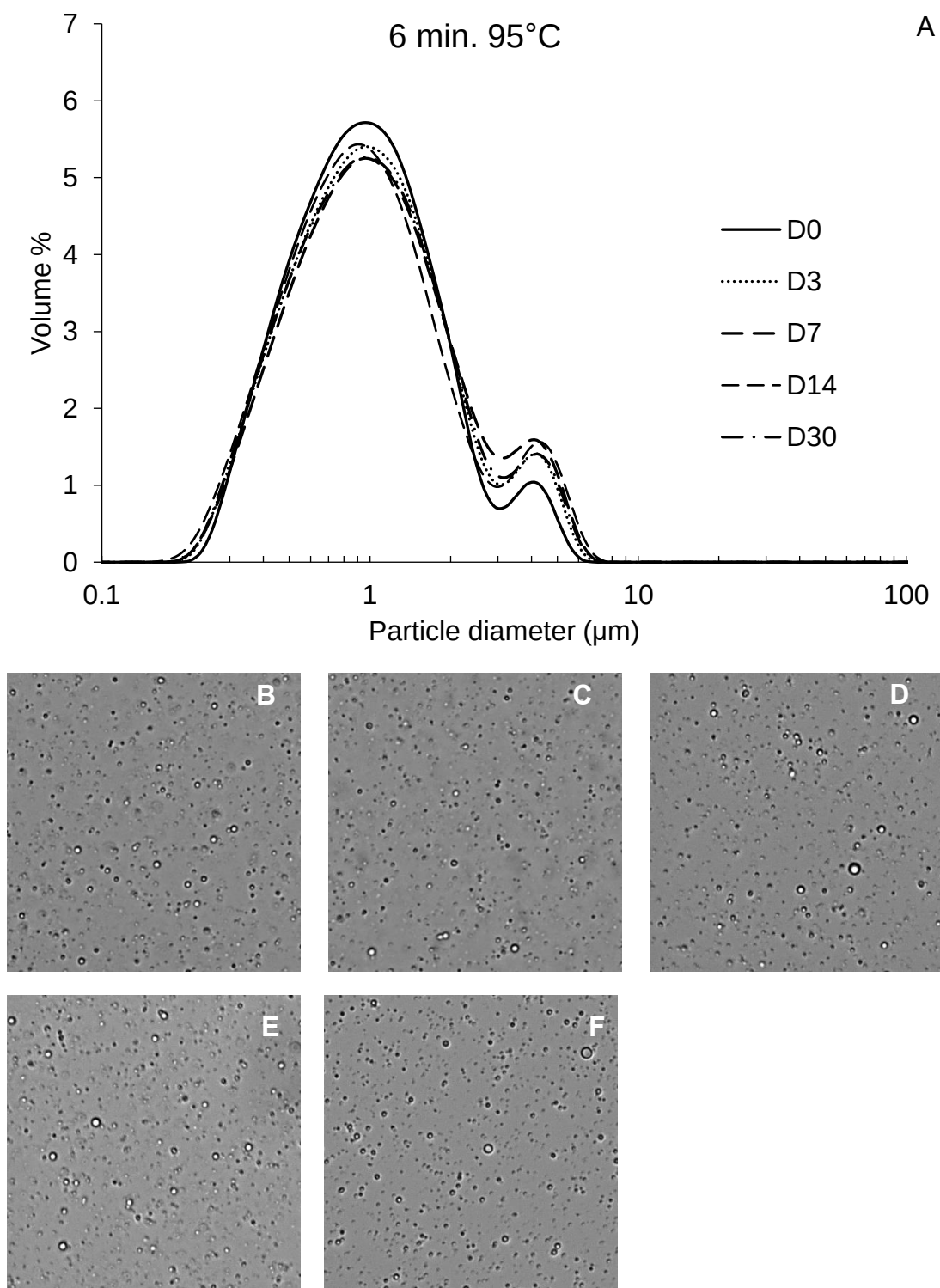


Figure 5.4: Particle size distributions (Panel A) and microscopy images (Panel B-F) of aged oil body emulsions recovered in NaHCO_3 (0.1 M, pH 9.5) and thermally treated (95 °C, 6 minutes). Emulsions (10%, lipid weight basis) were stored at 20 °C with the addition of NaN_3 (0.02 mM). Measurements were taken at day (D) 0/ 3/ 7/ 14/ 30 (corresponding to micrographs B/ C/ D/ E/ F, respectively). Scalebar represent 5 μm .

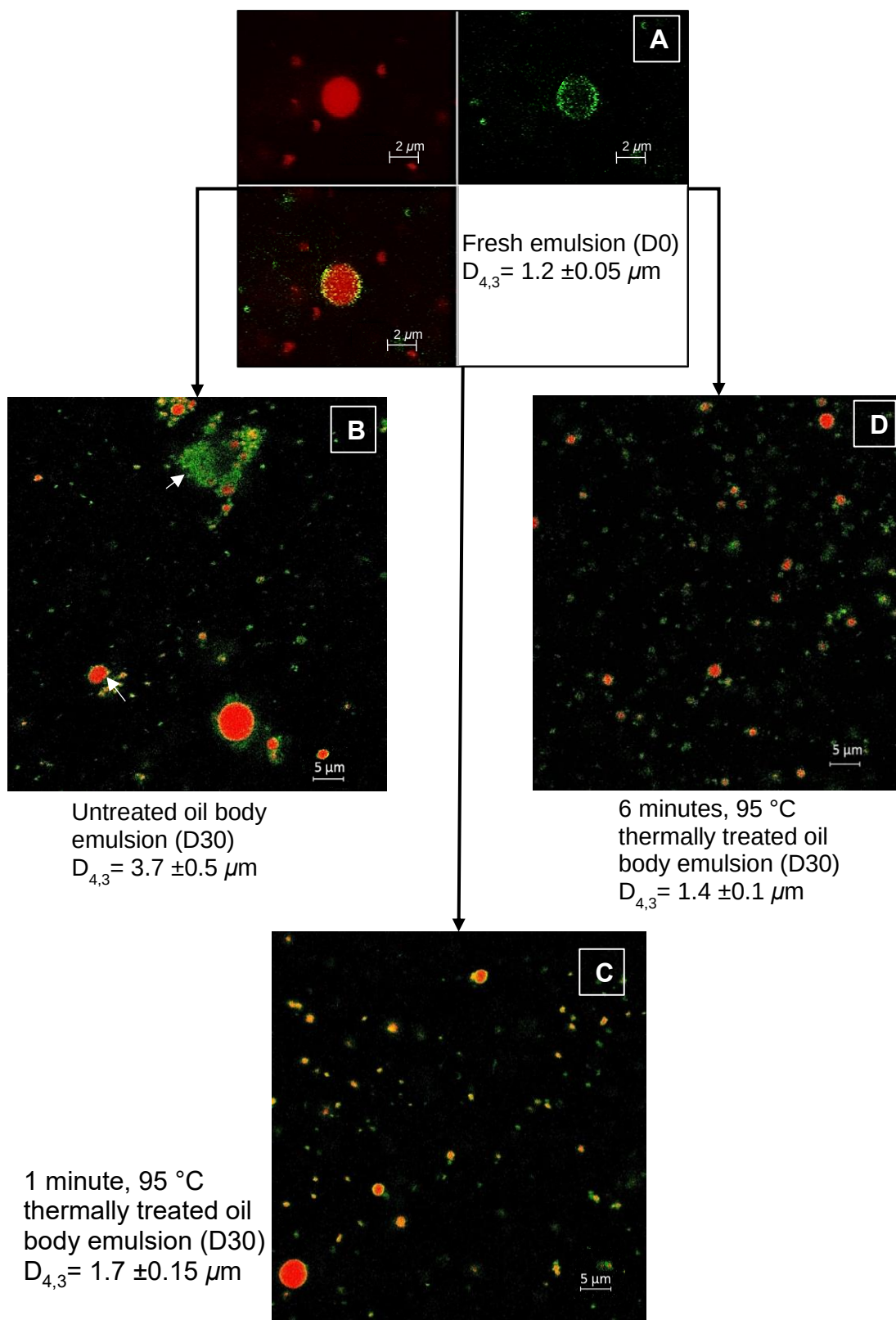


Figure 5.5: Confocal imaging aged (D30) emulsions. Panel B-C-D: untreated, thermally treated for 1/6 min. 95°C, respectively. Panel A: detail of oil body droplet at the start of the storage trial (D0). Green: protein; Red: oil. Arrows show oil bodies with altered droplet structure.

Bearing in mind that the addition of NaN_3 should limit microbial spoilage, the primary cause of the poor oil bodies stability *ex-vivo* (Figures 5.2-5.4), as well as *in-vivo* (Andreas Hoppe and Theimer, 1997), seems to be related to the carried over enzymes in the extract. In untreated emulsions, the instability may be triggered by oleosin proteolysis (Figure 5.1), which would cleave the hydrophilic domains (N- and C- terminus). Consequently, the electrical charge repulsions and steric hindrance which prevents oil bodies from coalescence (Tzen et al., 1993, 1992) will not be based on the exposed oleosin domains, but mainly on the zwitterionic properties of the exposed phospholipid layer. Moreover, the presence of lipolytic enzymes (as discussed in chapter 4) may promote the partitioning of the TAGs between the core and the surface of the oil bodies (Pieroni and Verger, 1979; Tzen and Huang, 1992). The release of acid molecules (fatty acids) in the vicinity of the oil body surface may affect the arrangement of oleosin hydrophilic domains. Altered surface chemistry is likely to reduce the steric barrier and make oil bodies more prone to coalescence.

On the other hand, despite thermal treatment significantly inactivating hydrolytic enzymes (>90% inactivation of lipase after 6 minutes at 95 °C), some coalescence was observed over the first days of the trial (Figure 5.3-5.4). Nikiforidis and Kiosseoglou (2009) found a direct correlation between content in exogenous proteins associated with the oil body surface, and storage stability. Nikiforidis and Kisseoglu (2009) hypothesised that, using a neutral pH solution, oil bodies covered by a secondary layer of exogenous maize proteins. This exogenous protein layer was believed to play an important role against coalescence over long term storage, through increase of the steric forces and strength of the surface membrane. However, the

recovery method developed in this thesis was designed to reduce the concentration of exogenous proteins to avoid high enzymatic load in the final oil body material (as discussed in Chapter 3). Taking into account that the recovered oil bodies using NaHCO_3 had an oil and protein content of (on a dry weight basis) 93 and 2.3%, respectively, and the mean diameter ($D_{3,2}$) was $0.85\ \mu\text{m}$, it was possible to calculate the surface protein load (McClements, 2004) per unit surface, which was approximately of $3.5\ \text{mg/m}^2$. This value is considered satisfactory for the stabilization of droplets against coalescence, and it is much lower to the one reported for oil bodies with the exogenous maize proteins ($12\ \text{mg/m}^2$) (Nikiforidis and Kiosseoglou, 2009). Moreover, bearing in mind that the oleosin molecules lay flat at the interface of oil bodies (Tzen and Huang, 1992), Nikiforidis and Kiosseoglou (2010) suggested that the steric forces operating between native oil bodies suspended in an aqueous medium was expected to be relatively weak, which could make the oil bodies more susceptible to coalescence (as shown in Figures 5.2-5.5). Therefore, the electrostatic repulsion between droplet is the driving factor that promotes oil body physical stability.

These results demonstrate that the thermal treatments enhanced the stability of oil bodies; a reduction of at least 90% of the lipolytic and proteolytic activity is necessary for long term storage. For a deeper understanding of the colloidal stability of aged emulsions, ζ -potential was measured (Section 5.2.3).

5.2.3. Zeta potential of aged emulsions

To better understand the destabilising phenomena of aged emulsions and how the enzymes can affect the surface charge, the ζ -potential of fresh and aged oil bodies was measured over 30 days at pH values from 3 to 10 (Figure 5.6).

At day 0, all the samples showed a zeta potential changing from about $+55 \pm 4.5$ mV at pH 3 to -60 ± 2.5 mV at pH 10 reaching zero between 6.3 and 6.5, which is a typical behaviour for protein-covered oil bodies (Tzen et al., 1993, 1992). As expected, ζ -potential profiles of the untreated samples changed significantly over time. While ζ -potential is similar at the extreme of pH values (3, 8, 9 and 10), a shift towards more negative ones was seen around the pH region of 4 to 7. Moreover, oil body preparations showed pI values at more acidic pHs (5.7, 5.1 and 5 for 7, 14 and 30 days of storage, respectively). These changes over time were diminished in a temperature/time dependent fashion, suggesting that a loss of hydrolytic enzyme activity preserved the surface chemistry (and so the charge) over storage. Most of the changes happened in the first 7 days of storage, reducing the pI at about 6. However, after 30 days the pI of the 1 minute treated samples was further reduced to 5.5, while the 6 minutes treated samples did not show further significant change. With a reduction of 90% of the total lipolytic activity, the changes in the zeta potential are quite limited, and only confined at pH values of 5, 6 and 7.

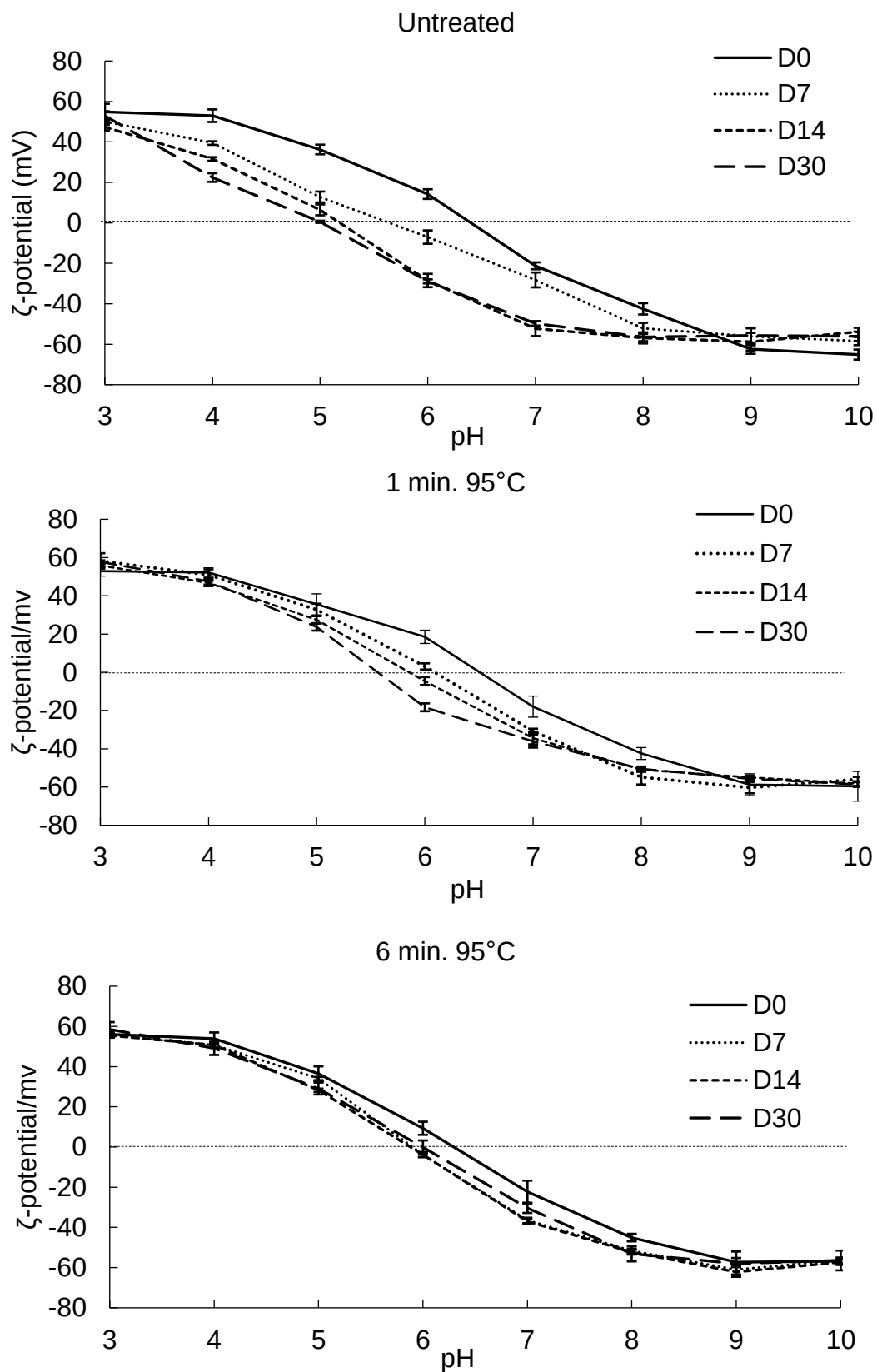


Figure 5.6: Zeta potential of aged untreated and thermally treated oil body emulsions (1 and 6 minutes 95°C) at day 0/ 7/ 14/ 30.

It is likely that the oil body-associated proteolytic activity is responsible for most of the changes that are seen in the ζ -potential of untreated emulsions. However, lipolytic activities may be also considered. The hydrolysis of oleosin exposes the charged hydrophilic heads of the PLs to the continuous phase, and the lipolytic activity releases negatively charged fatty acids to the exterior. As the steric protection of the oil bodies provided by oleosins is lost, the high negative charges seem not to be enough to overcome the attraction forces, leading to coalescence, as it is shown in Figure 5.2. The limited changes in zeta potential for thermally treated emulsion are probably due to the reduced enzymatic activity. However, using a short-time thermal treatment, the emulsion displayed a higher negative charged surface than the 6 minutes treatment. This is probably due to the residual lipolytic activity that may have released FAs at the exterior and proteolytic activity (not visible on SDS-PAGE in Figure 5.1) affecting both surface charge and oleosin conformation.

It is worth noticing that changes in the ζ -potential in the first 7 days for the 6 minutes treated emulsion is parallel to the shift in size that happens in the same period (see Figure 5.4). It was already discussed in Section 3.3.2.3 that the size of oil bodies might affect the electrophoretic mobility under the electric field, due to the drag force that is opposed to the movement (McClements, 2004). For this reason, it is probable that the decay of ζ -potential values might be due to the size increase that took place over the first days of storage trial.

5.3. Chemical stability

5.3.1. Fatty acid and composition of polar and neutral lipid from fresh oil bodies

Phospholipids, despite being a minor component of oil bodies, constitute up to 80% of the monolayer around the triacyclglycerol core (Tzen and Huang, 1992), and contribute to the stabilization of the droplets through interaction with oleosins. To analyse these components, a total lipid extraction (Folch et al., 1957) was performed on rapeseed seeds and fractionated with SPE (Section 2.4.4). The phospholipid and neutral lipids were isolated according to the method by Tzen and Huang (1992) from untreated/ thermally treated fresh oil bodies (1-6 minutes, 95 °C) and ran on thin layer chromatography (TLC, see Figure 5.7).

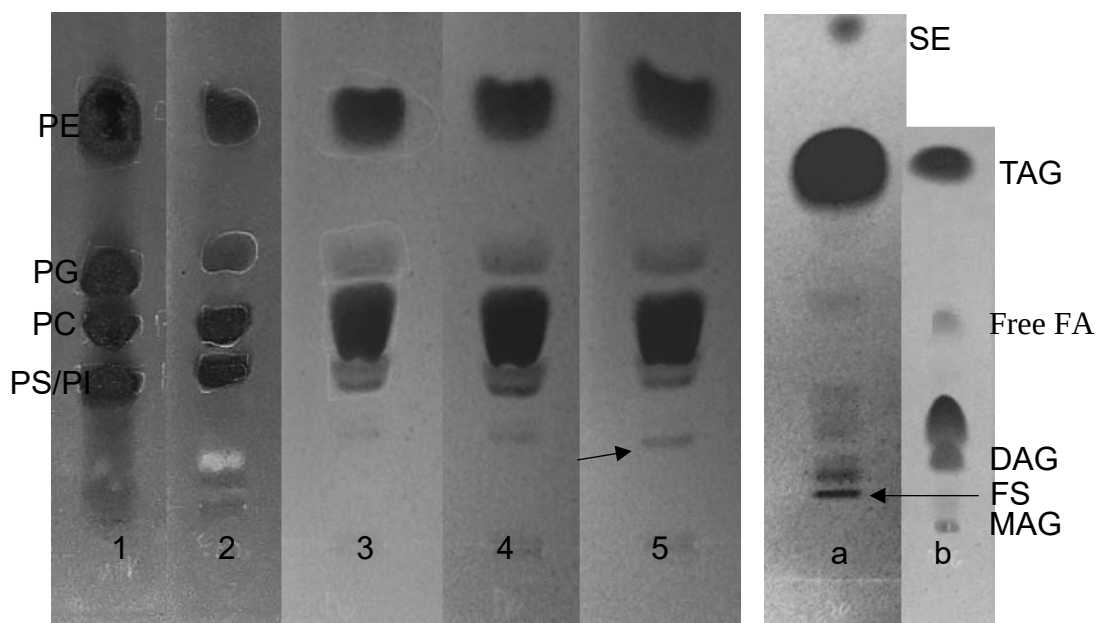


Figure 5.7: Polar and neutral lipid separation by TLC of rapeseed seeds and oil bodies. Lanes 1-5: phospholipid from seed (lane 2), and oil bodies (lane 3/ 4/ 5 for, respectively, untreated and thermally treated for 1 and 6 minutes, 95 °C). Lane 1 represent the standard mix containing: PE-phosphatidylethanolamine, PG-phosphatidylglycerol, PC-phosphatidylcholine, PS/PI- co-migration of phosphatidylserine and phosphatidylinositol. Lane a: neutral lipid from untreated oil bodies (representative of all the samples considered). Lane b represents the standard mix. **SE**: sterol esters, **TAG**-triacylglycerol, **DAG**- diacylglycerol, **MAG**-monoacylglycerol, **FreeFA**- Free Fatty Acids, **FS**- Free Sterols. Arrow indicates lyso-PC.

Phospholipid (PL) and neutral lipid (NL) groups could be effectively resolved by TLC (Figure 5.7), and Table 5.1 shows the composition of PL for each sample, based on imaging analysis of band intensities (in Figure 5.7). The most abundant phospholipid in seed was phosphatidylethanolamine (PE), followed by phosphatidylcholine (PC), phosphatidylserine/ phosphatidylinositol (PS/PI, which co-migrate in the solvent system considered) and phosphatidylglycerol (PG). However, in recovered oil bodies, the major class was PC accounting to approximately half of the phospholipid content, followed by PE, PS/PI and then PG. No difference was found for the neutral lipid composition between seed and oil body extracts, which were composed

majorly by TAGs, with small amounts of diacylglycerols (DAGs), Free Sterols (FE) and sterol esters (SE).

Table 5.1: Phospholipid and neutral lipid composition of rapeseed seeds and oil bodies (untreated and thermally treated for 1 and 6 minutes, 95 °C)

	Seed	Untreated	1 min. 95°C	6 min. 95°C
PE (%)	34.9 ± 1.5 ^a	37.5 ± 2.2 ^a	37.8 ± 3.2 ^a	36.9 ± 3.9 ^a
PG (%)	13.1 ± 1.4 ^a	5.4 ± 0.7 ^b	6.3 ± 0.8 ^b	6.6 ± 0.5 ^b
PC (%)	24.8 ± 2.1 ^a	48.1 ± 4.4 ^b	47.3 ± 5.1 ^b	49.1 ± 4.8 ^b
PS/PI (%)	27.1 ± 2.2 ^a	9.0 ± 0.8 ^b	8.5 ± 1.2 ^b	7.3 ± 0.8 ^b
SE (%)	1.1 ± 0.2 ^a	1.0 ± 0.3 ^a	0.8 ± 0.2 ^a	0.9 ± 0.2 ^a
TAG (%)	95.4 ± 0.5 ^a	95.8 ± 0.5 ^a	95.6 ± 0.7 ^a	95.2 ± 0.6 ^a
DAG (%)	1.2 ± 0.4 ^a	1.3 ± 0.4 ^a	1.4 ± 0.4 ^a	1.6 ± 0.3 ^a
FS (%)	2.3 ± 0.3 ^a	1.9 ± 0.6 ^a	2.2 ± 0.5 ^a	2.3 ± 0.3 ^a

PE- phosphatidylethanolamine, **PG**- phosphatidylglycerol, **PC**- phosphatidylcholine, **PS/PI**- comigration of phosphatidylserine and phosphatidylinositol, **SE**- sterol esters, **TAG**- triacylglycerol, **DAG**- diacylglycerol, **FS**- Free Sterols. Different (a, b) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the composition in PL/TAG of seed and recovered oil bodies was evaluated.

Phospholipid extracts from oil bodies also contained another band at the bottom of the TLC plate (lyso-PC, indicated by an arrow), which increased over thermal treatment. PL hydrolysis products may be a result of phospholipase A1 on PC (Richmond and Smith, 2011), which could have been active over seed soaking and/or oil body recovery, generating lyso-PC and fatty acids (FAs). However, this is not supported by the findings of Tzen and

Huang (1992), showing that the presence of oleosin limits phospholipase A2 and C activity, and phospholipid hydrolysis would occur only upon removal of the protein coat. Moreover, considering the time and temperatures applied during thermal process (6 minutes, 95 °C), it is unlikely that the increase in lyso-PC over heating is related to phospholipase activity. On the other hand, lyso-PC can also be produced from a different reaction. In fact, in addition to the Kennedy pathway for the generation of TAG molecules, involving three sequential transfers of acyl groups from acyl-CoA molecules to a glycerol backbone (Stymne and Stobart, 1987), TAG can also be synthesised by phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000; Ståhl et al., 2004). This reaction involves the transfer of an acyl group from a PL (e.g., PC) to a DAG molecule, to form TAG and lyso-PC. Furthermore, the presence of this band mainly in thermally treated samples, also suggests that PC may have been partially degraded to lyso-PC due to the high temperatures. As a support of this hypothesis, similar thermal degradation of PLs was described in egg yolk (Nemecz and Mennear, 1995), where the exposure of the egg to boiling water produced time-related elevations in the concentration of lyso-PC.

The lipid fractions on TLC for neutral and polar lipid were recovered and derivatised to be analysed by gas-chromatography (Figure 5.8). Canola oil is rich in oleic acid (C 18:1), plus contains linoleic (C 18:2) and α -linolenic (C 18:3) acid, with small amounts of palmitic (C16:0) and stearic (C 18:0) acid. The PL fatty acid composition of untreated and thermally treated oil body shows the same fatty acid species as the NL but the saturated: unsaturated fatty acid ratio varies depending on the PL class (Figure 5.8). Unsaturated fatty

acids make up to approximately 95% of total NL fatty acid composition. In comparison to this, it was found that PG and PS in untreated samples contain approximately 70% of unsaturated FAs and 30% saturated FAs, which proportion was not affected by heating. PC and PE from showed slightly higher percentage of unsaturated FAs (89 and 78%, respectively) which furtherly increased in thermally treated samples (up to +4 and +10%, respectively).

The difference in FA composition between the major PL groups suggests that thermal treatment followed by centrifugation may affect some PL species, not detectable in Table 5.1, probably due to the sensitivity of the analysis used. This phenomenon was also seen when oil bodies were exposed to urea over washing protocol (Millichip et al., 1996). However, it was already shown in Chapter 4 that thermal treatment did not lead to size increase of oil bodies. Fatty acids profile from oil body PL have already been characterised in *Echium plantagineum* (Payne et al., 2014), in two cultivars of rape seed (Katavik et al., 2006), and sunflower seed (Furse et al., 2013; Millichip et al., 1996). Payne et al. (2014) and Katavik et al. (2006) found that the saturated fatty acids were the predominant species in the oil body PLs; the lack of double bonds was believed to promote a firm anchoring of oleosin thus strengthening the surface (Payne et al., 2014). However, this hypothesis is in disagreement with the findings discussed in this thesis. In fact, oleosin was shown to be tightly associated with the oil body, so to resist the harsh treatment with urea (Section 3.3.1.1), despite the higher concentration in unsaturated fatty acids in PLs.

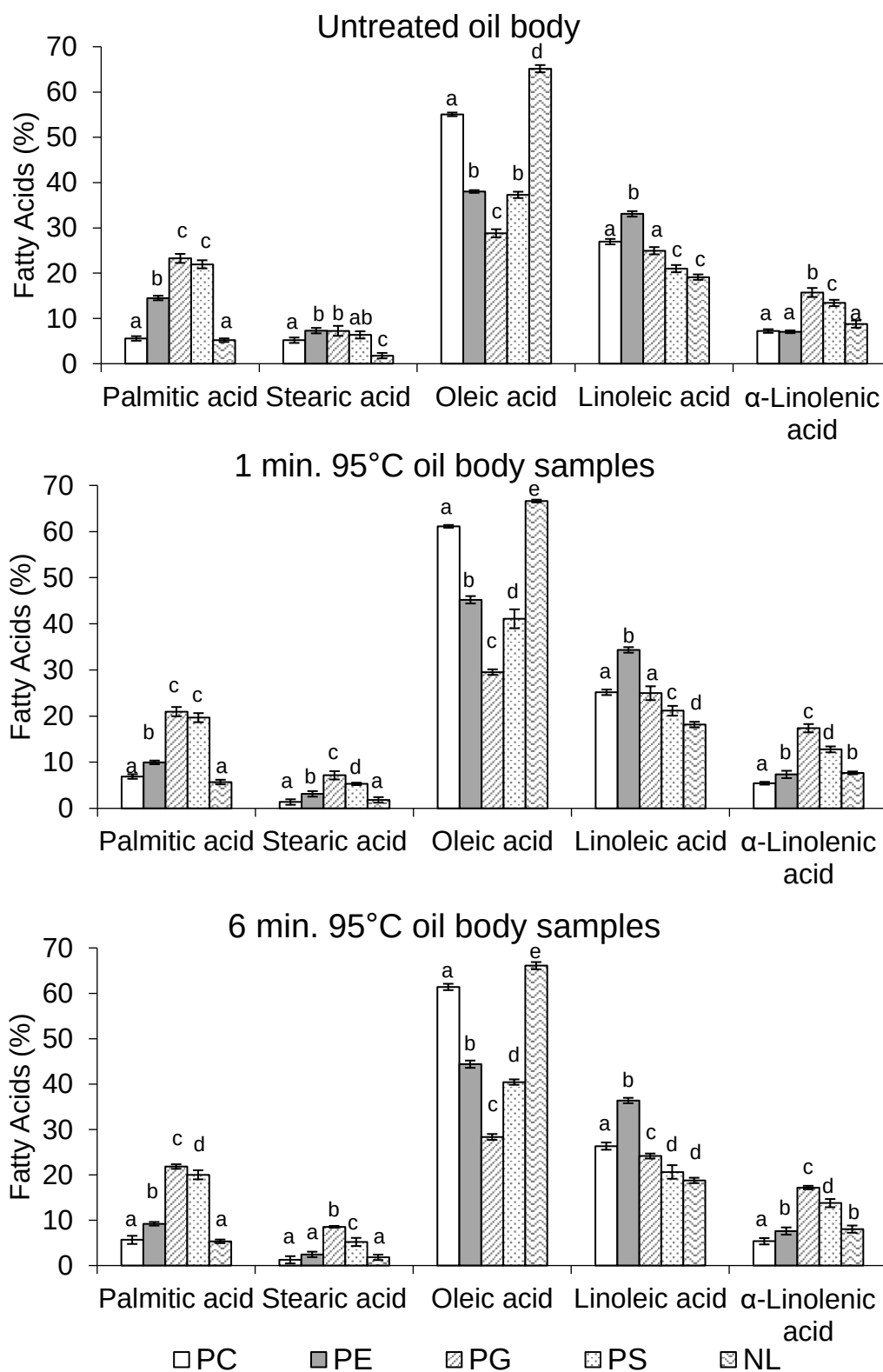


Figure 5.8: Fatty acid profile of phospholipids and neutral lipid from fresh rapeseed oil bodies. PE- phosphatidylethanolamine, PG- phosphatidylglycerol, PC- phosphatidylcholine, PS phosphatidylserine, NL- Neutral lipid. Different (a, b, c, d, e) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the fatty acid percentage among PLs was evaluated.

5.3.2. Fatty acid and composition of polar and neutral lipid from aged oil bodies

Oil body emulsions (10%, lipid weight basis) were recovered from rapeseed seeds using NaHCO_3 (0.1 M, pH 9.5) over grinding and washing step, then rinsed once using dH_2O , and stored at 20 °C for a period of 30 days. At day 7, 14 and 30 the emulsions were centrifuged to recover the aged oil body cream. The degradation of oil body proteins (e.g., due to proteases) could coincide with changes in PL and TAG composition. Therefore, the composition of these components over storage (Figure 5.9) was studied using TLC. Polar and neutral lipids were isolated from a total lipid extract recovered from 1 g of cream at each time point, to then analyse the lipid groups on thin layer chromatography (TLC) and fatty acids composition of the main lipid species through gas chromatography. Thermally treated samples (1 and 6 minutes, 95 °C) were used to study the impact of heating on the chemical stability of oil bodies against the enzymatic activity. All stored emulsions contained NaN_3 to avoid microbial spoilage (0.02 mM).

The analysis revealed that the amount of PE from untreated emulsions (panel A, left) was depleted gradually over the first two weeks, to then almost disappear at the end of the storage trial. In addition to PE, the concentration of other phospholipid groups (PC, PG, PS/PI) remained steady over the first two weeks, to then dramatically be reduced between 14 to 30 days. Moreover, the analysis of neutral lipid of these oil body samples (Figure 5.9 A, right) showed the gradual increase in TAG degradation products (fatty acids, FA; diacylglycerol, DAG; monoacylglycerol, MAG) over the storage trial. However, no dramatic changes were seen in heated emulsions for all lipid groups

considered (Figure 5.9, B-C), probably due to a reduced enzymatic activity after thermal treatment.

In addition to the usual phospholipids that compose the lipid monolayer of oil bodies (PE/ PC/ PG/ PS-PI), a band in untreated and 1 minute thermally treated sample started to appear between one to two weeks (Figure 5.9 A-B, indicated by arrows), suggesting that some enzymatic activity takes place even in short-time thermally treated samples.

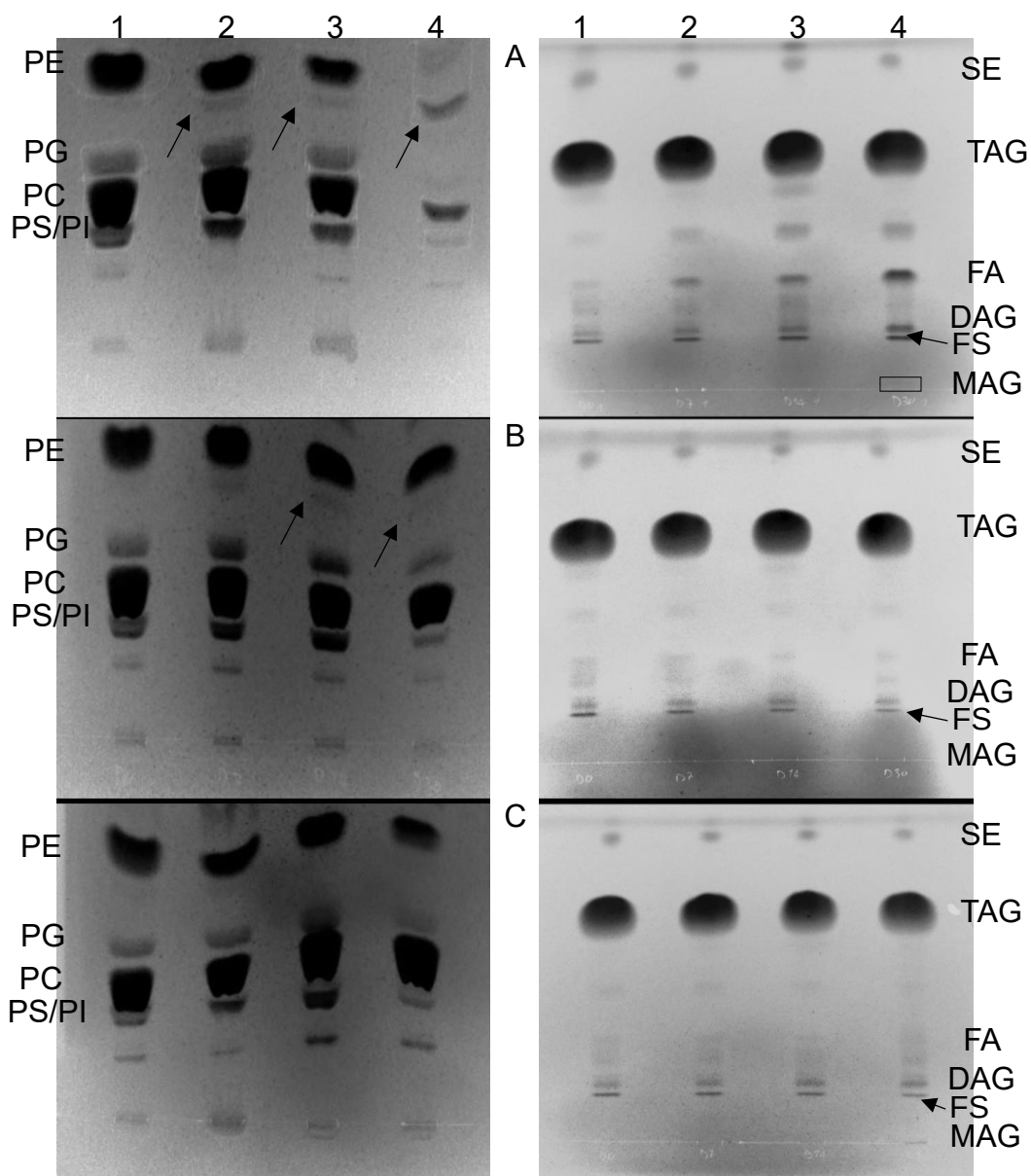


Figure 5.9: Polar (left) and neutral (right) lipid fractions of aged oil bodies analysed by TLC. A: untreated; B: 1 minute, 95°C; C: 6 minutes, 95 °C. Lanes 1/ 2/ 3/ 4 represent 0/ 7/ 14/ 30 days, respectively. Arrows indicate degradation products of phospholipid.

Under the right conditions (e.g., temperature, substrate availability, pH) each phospholipase may generate a different product depending on the ester bond that is cleaved (Figure 5.10).

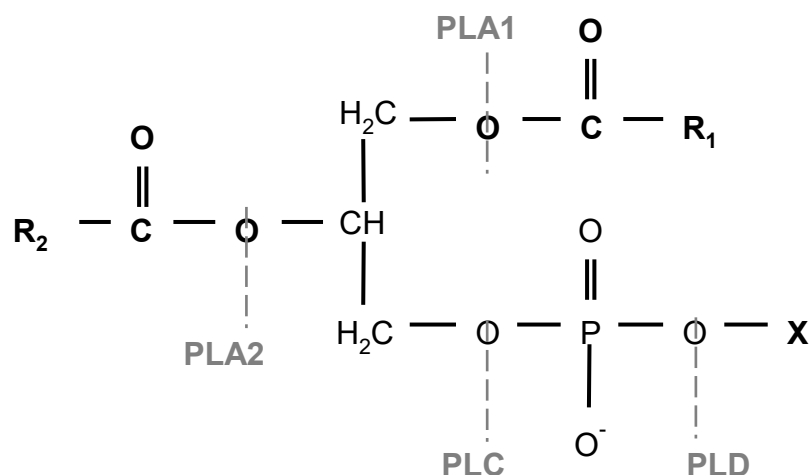


Figure 5.10: Phospholipid structure and the cleave sites of phospholipases. Phospholipid molecule consists of a glycerol-3-phosphate molecule esterified at *sn*-1 and *sn*-2 positions to non-polar fatty acids (in bold, R_1 and R_2) and at its phosphoryl group to a polar head group (X). Cleavage site and phospholipases are indicated in grey. PLA1-2- phospholipase A1 and phospholipase A2; PLC-phospholipase C; PLD-phospholipase D. Image adapted from Maroun et al. (2010).

Phospholipase A cleaves the acyl ester bond at *sn*-1 (A1) or *sn*-2 (A2) position, generating lyso-phospholipids; phospholipase C cleaves the glycerophosphate bond, generating DAG; phospholipase D removes the polar head group generating phosphatidic acid. Bearing in mind that the hydrolysis products of phospholipase A (lyso-phospholipids) should migrate at the bottom of the TLC plate in this solvent system, the presence of a band just below PE could be related to the formation of phosphatidic acid (PA). However, these bands may also correspond to oxidized species of PLs (Section 5.3.3).

The presence of increasing concentrations of FA, DAG and unusual phospholipids (presumed PA) from untreated and short time (1 minute) thermally treated samples (Figure 5.9 A-B), can, therefore, be explained by the catalytic activity of lipolytic enzymes on oil bodies. However, when thermal treatment is extended to 6 minutes, corresponding to more than 90%

inactivation of lipase (as discussed in Chapter 4), no clear sign of enzymatic activity *ex-vivo* can be seen; thus, confirming the lipase activity as a good marker for the inactivation of other lipolytic enzymes.

Neutral lipid, PC and PE bands (corresponding to the major classes of phospholipids) from untreated and thermally treated samples were recovered from TLC plates and the extracts were derivatised for gas chromatography analysis. Figures 5.11 and 5.12 show the composition percentage in unsaturated and saturated fatty acids at each time point for PC/PE and neutral lipid, respectively. After storage at 20 °C, only untreated emulsions showed changes in the saturated: unsaturated ratio. Unsaturated FAs from PE were the most depleted after four weeks, making up only 38% of the total FA composition. Despite PC showing similar trends, the decrease in unsaturated FAs was not as dramatic (Figure 5.10). Moreover, the analysis of NL from untreated emulsions after storage (Figure 5.11), showed a gradual decrease in unsaturated FAs, and relative increase in saturated FAs, suggesting that some oxidation took place over storage. Some changes to a much lesser extent were seen in thermally treated emulsions between 3 and 4 weeks of storage. The oxidative stability of untreated and thermally treated samples will be discussed in Section 5.3.3.

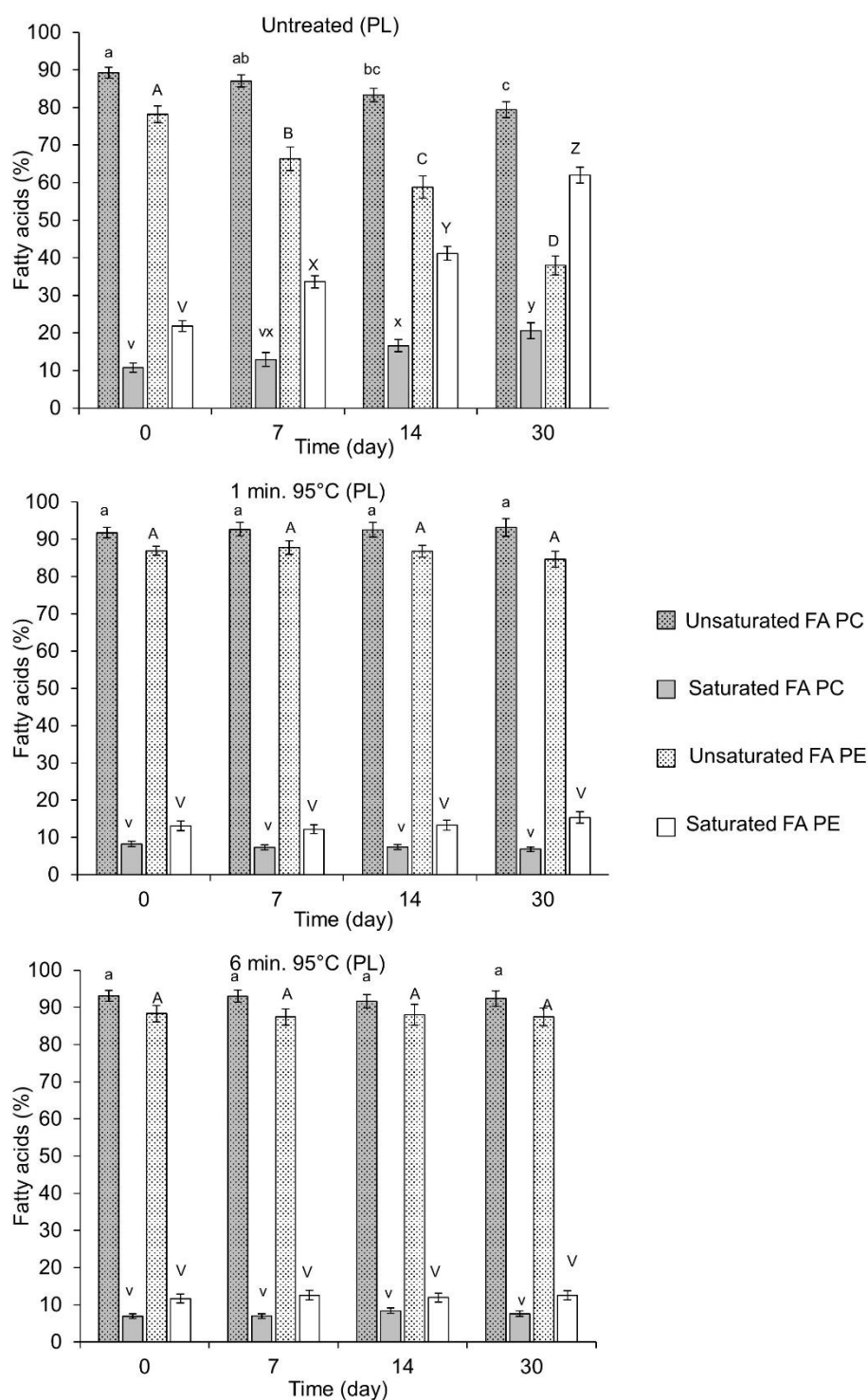


Figure 5.11: Composition in unsaturated and saturated fatty acids of PE and PC from aged oil body emulsions. PC and PE bands were scraped off from TLC plated after separation of phospholipids extract of untreated and thermally treated (1- 6 minutes, 95 °C) of aged oil body emulsions, and analysed by gas chromatography. Different (a, b, c, d, e) and (v, x, y, z) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the unsaturated and saturated fatty acid percentage of PC were evaluated. Different (A, B, C, D, E) and (V, X, Y, Z) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the unsaturated and saturated fatty acid percentage of PE were evaluated.

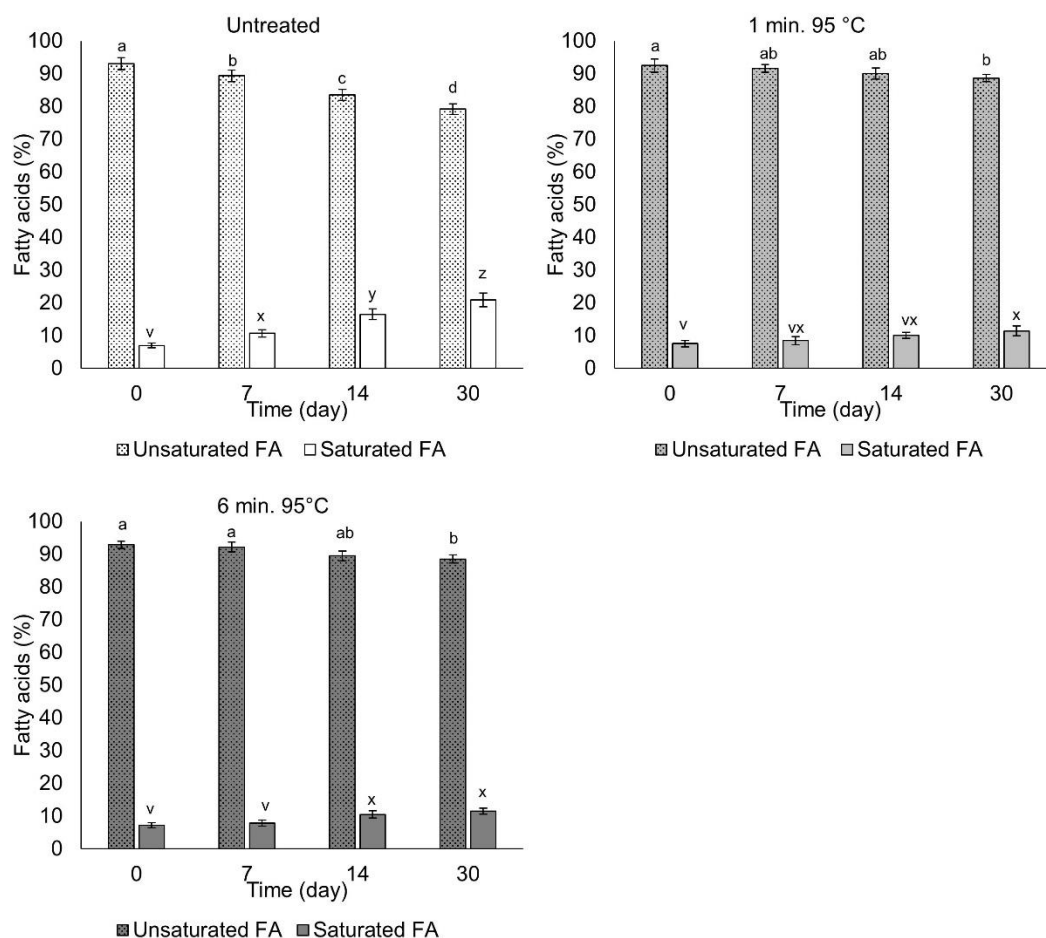


Figure 5.12: Composition in unsaturated and saturated fatty acids of neutral lipid from aged oil body emulsions. Neutral lipid extracts of untreated and thermally treated (1- 6 minutes, 95 °C) of aged oil body emulsions, were derivatized and analysed by gas chromatography. Different (a, b, c, d, e) and (v, x, y, z) letters indicate non-homogenous groups established by ANOVA ($P < 0.05$) when the unsaturated and saturated fatty acid percentage of TAGs were evaluated.

Oleosin degradation is a major factor regulating the activity of other enzymes in oil body extracts (Matsui et al., 1999; Sadeghipour and Bhatla, 2002). Tzen and Huang (1992) showed that the presence of oleosin protected PLs against the activity of phospholipase A2 and C; and phospholipid hydrolysis only occurred when oleosin was degraded by a commercial protease. The phospholipase A2 was shown by May et al. (1998) to be in association with the oil body during TAG mobilization in cucumber. Moreover, Abdelkarim et al. (2004) suggested that a phospholipase D may also be

involved in the degradation of oil bodies from sunflower seeds. Because untreated samples are the ones that show higher proteolysis (Figure 5.1), they are clearly expected to be the ones that are most susceptible to lipolytic activity (see Figures 5.9 and 5.11-5.12).

5.3.3. Effect of thermal treatment on the oxidative stability of oil body emulsions

Oxidation of oil is a serious concern in many sectors of the food industry, which can lead to the development of undesirable off-flavours and toxic compounds (Guillén et al., 2005), altering the sensory quality of the product (Frankel, 2005). This phenomenon is sometimes enhanced by the tendency of food industries to discourage the use of synthetic antioxidants, or fortifying food with pro-oxidants ingredients (e.g., iron).

The production of lipid radicals is the first step of oxidation, which are formed through the free radical chain reaction in the presence of an initiator, followed by a propagation step fuelled by atmospheric oxygen. Firstly, lipid hydroperoxides are formed, which are then broken down to aldehyde, ketones and hydrocarbons producing the characteristic off-flavours (Frankel, 2005). However, oil bodies recovered through aqueous extraction from a wide range of oilseeds have been proved to be highly stable against oxidation (Chen et al., 2012; Fisk et al., 2008; Gray et al., 2010; Karkani et al., 2013). Fisk et al. (2008) showed that the production of hydroperoxides and secondary oxidation products was significantly lower in oil bodies compared to emulsions stabilised with other synthetic emulsifiers (DTAB, SDS, Tween). Moreover, the chemical

stability could be further increased in oil bodies with high exogenous protein content (Karkani et al., 2013).

5.3.3.1. Primary and secondary oxidation

The aim of this section is to study the effect of thermal treatment on the oxidative stability of oil bodies in accelerated storage condition (40 °C). These data were correlated with particle size (Figure 5.14) and tocopherol content (Figure 5.15) to study their effect in the antioxidant stability. For this reason, oil bodies were recovered through grinding of rapeseed seeds in NaHCO_3 (0.1 M, pH 9.5), and washing in the same media. After a final rinse in dH_2O , untreated oil body creams were produced. Thermal treatment (1-6 minutes, 95 °C) was applied on oil body emulsions prior to the last centrifugation step. Untreated and thermally treated WOB II creams were re-suspended in sodium phosphate buffer (10%, lipid weight basis) and stored at 40 °C for up to 10 days. All emulsions were protected from light and contained NaN_3 (0.02 mM) to avoid microbial spoilage. Over storage, peroxide value (PV) and 2-Thiobarbituric (TBA) acid tests were performed to measure hydroperoxide and secondary products, respectively. The ferric thiocyanate method for PV is based on the oxidation of ferrous to ferric ions, which are determined colorimetrically as ferric thiocyanate. TBA test is based on the measurement of the pink-coloured solution after reaction of TBA with secondary products of polyunsaturated fatty acids (mostly malondialdehyde, MDA). Despite polyunsaturated fatty acids are not the major group in lipid extract from rapeseed oil bodies (Figure 5.8), they are 40 times more reactive than oleate in oxidative reactions, due to the

methylene on carbon 11 between two double bonds that can lose a hydrogen very readily (Frankel, 2005). For this reason, TBA value was chosen as marker for secondary product production.

Figure 5.13 shows the impact of heating oil body emulsions on hydroperoxide formation (A) and TBARS content (B) over storage at 40 °C. At the start of the experiment, hydroperoxide concentrations were close to zero in all oil body emulsions; and no significant difference was seen after heating the samples for up to 6 minutes (95 °C). Untreated emulsions had very low amounts of hydroperoxides over the first two days, which then increased steadily until the end of storage. Thermally treated samples showed higher concentrations of hydroperoxides during the first 6 days, but no difference was found between the two treatment times ($P < 0.05$). However, after day 6, oil bodies that were heated for 6 minutes showed a higher increase rate of hydroperoxides than the untreated and 1 minute heated samples (Figure 5.12 A).

TBARS formation was low for all oil body samples over the first two days. However, despite untreated emulsions showed low level of hydroperoxides, the formation of TBARS from day 2 were remarkably higher compared to the thermally treated samples, thus suggesting that the primary products were readily decomposed to secondary ones. Thermally treated emulsions showed little increase in TBARS content, and no difference was found between the two samples ($p < 0.05$).

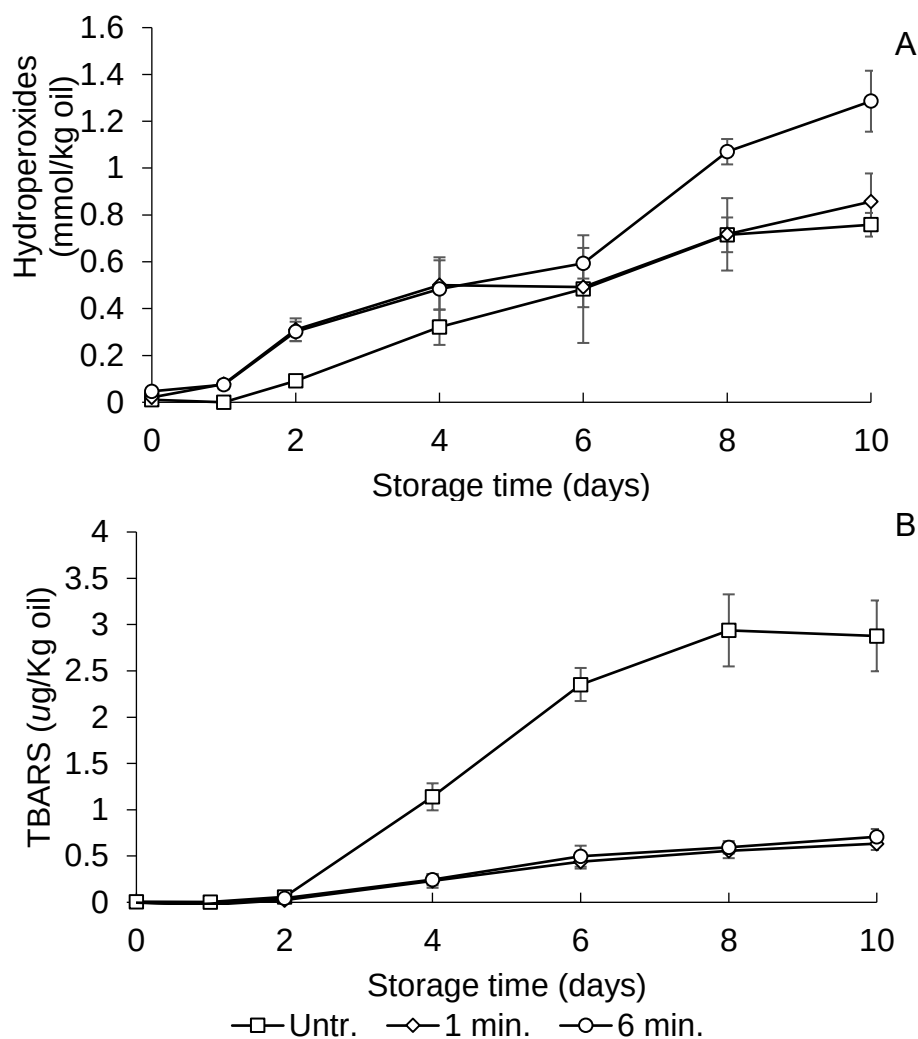


Figure 5.13: Hydroperoxide (A) and TBARS (B) content of rapeseed oil body emulsions. Untreated and thermally treated (1, 6 minutes, 95 °C) oil body emulsions (10%, lipid weight basis) were stored at 40 °C, measuring the hydroperoxides (A) and TBARS (B) at day 1/ 2/ 4/ 6/ 8/ 10. All emulsions contained NaN₃ to avoid microbial spoilage.

Due to the composition in fatty acids of rapeseed oil, the low stability against oxidation usually limits its application into food products (Gunstone, 2004). However, the results shown in this section revealed that the oxidative stability of oil bodies is satisfactory, having levels of peroxides below the limit for unsaturated oils (< 2.5 mmol/Kg) (Frankel, 2005); moreover, thermal treatment significantly improved the overall oxidative stability. The trends in

oxidation rates for untreated and thermally treated emulsions may be due to a combination of several factors, such as droplet characteristics (e.g., protein content, surface charge, particle size, aggregation patterns), and tocopherol content.

5.3.3.2. *Droplet characteristics*

Surprisingly, untreated emulsions were the only samples that showed a level of oxidation close to zero in the first two days of storage. The presence of higher levels of exogenous proteins associated with the surface of oil bodies (as discussed in Chapter 4), may have affected the oxidative rates of the oil. These proteins may be preferentially oxidised or may quench free metal ions protecting the oil from oxidation. Moreover, the formation of aggregates over storage in untreated emulsions, which has been discussed in Section 5.2.2, may have altered the oxidative properties of oil bodies as well. When large aggregates are formed, the droplets at the exterior may protect the ones in the interior from initiators or pro-oxidants. However, oil bodies in thermally treated emulsions were present as small, discrete droplets over storage, which may have made them more susceptible to initiators.

To study the involvement of droplet integrity on oxidation rates, the mean volume diameter ($D_{4,3}$) was measured over storage (Figure 5.14). It can be clearly seen from the graph that untreated and thermally treated emulsions were stable until the second day. However, from day 2 untreated emulsions showed enhanced coalescence, probably due to the catalytic activity of the oil body-associated enzymes. The increase in size clearly coincides with a

change in trend for the TBARS formation, suggesting a correlation between droplet integrity and low oxidative rates (Figure 5.13).

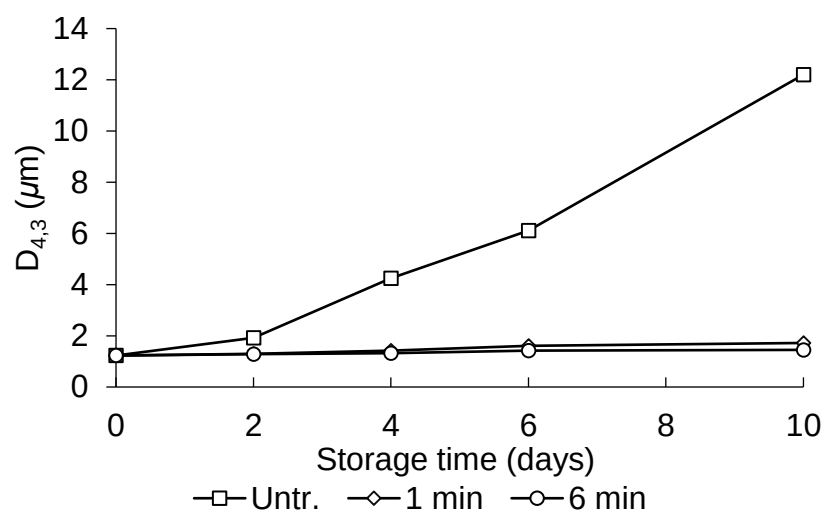


Figure 5.14: Mean volume diameter ($D_{4,3}$) of untreated and thermally treated emulsions stored at 40 °C.

The unique surface structure of oil bodies, with oleosin proteins imbedded into the phospholipid monolayer, may not only be responsible for droplet integrity, but also act as a physical barrier against pro-oxidants. When this structure is lost by hydrolytic enzymes, the surface-active hydroperoxides that reside on the droplet surface (McClements and Decker, 2000), get more exposed to the external environment. As a consequence, they can be readily degraded into highly reactive peroxy and alkoxy radicals by pro-oxidants, which is the most likely step that accelerates oil oxidation (McClements and Decker, 2000; Mei et al., 1998a, 1998b).

It is worth considering the effect of pro-oxidant enzymes (e.g., lipoxygenases) in the formation of hydroperoxides, which may have affected

the results shown in Figure 5.13. Unfortunately, the assessment of this enzymatic activity was not possible.

5.3.3.3. *Tocopherol content*

To study the involvement of the intrinsic antioxidants in the oxidative properties of oil body emulsions, tocopherols were measured in aged emulsions using HPLC (Figure 5.14). In chapter 4 the effect of thermal treatment on tocopherol degradation was already discussed, showing that heating oil bodies for up to 6 minutes at 95 °C reduced their concentration of about 25%. Despite the lower content in tocopherols due to heating, no further decline was seen in thermally treated emulsions over storage. However, total tocopherols content in untreated samples showed a decay from day 2, decreasing of about 50% by the end of storage trial.

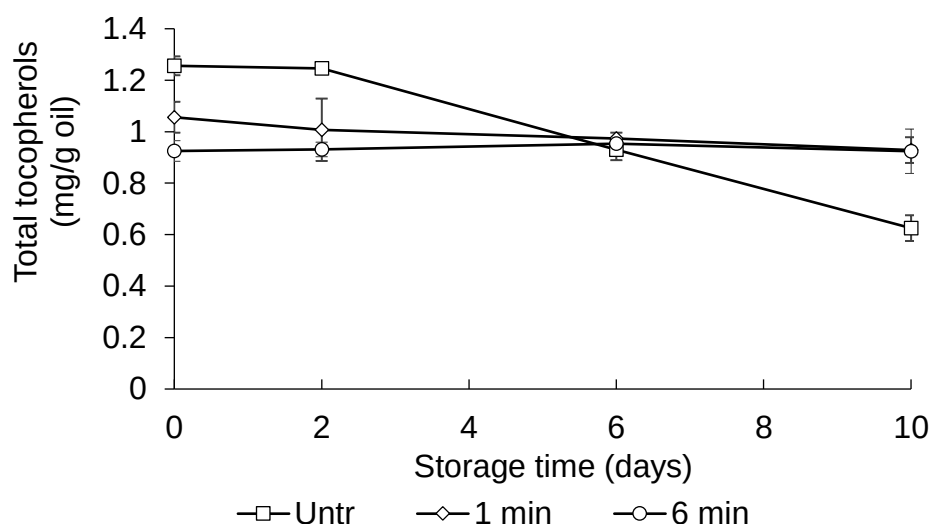


Figure 5.15: Tocopherol content of oil body emulsions over storage at 40 °C.

Tocopherols are chain breaking antioxidants, which can delay the initiation step or interrupt the propagation by readily donating hydrogen atoms to lipid alkyl, alkoxyl and peroxy radicals (Frankel, 2012). In oil-in-water emulsions, the effectivity of tocopherols as antioxidants is dependent on the concentration (Frankel, 2012), and on the type (Wagner et al., 2004; Zaunschirm et al., 2018). However, tocopherols should be effective antioxidants for their amphipathic nature (McClements and Decker, 2000), as the hydrophobic side chain would be included in the PL layer, while the phenolic head would be located on the surface, protecting the oil under oxidative stress.

5.4. Summary of results

In chapter 4, lipase enzymes were recovered from oil bodies (before and after heat treatment) and its activity was measured in vitro; there was a clear reduction in activity after heat treatment of oil body emulsions. In this study, the possible correlation between enzyme activity, altered by heat treatment, and oil body stability (physical and chemical) was explored. Proteolytic activity, which is thought to have the highest impact on oil body integrity, was significantly reduced with a thermal treatment corresponding to either 60 or 90% of lipolytic enzymes inactivation. Therefore, droplet integrity and surface charge stability were enhanced. However, a reduction of 90% in lipolytic activity was necessary to stabilise the emulsion against physical destabilisation, chemical oxidation and loss of tocopherol over storage.

Biochemical studies revealed that rapeseed oil was high in oleic, linoleic and α -linolenic acid, with major phospholipids class being PC and PE. Over storage, untreated emulsions showed major changes in both lipid and fatty acid composition, probably due to lipolytic activity. However, only the samples heated for 6 minutes, 95 °C (corresponding to <90% inactivation of lipolytic activity), did not show significant changes. Moreover, thermal treatment significantly improved the oxidative stability of oil bodies.

The work discussed here shows a clear correlation between enzyme inactivation and improved stability of oil bodies, and that lipase activity measurement is a good marker for general enzyme inactivation during thermal treatment.

CHAPTER 6.

GENERAL DISCUSSIONS

6.1. Background

The grinding of oleaginous seeds in aqueous buffer leads to the formation (recovery) of a natural oil-in-water emulsion (oil bodies). In this study, oil bodies (Figure 6.1) were effectively recovered from rapeseed seeds by wet milling, filtration, and centrifugation of the buoyant fraction.

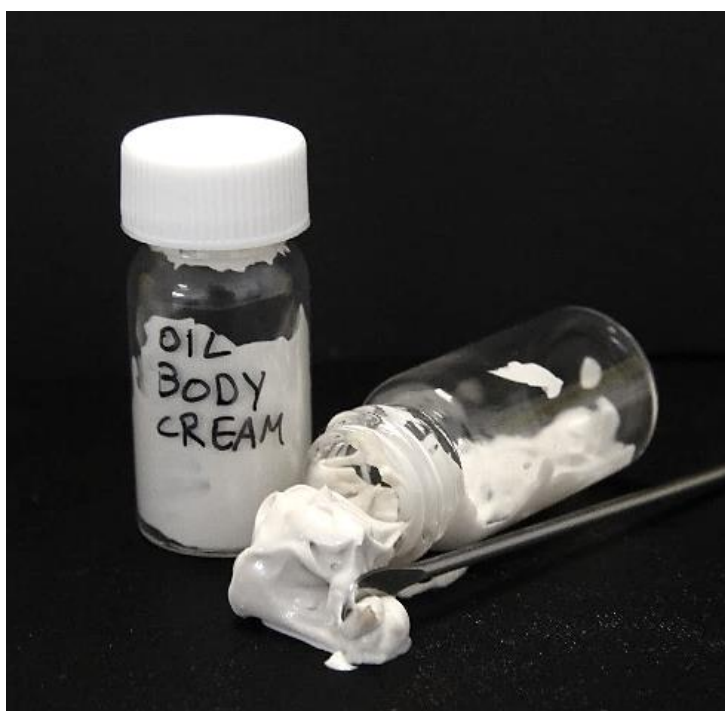


Figure 6.1: Oil body cream.

Because oleaginous seed oil bodies provide an emulsion of pre-dispersed oil, the recovery of these droplets is believed to be a greener method when compared to the current process of oil extraction, which involves the pressing of the ground seed material with the use of organic solvents (e.g., hexane). Moreover, the refining step implies the extensive use of chemicals and high temperatures to remove undesirable materials (e.g., phospholipids,

free fatty acids). Due to their composition and physical properties, oil bodies have the potential to be used widely in food, pharma and personal care products; to date only niche personal care applications have gone to market. Preparation of stable oil bodies is essential for any commercial application; two areas that are not well studied are the relationship between oil body stability and enzyme carry over, and the impact of thermal treatment on enzyme inactivation. In the case of the food industry, the pre-dispersed state of oil in the seed is of particular interest, as it could evade additional cost for synthetic emulsifiers and emulsification processes.

6.2. Oil body quality is affected by the recovery protocol

6.2.1. Effect on droplet integrity

When oil bodies are recovered through grinding of the seed matrix in an aqueous solution, a natural oil-in-water emulsion is formed. Oil bodies can be damaged during grinding, and the surface-active molecules in seed can stabilise the lipid droplets. Therefore, it is difficult to decide whether the recovered droplets are intact oil bodies (presenting only oleosin on the surface), or artefacts with a mixed surface composition, and thus with different physical properties. In Chapter 3, it is shown that the soaking and grinding step have the highest impact on the physical properties of the final oil body product. In regard of the grinding step, the choice of an appropriate solution at the right seed:volume of media ratio is fundamental to recover intact droplets. In literature, oil body integrity is usually measured by looking at the droplet size, which should be within the in-vivo range (0.5-2.5 μm) described by Tzen et al.

(1993). However, it is possible that the surface-active molecules in seed can stabilise the oil body with a diameter similar to the ones present in seed, thus making the size measurements doubtful for integrity tests.

The ability of urea to remove the exogenous proteins from oil body preparations has been reported in the literature (Lacey et al., 1998; Millichip et al., 1996). However, Nikiforidis and co-workers (2013) also found that oil bodies went under extended coalescence and release of oil after treatment with urea, probably due to protein (oleosin) denaturation. In this thesis, it is shown that oil bodies that have not been damaged over grinding step, retain their integrity after exposure to urea (9M), thus demonstrating to be a more appropriate methodology for integrity tests compared to size measurements. As detailed in Chapter 3, the grinding of seed in low viscosity systems (1:7 seed:grinding media volume ratio) and alkaline media (NaHCO₃, 0.1M, pH 9.5) limits the damage to the oil body, by creating enough repulsion forces and thus limiting coalescence.

6.2.2 Effect on oil body purity from exogenous seed material

One of the downsides of using oil bodies is related to their physiological role in the seed. Over germination, oil bodies are subjected to hydrolysis by endogenous seed enzymes (e.g., proteases, lipases and phospholipases) to convert TAGs into FFA and energy by β -oxidation. Although it is highly probable that these enzymes will be carried over in the oil body material using the aqueous recovery, they have not been the main focus in the latest research. In fact, to the best of our knowledge, the literature focuses only on

the physicochemical properties of oil bodies, without consideration of the effect of enzymatic activities on these characteristics.

The use of a specific grinding solution is the most efficient way to significantly reduce the exogenous seed protein in crude oil body extracts. In rapeseed, some exogenous proteins can be removed by grinding seeds and/or washing suspensions of oil bodies in solutions at pH values greater than 7.0 using either Tris-HCl (0.1 M, pH 8.5) or a 0.01 NaHCO₃ solution (pH 9.5). Moreover, highly purified oil body material can be obtained by raising the pH of these media to values ≥ 8.8 (e.g., 0.1 M NaHCO₃, pH 9.5). However, these parameters may be valid only for the Canola variety used in this thesis, as the pH value of the recovery solution relies on the protein composition of the oilseed species (Zhao et al., 2016). For example, comparative studies of maize germ and sunflower oil bodies revealed that the same recovery protocol lead to different results: while maize germ oil bodies are free of any exogenous protein after washing with sucrose at a mildly alkaline pH value, residual seed proteins are detected in sunflower oil bodies (Nikiforidis et al., 2013).

6.3. Enzymatic activity in oil body extracts

In this work, it was shown that essential knowledge of the enzymatic activity associated with the oil body could be of significant advantage for the development of a successful protocol for the recovery of oil bodies with enhanced physical and chemical storage stability. In rapeseed, enzymes are tightly associated with the oil body and have been proved to be resistant to washing protocols. It was shown in Chapter 4 and 5 that despite the use of

alkaline buffers during grinding and washing steps leads to a protein content similar to a preparation washed with urea (9 M), the lipolytic and proteolytic activity was still relatively high, confirming that these enzymes are strongly associated to the oil body. Moreover, because the enzymatic activity may be affected by cofactors (e.g., ions), the development of recovery protocol must take into consideration the effect of inorganic ions in the recovery solution used. For example, one of the standard procedures is to use NaCl to reduce the content of exogenous proteins over washing (as detailed in Chapter 3); however, the addition of NaCl, even at very low concentrations (>0.1 mmol/l), may lead to a several-fold increase in lipolytic activity in vitro (Rosnitschek and Theimer, 1980).

In this thesis, a correlation between oil body purity from exogenous seed material and physical stability over storage was demonstrated. Preparations of oil bodies with increased shelf life were produced using alkaline solutions, which limited coalescence for up to four weeks. Improved stability was achieved after thermal treatment, which appears to be linked to the inactivation of enzymes. Comparative analysis of the physical and chemical stability of oil body preparations with different protein content reveals that the presence of active enzymes is the major destabilising factor. Oleosin is completely hydrolysed in the first weeks of storage, even in purified extracts (recovered using NaHCO_3), which relegates the phospholipid layer to stabilise the oil body. Without a physical barrier, the electrostatic repulsions are not strong enough to avoid coalescence, and emulsion destabilization takes place. Loss of oil body morphology was confirmed using confocal microscopy after 30 days. This effect is even more enhanced in high protein content oil bodies

(such as COB recovered in dH₂O), where extended droplet destabilization was seen over the first days of storage. Once proteolysis removes the physical barrier that protects oil bodies, the lipolytic enzymes have more easy access to their substrate (Matsui et al., 1999; Sadeghipour and Bhatla, 2002). Lipid degrading enzymes hydrolyse phospholipid to their respective lyso-phospholipid, and triacylglycerol to free fatty acids and glycerol, as shown by chromatography analysis of oil bodies during four weeks of storage (20 °C). Moreover, lipid oxidation of free fatty acids generates hydroperoxides, which are further transformed into various secondary products. The lipid hydrolysis and oxidation cause an increase of acidity, unacceptable functional properties and undesirable organoleptic characteristics which would limit the use of oil bodies as a food ingredient. Moreover, oxidative enzymes may potentially enhance this process. However, further analysis is needed to measure the magnitude of these activities.

6.4. Effect of thermal treatment on fresh oil body extracts

6.4.1. Effect on enzymatic activity

As enzymes seem to have a strong association with the oil body, thermal treatment is a strategy to inactivate enzymes and thus extend the shelf life. There have been no systematic studies into the effect of heat on oil body-associated enzymes, or on the physical and chemical stability of these organelles ex-vivo. Lipolytic activity in oil body extracts was used as a marker of enzymatic response to thermal treatment. WOB I emulsions (produced from WOB I creams dispersed in dH₂O) were heated up to 95°C and held for a range

of times (0 – 6 minutes); the emulsion was cooled on ice and centrifuged to recover the thermally treated WOB II cream. Analysis of the lipolytic activity in both thermally treated WOB II cream and serum phase (coming from the separation of WOB I emulsion), suggested that lipolytic enzymes may exist (presumably as different isoforms) with different tendencies to remain associated on the oil body, supporting the findings of Hills and Murphy (1988). However, the trends of lipolytic activity in oil body creams may also suggest that the reduction is due to a solubilisation of the enzyme molecules into the serum phase due to heating, followed by thermal denaturation.

The location of lipolytic enzymes in the seed is plant specific, with rapeseed being one of the few species to present an oil body-associated lipase (Hills and Murphy, 1988), together with others such as Arabidopsis (Eastmond, 2006) and castor bean (Ory, 1969). It has been suggested that lipases may contain a hydrophobic domain which should be responsible for their association with the oil body surface (Quettier and Eastmond, 2009). However, it has also been suggested that the structural proteins (such as oleosin and caleolosin) may be involved in regulating lipolysis, by facilitating or blocking the access to the substrate (Frandsen et al., 2001).

In this thesis, it was also shown that the thermal treatment of the oil body emulsion had a significant effect on the quantity of contaminating material in the final cream. Heating the emulsion, denatured the bonds between exogenous protein and oil body surface, which were then removed by the following centrifugation. This was confirmed with an increase in protein content in the serum phases.

6.4.2. Effect on physical stability

Analysis of particle size and zeta potential of thermally treated oil bodies revealed that oleosins were not affected by high temperatures, and that the droplets had a very similar size and surface charge to the native one: $D_{4,3}$ of 1.4 μm and a zeta potential ranging between about +55 to -60 as the pH increased from 3 to 10 (pI 6.0). The occurrence of protein denaturation is mainly a function of the intensity of heat exposure. Differential scanning calorimetry revealed oleosin thermal denaturation at 50 and 59 °C (Cabanos et al., 2011), which is far below the temperatures used in this thesis (95 °C). However, Cabanos et al. (2011) analysed the oleosin outside the native location, compromising its original conformation, which could have affected the results. The exposure to high temperature, followed by the centrifugation of the emulsion, led to minor changes of the fatty acid composition of oil body phospholipids. The major phospholipid (PL) class was PC, followed by PE, PS/PI (that comigrate in the solved system used on TLC) and PG. After thermal treatment, the portion of unsaturated fatty acids increased mainly in PC and PE, which may be due to either removal of some phospholipid species, or thermal degradation of lipids. However, these changes did not influence droplet stability.

The functionality and stability of oil bodies ex-vivo seem to be related to the surface composition of the recovered droplets. It is indeed proposed that the synergistic interaction between phospholipid and proteins allows the formation of a strong interphase that would prevent coalescence within the seed over maturation and makes oil body physically stable. The secondary structure of oleosin is not random: the positively charged residues at the N-

and C- terminus interact with the negatively charged phospholipid head groups to form a structure that covers the surface of oil bodies. The synergy of these molecules has been described in reconstituted oil bodies produced with oleosin recovered from oleaginous seeds (Deleu et al., 2010; Tzen et al., 1998). Droplets surrounded only by phospholipids have high repulsion forces; however, after overcoming the electrostatic repulsion, without a coalescence barrier, the resultant flocculation leads to quick emulsion destabilization. In droplets rich in oleosins, flocculation takes place fairly quickly, but are resistant to coalescence by steric hindrance and electrostatic repulsion provided by the protein. When an appropriate amount of oleosin is added to cover the surface of the oil body effectively, coalescence and fast flocculation are prevented. This effect was thought to be because of the synergistic interactions between the oleosin proteins and the phospholipid monolayer producing a stable interface (Deleu et al., 2010).

6.6. Effect of thermal treatment on oil body stability over storage

The use of lipolytic activity as a marker of enzymatic inactivation, used to develop an appropriate thermal treatment, was tested through a study of the physicochemical characteristics of oil bodies over storage (1 month, 20 °C). Thermal treatment significantly improved the physical stability of oil bodies. Holding the WOB II emulsion at 95 °C for 6 minutes is the ideal process to maintain size, morphology and surface chemistry of oil bodies recovered in this work. However, milder treatments (1 minute, 95 °C) create a reasonably stable emulsion which may be economically interesting for the industry due to

the short holding time. Over storage, untreated samples showed evident lipolytic and proteolytic activity, with the formation of DAGs and FFAs from TAGs, and hydrolysis of oleosins; looking at the FA composition of PLs, PE was the most subjected to changes, with a significant decrease in unsaturated FAs over four weeks. Oil bodies thermally treated for 1 minute showed lipolytic activity as well, but to a lesser extent than the untreated samples.

The shelf life of other raw materials (e.g., milled cereals) and the usability of these in food formulations is greatly influenced by the lipid-associated deterioration. For example, rice bran and oats both have high lipase activity, and the stability of the products made from them relies entirely on the inactivation of these enzymes (Lehtinen et al., 2003). Inactivation of lipase in cereals is usually carried out on the milled dry material using a steam flow (at ambient or high pressure, for up to 40 minutes) (Ekstrand et al., 1993; Lehtinen et al., 2003), or using microwaves (Vetrimani et al., 1992). Steam treatment is also used to inactivate lipolytic enzymes in rapeseed seeds, at temperatures between 77 to 100 °C for 15-20 minutes, depending on the rapeseed variety (Ohlson, 1992). Thermal treatment on whole rapeseed seeds has also been used by Ponne et al. (1996) to inactivate lipase, proving that the denaturation of the thermostable isoform occurred only at 130 °C. The thermal treatment used in this thesis (95 °C for up to 6 minutes) is a relatively mild process compared to these methodologies used in literature to stabilise oil bodies. In fact, these vary between 10 to 30 minutes, at temperatures ranging between 70 to 100 °C (Chen et al., 2012; Iwanaga et al., 2007; Karkani et al., 2013; Naziri et al., 2017; Wu et al., 2012).

The oxidative stability of lipids may be increased with tocopherols, by scavenging lipid peroxy radical and thus increasing induction period (Frankel, 2012). It has been demonstrated that in soybean oil bodies, tocopherols are an intrinsic component of the droplet (Fisk et al., 2008). This hypothesis was confirmed in this thesis, finding these molecules in the oil body extract, without being influenced by the recovery and washing protocol used. Although tocopherols were thermally degraded (up to 25% after 6 minutes), no further change was seen over storage (40 °C). However, untreated emulsions showed extended production of oxidative products with a concomitant reduction in tocopherols. The higher oxidative stability in the thermally treated emulsion is believed to be due to the maintained natural structural integrity of oil bodies.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1. Conclusions

The research presented here has provided an insight on the driving factors that affect the physicochemical stability of oil body material over recovery protocol and storage. Oil body droplet integrity is deeply affected by seed soaking time, seed:volume of grinding media ratio and pH of the milk. Grinding soaked seeds (≥ 16 hours) in a low solid-to-media ratio (1:7 w/w), using 0.1 M NaHCO_3 (pH 9.5), significantly enhances the integrity of recovered oil bodies compared to deionised water. Following this methodology, more than 90% of intact oil bodies were effectively recovered from oilseed rape seeds.

Alkaline media have also the ability to interfere with the bonds between exogenous proteins and the surface of oil bodies, reducing the amount of contaminant material in the final cream. The washing of oil bodies using NaHCO_3 solution (0.1 M, pH 9.5) with a further rinse in dH_2O , removes residual seed proteins reaching a protein content similar to a preparation washed using urea (9 M). The composition at the interphase of the droplet affects the ζ -potential, with an increase in positive and negative charges at the extreme of pH values in highly purified oil bodies. The removal of exogenous seed proteins enhances the physical stability over storage, mainly due to the reduction of seed enzymes. However, these proteins seem to resist the washing protocol using NaHCO_3 solution, leading to some coalescence over storage (20 °C, 4 weeks).

This thesis shows how the lipolytic activity carried over in the oil body extract is affected by the recovery protocol applied. The use of alkaline

solutions leads to a reduction of the enzymatic activity measured in oil body creams, but to a lesser extent than the urea washing protocol. This may be due to the presence of different lipolytic enzymes having different tendencies to be associated with the oil body, or by cofactors that may be more efficiently removed using urea. Due to the resistance against washing, thermal inactivation of enzymes is necessary. The assessment of lipolytic activity over heating (95 °C) was successfully used in this thesis as a marker of thermal stabilization efficiency of the emulsion, without leading to alteration of droplet integrity due to the high temperature. A relatively short-time thermal treatment (1 minute, 95 °C) of the oil body emulsion, followed by centrifugation, reduces by 60 % the total lipolytic activity in the recovered cream. Longer treatments (up to 6 minutes, 95 °C) enhances the inactivation of enzymes up to 90%.

This work showed that the presence of proteolytic activity in oil body emulsions have the highest impact on oil body integrity over storage (20 °C, 30 days). Upon hydrolysis of the oleosin, the oil body physical stability depends on the electrostatic repulsion created by the phospholipid layer exposed to the continuous phase. However, this work demonstrated that these forces were not sufficient to limit emulsion destabilisation over storage. Moreover, the presence of lipolytic enzymes led to the formation of FFA and DAG molecules from TAGs. Thermal treatment significantly enhanced the physicochemical stability of oil bodies through inactivation of enzymes. The resultant emulsion showed little variation in size and surface charge during one month of storage (20 °C), and no significant change in TAG and PL composition was seen over this period.

In this thesis, it was shown that tocopherols were intrinsic components of rapeseed oil bodies, being resistant to recovery and washing protocol using NaHCO_3 (0.1 M, pH 9.5). Although thermal treatment reduced the amount of tocopherols (up to 25% after 6 minutes, 95 °C), the oxidative stability of these oil body preparations was significantly improved compared to untreated samples. The higher oxidative stability is mainly related to the preservation of the original structural integrity during storage.

In conclusion, using the optimised recovery protocol and thermal treatment, the oil body cream is i) made of intact and pure oil bodies, ii) enzymatically stable, iii) physically and chemically stable during aging.

7.2. Future works

The work of this thesis focused on the recovery and stabilisation of oil bodies from oilseed rape seeds, providing insights into what factors affect droplet integrity, extract purity, and storage stability. However, oil body research for food purposes is still at its early stages, and further work would add significant value.

An area of work may be related to the optimisation of recovery protocol for industrial scale application. The current lab scale aqueous recovery of oil bodies yields only 50% of the rapeseed oil compared to industrial solvent extractions (over 90%). Therefore, a cost-effective process should be designed. For example, water usage could be one of the first points that could be optimised. It was shown in Chapter 3 that oil body integrity is maintained when seeds are ground into large quantities of aqueous solution. New

methodologies to reduce the water consumption (e.g., dry cryo-milling or solvent re-usage) would be of great advantage. Moreover, the waste seed-cake may be a source of highly valuable molecules (e.g., plant proteins) that could be recovered in a separate process.

As well as focusing on the reduction of water usage for the oil body recovery, a systematic characterization of the serum phases produced during this method could be of interest. In Chapter 4 it was shown a level of lipase loss to the supernatant after heating and/or centrifugation. Lipolytic enzymes have found great uses in a wide range of application, such as dairy, pharmaceutical, textile and detergent industry. The question then arises, “can the oil body recovery be used to “harvest” enzymes of interest?”

Despite in Chapter 4 it was shown a reduction in hydrolytic enzymes washing crude emulsions of oil bodies, the forces that encourage this detachment, or by what means the enzymes are associated to the oil body surface is currently unknown. Further studies on this matter could help in the development of a more appropriate protocol to further reduce the amount of enzymes from oil body extracts.

The “quality” of seeds, in terms of storage condition or structural integrity, may influence oil body quality (e.g., size, surface chemistry, oil composition or oxidation state). The loss in seed viability takes place with long term storage of oilseeds, but the effect on oil bodies has never been explored. It would be of interest to study what are the effects of seed damage, or seed storage condition (temperature, relative humidity, oxygen) on oil body quality.

Nowadays costumers are concerned about the toxicological implications and in a great demand of data about residues and contaminants in vegetable oils. The absence of a refining step in the recovery of oil body may lead to the presence of pesticides, mycotoxins, and heavy metals in the final material, which are extremely harmful for human health. At the moment, there is no research measuring how the recovery protocol may affect the final concentration of these contaminants in oil body material.

CHAPTER 9. REFERENCES

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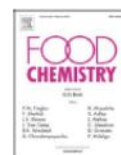
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APPENDIX 1

PUBLISHED PAPER

**Enhancing the recovery of oilseed rape seed oil bodies (oleosomes)
using bicarbonate-based soaking and grinding media.**



Enhancing the recovery of oilseed rape seed oil bodies (oleosomes) using bicarbonate-based soaking and grinding media

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ABSTRACT

An aqueous process for the recovery of oil bodies from rapeseed using sodium bicarbonate-based soaking and grinding media (pH 9.5) was investigated. The effect of the ratio between seed and mass of media during grinding and molarity of the medium used on oil body integrity, purity and storage stability have been studied. The grinding of seeds in solution at a ratio of 1:7 (w/w) significantly improved the quality of oil body suspension to a size more in-line with that seen in vivo (average $D_{4,3}$ of 1.19 μm). The purity and the composition of the recovered oil bodies depends on the molarity of medium used; the use of a sodium bicarbonate solution (pH 9.5, 0.1 M) in the grinding and washing steps produced oil body preparations with the same purity as that resulting from washing a crude preparation with 9 M urea. The resultant emulsion had improved physical stability over a storage period of one month.

1. Introduction

Oil bodies (OBs, denoted often also as oleosomes or lipid bodies) provide the seed with energy during germination; they are generally between 0.2 and 2.5 μm in diameter (Tzen & Huang, 1992). When viewed under an electron microscope, an electron-opaque matrix of triacylglycerols (TAGs) surrounded by one electron-dense layer of phospholipids (Tzen & Huang, 1992; Yatsu & Jacks, 1972) with embedded surface active proteins (e.g. oleosin) can be seen (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). The oleosin contains three basic structural domains: an amphipathic domain present at the N-terminal and C-terminal, residing on the OB surface ensuring stability via steric hindrance and electronegative repulsion, and a highly hydrophobic domain penetrating into the TAG matrix (Frandsen, Mundy, & Tzen, 2001; Murphy & Cummins, 1989; Qu & Huang, 1990). The unique structure of the oleosin is thought to prevent coalescence of OBs both in the cytosol of oilseed cells and *ex vivo* (Tzen & Huang, 1992).

The conventional process of oil extraction from oleaginous seeds using organic solvents and the subsequent oil refining has a high environmental impact, and it is hazardous due to flammability and risk of explosion. During this process, OBs are destroyed as the neutral oil (triacylglycerol) partitions into the organic solvent. The production of oil from vegetable sources is estimated to be approximately 150 million tonnes per annum, of which 30% is processed into emulsions using high energy homogenisation and the addition of emulsifiers/surface active

agents (Gunstone, 2004). Many natural and processed foods are considered either partly or wholly as oil-in-water emulsion (McClements, 2004). When OBs are released (recovered) from the cells of oilseeds into an aqueous medium (a relatively benign wet milling process) they may form a stable oil-in-water emulsion, depending on the solution implied. Compared with conventional oilseed processing, the recovery of OBs as a natural source of pre-emulsified oil, to be used in a range of food applications, is likely to reduce the impact on the environment. OBs have been recovered from a number of different seeds, such as pumpkin (Adams et al., 2012), soybean (Chen, McClements, Gray, & Decker, 2012; Chen & Ono, 2010; Iwanaga, Gray, Decker, Weiss, & McClements, 2008), and maize (Nikiforidis & Kiosseoglou, 2009). Despite the differences between the authors, the key points for the extraction of OBs are similar, comprising: disruption of the seed matrix in aqueous media; collection of a "crude" OB cream after centrifugation and washing of the cream. The latter stage is aimed to remove the polysaccharide, seed debris and exogenous proteins that are not covalently bonded to the OB (Tzen, Peng, Cheng, Chen, & Chiu, 1997), using buffers, detergents or chaotropic agents, to obtain a purified OB cream free from exogenous material. As detailed by Millichip et al. (1996) urea (9 M) is a rigorous washing agent that produces an OB preparation free from exogenous seed proteins without destabilising the integral oleosin proteins necessary to retain OB integrity (Lacey, Wellner, Beaudoin, Napier, & Shewry, 1998).

Some studies have identified enzymes associated with recovered oil

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bodies (Chen & Ono, 2010; Chen, Zhao, Cao, Kong, & Hua, 2014; Karkani, Nenadis, Nikiforidis, & Kiosseoglou, 2013; Zhao, Chen, Chen, Kong, & Hua, 2016). The carry-over of enzyme activity (proteolytic or lipolytic) could cause destabilisation of OBs ex-vivo. The removal of these enzymes would therefore reduce the temperature and time required for effective biochemical stabilisation of the OB preparation. Urea is not a food grade material, so cannot be used to wash OBs and remove enzymes. However, as the enzymes contain multiple charged groups, they can be solubilised in salt solutions, and their solubility can increase with increasing salt concentration, in a phenomenon called “salting-in” (Arakawa & Timasheff, 1985). We hypothesised that a salt such as sodium bicarbonate, could be a suitable food grade replacement of the urea. The aim of this work was to test the ability of a sodium bicarbonate solution (pH 9.5) to recover intact OBs with reduced carry-over of exogenous proteins. The effect of changing the molarity of the bicarbonate solution, and the ratio between seed mass and mass of the medium during grinding were tested.

2. Materials and methods

Oilseed rape seeds (variety Quartz) were sourced from a local farm at time of harvest (2015), and stored at 20 °C and RH 50% until use. All chemicals and reagents were purchased from Sigma-Aldrich Trading Co., Ltd. of analytical reagent grade or higher. Precision Plus Protein Dual Color Standard (Bio-Rad, USA) was used as protein marker. Ultrapure water (Nanopure Infinity system, Barnstead International, IA) was used for all media.

2.1. Oil body recovery and purification

OBs were isolated from oilseed rape seeds and purified according to the method of Tzen et al. (1997) with the following modifications. Seeds (200 g) were soaked in sodium bicarbonate pH 9.5 (0.01, 0.1 or 0.3 M, adjusted with 0.1 M NaOH) or water, at ratio of 1:4 (w/w) at 4 °C for 16 h (unpublished data) and the soaking medium was then discarded. The soaked seeds were then ground in the same medium type as used on soaking (4 °C) at ratios (dry seed weight based) of 1:4 (w/w) or 1:7 (w/w) in a Kenwood blender (BLX52) at full power for 90 s. After grinding, the slurry was left stirring for 24 h at 4 °C, to improve the yield of oil bodies, and then filtered through three layers of cheesecloth. The filtrate was transferred in 400 mL tubes and centrifuged at 10000g for 30 min at 4 °C using a Beckman J2-21 centrifuge, fixed rotor JA-10. The upper layer was isolated using a spatula, drained on filter paper (Whatman, grade 5) and called crude oil body fraction (COB). Sodium azide (0.02 mM) was added to all media to avoid microbial spoilage. The COB preparation was dispersed in washing solution (0.1 M NaHCO₃, pH 9.5 or 9 M urea, 1:4 w/w) and centrifuged (10000g, 30 min). To remove any residue of the washing solution, the fat pad was isolated using a spatula, drained on filter paper, suspended in water (1:4 w/w) and centrifuged (10000g, 30 min). After centrifugation, the cream layer was collected and designated as urea-washed oil body cream (Urea-WOB) or sodium bicarbonate washed oil body cream (NaHCO₃-WOB). All creams were stored at 4 °C and analyses were completed within 24 h.

2.2. Particle size measurement

The particle size of OB suspensions was measured with a LS 13320 laser diffractometer (Beckman-Coulter, USA) using the Mie theory of the scattering of light by spherical particles. The samples were diluted as appropriate prior to measurement. The real part of the refractive index, was 1.462, corresponding to the refractive index of rapeseed oil. The imaginary part, corresponding to the ‘attenuation coefficient’ that describes the turbidity of a sample, was set to 0.01 according to the laser diffractometer guidelines for lightly coloured translucent materials. For the description of particle size distribution (PSD), the volume

frequency distribution (%/μm) and the volume mean diameter ($D_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$) have been reported. The fraction of intact droplets ($\leq 2.5 \mu\text{m}$ in diameter), expressed as percentage, was found using the LS 13320 software from the cumulative volume frequency distribution curve.

2.3. Preparation of oil body emulsions for stability test

Oil body emulsions were prepared suspending the washed cream in water (10% lipid weight basis) using a vortex at maximum speed (1 min). Sodium Azide (0.02 mM) was present in all emulsions to avoid microbial spoilage. The $D_{4,3}$ was measured at time 0, 7, 14, 21 and 28 days storing the samples at 20 °C.

2.4. Lipid extraction

COB or WOB creams (approximately 0.2 g) were dried in a vacuum oven (40 °C, −900 mbar) for at least 48 h until constant mass. Dry matter was ground (mini-bead beater, 3450 rpm) for 2 min in isooctane (1 mL) to extract the oil (Gray, Payne, McClements, Decker, & Lad, 2010). Samples were centrifuged (Thermo Heraeus Fresco 21) at 17,000g for 5 min at 4 °C and the upper phase was removed with a pipette; this process was repeated three times and the recovered lipids dried under nitrogen. The resultant pellet after delipidation was used for protein extraction and analysis as described in Section 2.6.

2.5. Protein content

The proteins were extracted from delipidated dry material (as detailed in Section 2.4) using 2% sodium dodecyl sulfate solution (SDS, 1 mL) and incubated for 30 min at 60 °C in a water bath. Protein extracts were vortexed for 1 min and centrifuged at 17000g (5 min, 4 °C). The supernatant was collected and assayed on the same day for protein content using BCA assay (Smith et al., 1985). On performing the analysis, samples were diluted to obtain absorbance values within the range of the standard curve.

2.6. SDS-PAGE

To compare the degree of exogenous protein carried over in the extracts, protein samples were appropriately diluted in 2% SDS solution normalised by the amount of oleosin. 20 μL of protein extract was then mixed with an equal volume of sample buffer (Laemmli buffer (Biorad, UK) + 5% β-mercaptoethanol), heated at 95 °C for 5 min and then cooled on ice. Proteins were resolved by SDS-PAGE using 4–15% polyacrylamide gels (Mini-Protein TGX Gels, 10-well, 50 μL, Bio-Rad, Hercules, USA); gels were positioned within a SE 600 BioRad separation unit and suspended in tank buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was run at 100 V for 40 min. After electrophoresis, the gels were washed three times (10 min, H₂O). The gel was then stained using comassie brilliant blue R-250 (1 h) and de-stained with de-staining solution (2 h). Gels were imaged using Bio-Rad Gel Doc XR System.

2.7. Zeta potential measurement

A series of OB emulsions were prepared in ultrapure filtered (0.2 μm Millex syringe filter unit, Millipore) water at a concentration of 0.008% (lipid weight basis) and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH solutions. After stabilization of the pH, the emulsions were injected into the measurement chamber of the particle electrophoresis instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA). The instrument settings used were: temperature = 25 °C; refractive index of dispersant = 1.333; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm; voltage = 35 V. The ζ-

potential was then determined by measuring the direction and velocity of the OBs moving under the applied electrical field. The ζ -potential value was reported as the average and standard deviation of two independent samples from each emulsion replicate, with two readings taken per sample.

2.8. Light microscopy

The microstructure of OB emulsions was investigated using light microscopy (EVOS, UK). A small drop of oil body emulsion was placed on a glass slide, covered with a cover slide and imaged at a magnification of $40\times$.

2.9. Statistical analysis

All extraction experiments were carried out at least in triplicate and analyses were made on at least three emulsion preparations. Statistical analysis was performed by one-way ANOVA using SPSS 22. Assessment of significance difference was based on a 95% confident limit ($P < 0.05$).

3. Results and discussion

3.1. Effect of the seed:medium ratio and type of media during grinding on recovered OB integrity

The viscosity of a system with dispersed solids is reduced on increasing the volume of the medium (Austin & Trass, 1997), which may lead to a reduction in the forces experienced by OBs during their recovery, and to a reduction in the concentration of seed particulates that might collide with and damage OBs. In this set of experiments, seeds soaked in water or sodium bicarbonate (0.1 M, pH 9.5) for 16 h (54% weight increase, unpublished data) were ground using two ratios of seed mass to mass of media to investigate the effect of seed loading during grinding on OBs integrity. Based on preliminary data, a 1:4 and 1:7 ratio (w/w), was selected.

To evaluate whether a specific seed-to-medium ratio would damage the OBs, the crude oil bodies (COBs) were exposed to urea (9 M) to remove the exogenous seed proteins that could be adsorbed at the interface of damaged droplets, temporarily stabilising them. After exposing the cream to urea, intact OBs will remain small discrete entities (Tzen et al., 1997), whereas hybrids (damaged OBs with altered surface chemistry) will rapidly destabilise and coalesce forming larger droplets. No change in the size after wash of COBs with urea (9 M) has been used in this study as a diagnostic method for the detection of intact OBs.

Fig. 1 shows the particle size distribution of urea-WOBs extracted using water and sodium bicarbonate (0.1 M, pH 9.5) at both seed-to-medium ratios. At high solid loading ratios (i.e. 1:4 ratio w/w) the OB particle size distribution (PSD) showed a broad distribution after grinding seed in water and sodium bicarbonate, with a major peak at 12 and 6 μm , respectively. The presence of peaks at diameters above 3 μm is due to coalescence of droplets as confirmed by optical images (Fig. 1A–B). In a more diluted seed grinding system (1:7 ratio w/w) (Fig. 1) smaller droplets were observed after grinding seed in either water or sodium bicarbonate media then washed with urea; this confirmed that the low solid load of the grinding media improved OB integrity. With a low solid loading during grinding, the volume mean diameter ($D_{4,3}$) of urea-WOB extracted in water (3.6 μm) was larger than the urea-WOB extracted in sodium bicarbonate (1.2 μm). The presence of OBs with higher diameter after grinding seeds in water, suggests that an alkaline pH plays an important role in the maintenance of the droplet integrity, as it is discussed in Section 3.2. The results shown here suggest that the optimal grinding condition to recover intact OBs is the sodium bicarbonate solution at 1:7 ratio, as the lower density of seed solids in this grinding medium reduced the damage to OBs.

3.2. Effect of sodium bicarbonate solution strength during seed grinding on recovered oil body integrity

The charged surface of OBs held in an aqueous solution at certain pH values, can cause electrostatic repulsion that promotes the maintenance of droplets as discrete organelles, leading to an increased stability of the emulsion against aggregation (Iwanaga et al., 2008). However, at pH values close to the isoelectric point (5.5–6.6), the overall charge is zero, and in absence of repulsion forces, aggregation occurs (Tzen & Huang, 1992; Tzen et al., 1993; White et al., 2008). We hypothesized that if the pH of the grinding solution was far from OB pI, coalescence could be reduced. Therefore, in this set of experiments, the buffering capacity of the extraction media (to intend as the ability to hold the pH value far from the pI during grinding), and its effect on the recovered OB integrity, was tested. Sodium bicarbonate solutions with increasing molarity (0.01 M, 0.1 M, 0.3 M) were prepared and compared to water and the PSDs of the recovered OB was analysed. Subsequently, all COB preparations were exposed to urea (9 M) to reveal the presence of hybrids (as detailed in Section 3.1), and the following PSD changes compared to COB distributions were used to assess droplet integrity. The COBs recovered in sodium bicarbonate solution showed similar droplet size (Fig. 2A) for all molarities considered. However, when water was used as extraction media, the PSD showed a similar size range but with higher percentage for bigger droplets, due to the formation of aggregates (Fig. 2C). The buffering capacity of the system changes using different molarities of the medium, reaching a pH value following seeds grinding of 8.5, 7.0 and 6.0 for 0.1 M and 0.01 M sodium bicarbonate, and water, respectively. At higher buffering capacity (0.3 M), the solution is able to hold the pH value at 9.5. After exposure to urea (urea-WOB, Fig. 2B), the quality of OBs recovered using sodium bicarbonate (at all concentrations tested), improved considerably with those recovered using water. When grinding seeds in water, the pH value of the media is within the pI of OBs (5.5–6.6), neutralising the surface charge of the droplet and causing the formation of aggregates, which may enhance coalescence during the grinding process. As a result, a remarkable droplet size shift to bigger droplets occurs when water is used (Fig. 2B–C–G). A similar size shift was registered for 0.01 M solution, probably due to the weak repulsion forces during grinding, as the pH value was close to the pI (described in Section 3.4) that were not enough to avoid the OBs to coalesce (Fig. 2B–D–H). Among the different molarities of sodium bicarbonate used, only the 0.1 M did not show any shift to particles of higher diameter after urea exposure, and the recovered droplets maintained their singularity (Fig. 2B–E–I). However, despite the pH being far from the pI using higher molarities of the sodium bicarbonate solution (0.3 M), the PSD of urea-WOB showed the presence of coalesced droplets (Fig. 2B–F–J).

Those results demonstrate that holding the pH value above 8.5, the OB surface may create enough repulsion forces that prevent the aggregation of droplets during the grinding step, limiting the coalescence. Moreover, the alkaline pH value of the system (≥ 8.5) is a suboptimal environment for rapeseed enzymes, such as lipases which optimum pH value is 5 (Hoppe & Theimer, 1997), resulting in the inhibition or delay of their activity on the OB over the extraction process. However, the prolonged exposure to high alkaline pH (> 8.5) and high salt concentration (0.3 M), may have contributed to reduce the number of salt bridges between deprotonated oleosin branches and phospholipid heads (Cao et al., 2015), facilitating coalescence of OB droplets during grinding. Those results suggest that the optimum salt concentration to extract intact OBs is the 0.1 M, as the buffering capacity of the medium is able to hold the pH value far from the pI, without destabilising the integral proteins. Using this solution, over 90% of OBs were within the range of intact droplets ($< 2.5 \mu\text{m}$) (Tzen et al., 1993), while the rest of the population at higher diameters can be attributed to limited coalescence.

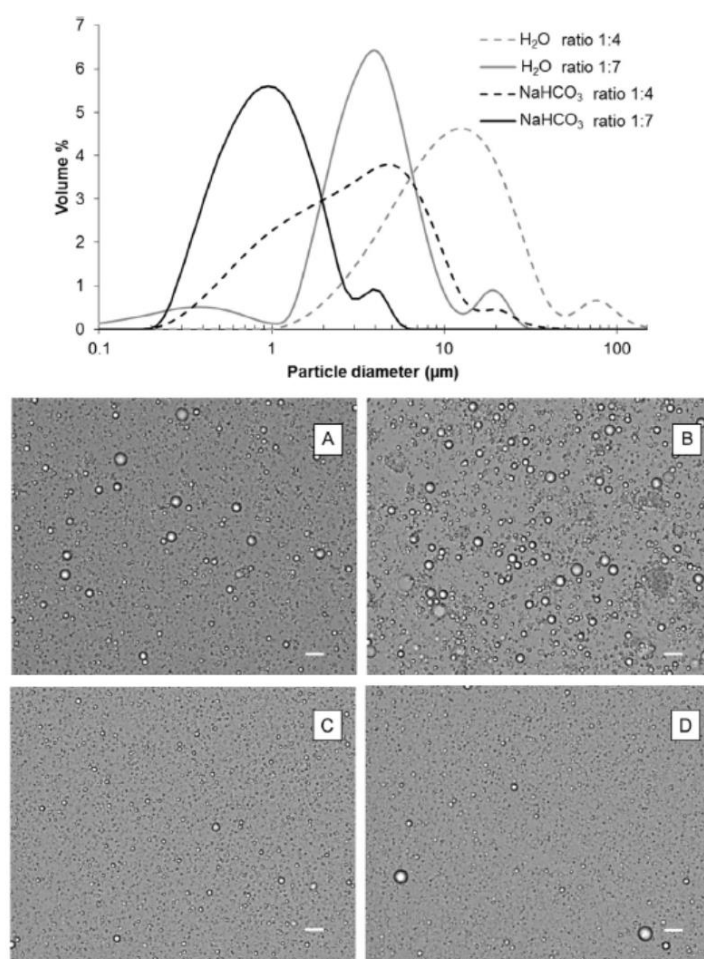


Fig. 1. Above: Particle size distribution of OB extracted in 0.1 M NaHCO_3 and then exposed to urea (9 M) (urea-WOB) to reveal the presence of hybrids. Dashed and solid lines are for cream recovered at ratio seed-to-grinding medium mass of 1:4 and 1:7, respectively, using H_2O (grey lines) and NaHCO_3 (black lines). Below are shown optical images of Urea-WOB extracted using different ratios seed mass to mass of grinding media (w/w). Panel A and B: urea-WOB extracted at 1:4 ratio in NaHCO_3 and H_2O , respectively. Panel C and D: urea-WOB extracted at 1:7 ratio in NaHCO_3 and H_2O , respectively. Scale bar represent 10 μm .

3.3. Protein carry-over in crude oil body emulsions (COB)

In addition to the integral protein of OBs (e.g. oleosins), many seed exogenous protein molecules (located elsewhere in the cell) may bind to the OB surface during their recovery (Tang, Ono, & Mikami, 1997). As mentioned above, the protein solubility is influenced by the nature of the grinding medium: ionic strength, type of solvent, pH, temperature (Zayas, 1997). The effect of grinding medium (water and sodium bicarbonate at different molarities) on the carry-over of exogenous proteins in the COB extract was tested. Fig. 3A shows the protein content (as% of dry matter) of COB and WOB extracts. The COB recovered in water had the highest fraction of total protein ($9.8 \pm 0.74\%$), which decreased to $8.5 \pm 0.27\%$, $4.5 \pm 0.17\%$ and $3.8 \pm 0.25\%$ when using 0.01, 0.1 and 0.3 M sodium bicarbonate, respectively. After exposure to urea (9 M), the washed preparation recovered using water or sodium bicarbonate had a protein content of $2.60 \pm 0.05\%$. This value is in line with the one reported by (Tzen et al., 1993) in different varieties of seeds including rapeseed (0.59–3.46%, dry basis), confirming that the urea-WOB represent the purest preparation of OBs. In order to confirm the washing effect of the bicarbonate solution, protein

samples of COBs and WOB were analysed by SDS-PAGE (Fig. 3B). Lane 2, corresponding to the protein extract of OBs recovered in water, showed several bands of exogenous proteins (at higher molecular weight of the oleosin, 18 kDa) carried over in the extract. By increasing the molarity of the sodium bicarbonate solution (lanes 3, 4 and 5), the majority of bands at high molecular weight lost intensity, suggesting that they had been removed from the OB emulsion.

The removal of exogenous proteins in the presence of sodium bicarbonate is probably due to a combination of pH and salt concentration effect. As detailed by Wanasundara and McIntosh (2013), at a pH value between 3 and 5 the solubility of *Brassicaceae* oilseed proteins is the lowest, but increases at values that are above and below this range. Therefore, the trend of protein content in Fig. 3A is related to the pH value during grinding as discussed in Section 3.2. Similar results were reported by Chen and Ono (2010), where by neutral pH extraction the surface of oil bodies from soybean was characterised by the presence of several proteins and enzymes, like lipoxigenase, β -amylase, γ -conglycinin, and some not well known proteins; while at alkaline values (up to pH 11) a washing effect (removal of exogenous proteins) occurred. In addition to this, the increase in salt concentration, causes an

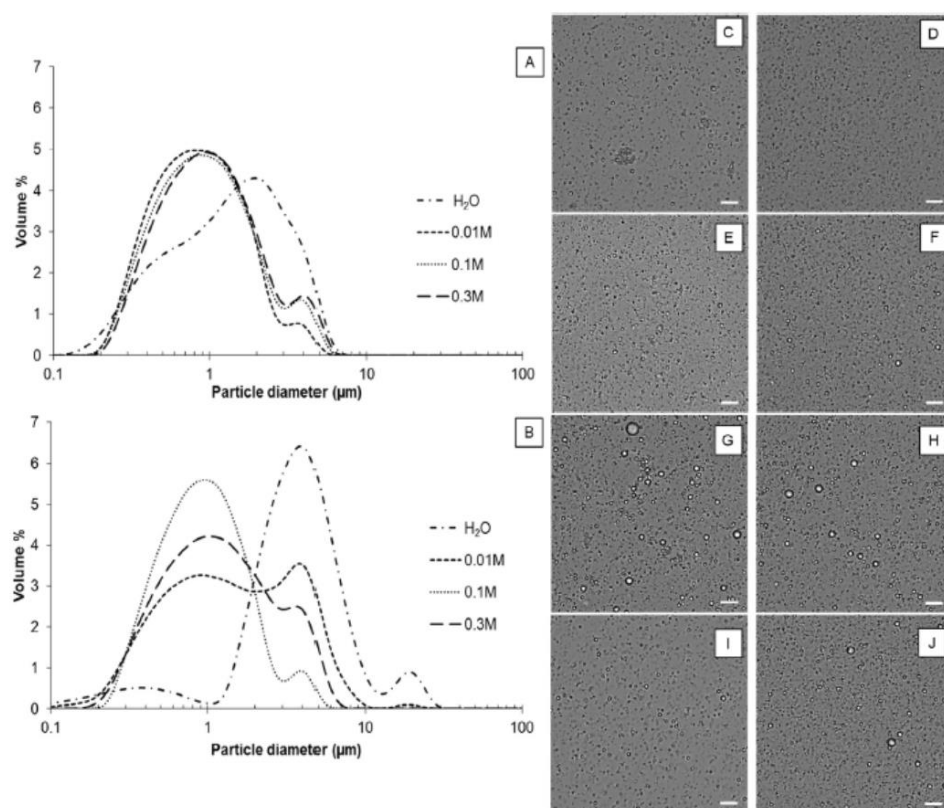


Fig. 2. A) Particle size distribution and microscopy images (Panel C, D, E, F) of crude oil bodies (COB) recovered on grinding oilseed rape seeds (1:7 seed:grinding medium) in H_2O , or NaHCO_3 pH 9.5, at molarities of 0.01, 0.1, 0.3, respectively. B) Particle size distribution and microscopy images (Panel G, H, I, J) of the same COB preparations exposed to urea (9 M) to reveal the presence of hybrids. Scale bar represent 10 μm .

increase in protein solubility (Arakawa & Timasheff, 1985). pH and salt concentration in our system may be working in synergy to remove exogenous proteins without bringing the system to high pH values.

The different composition and quantity of protein on the surface affects the OB properties. Therefore, the surface potential in the different emulsions was measured.

3.4. ζ -potential of COB preparations

The ζ -potential is a measure of the surface potential of a colloidal system, measured when exposed to an oscillating voltage across a measurement cell. In this study ζ -potential was used to predict how the protein carry over affects the droplet stability at different pH values, due to the type of extraction solution, as detailed in Section 3.2. The extracted COBs were suspended in ultra-pure water and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH. The surface charge of COBs (Fig. 4) moved from positive to negative values when the pH was increased, which is typical for protein-covered oil droplets (Guzey & McClements, 2006). The charge was negative (< -40 mV) with a similar ζ -potential for all emulsions at pH from 8 to 10, but differences were observed at pH's 3 to 8, due to the different protein composition at the interface of the extracted OBs (Fig. 3B). As detailed in Section 3.3, the use of sodium bicarbonate solution system, led to a purification of the OB extract from exogenous protein, increasing the

molarity of the salt solution used reaching values close to the urea-WOB. However, among all emulsions tested, only recovering the OB in sodium bicarbonate at a molarity of 0.3, the ζ -potential had a comparable trend to the urea-WOB ($P < 0.05$) in all pH considered, suggesting that the two emulsions had similar proteins at the interface. Similar findings have been registered in Payne, Lad, Foster, Khosla, and Gray (2014), where the differences in ζ -potential was also attributed to proteins that become associated with the surface of the oil bodies, altering also the pI of the organelle *ex vivo*. When the surface charge reaches the neutrality, the ζ -potential value is equal to zero, which corresponds to the pI of the droplets. The pI of COBs depended on the extraction medium used, ranging from 4.2 using water to 5.0, 5.7 and 6.2 for COBs extracted with, respectively, sodium bicarbonate at molarities of 0.01, 0.1 and 0.3. The pI values of the cleanest preparations form exogenous protein (urea-WOB and COBs extracted in 0.3 M sodium bicarbonate) are higher than the ones registered in other studies on different seeds (Chen et al., 2012; Iwanaga et al., 2007; Nikiforidis & Kiosseoglou, 2009), which may be due to the different methodology used, but in line with the work of Tzen, Lie, and Huang (1992) and Tzen et al. (1993) on rape seeds.

The ζ -potential can be used for evaluation of colloidal interaction, classifying the emulsion stability depending on its value (Patel & Agrawal, 2011). The presence of high amount of exogenous proteins for COBs recovered in water and 0.01 M sodium bicarbonate,

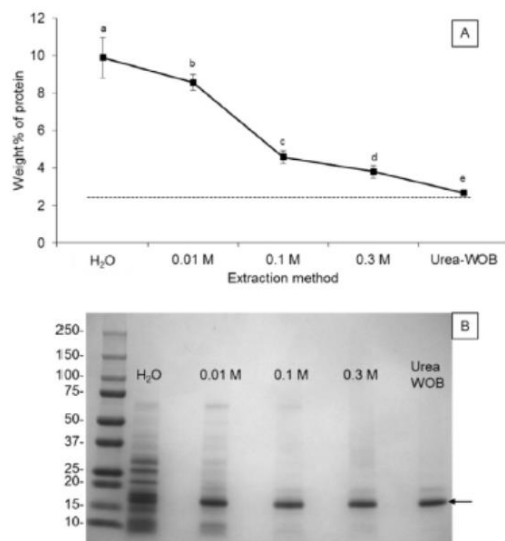


Fig. 3. A) Protein content of COB recovered using H₂O or NaHCO₃ (0.01, 0.1, 0.3 M) and urea-WOB (from COB recovered in 0.1 M NaHCO₃), expressed as percentage based on dry weight. Values are means $\pm$ SD, with P value < 0.05 . B) SDS-PAGE of protein extract from COB extracted with H₂O or NaHCO₃ (0.01, 0.1, 0.3 M) and of urea-WOB. Oleosin band is indicated by an arrow.

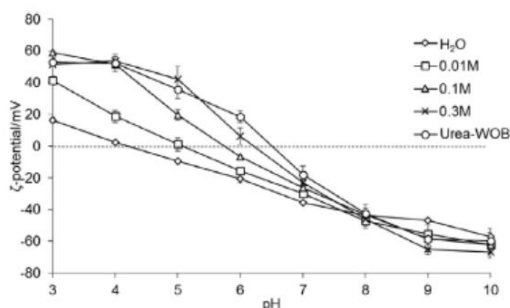


Fig. 4. ζ -potential of COB extracted from oilseed rape using H₂O (○) and NaHCO₃ at molarity of 0.01 (□), 0.1 (Δ), 0.3 (x). Urea-WOB (○, from COB recovered in 0.1 M NaHCO₃) is shown as reference, representing the purest preparation.

results in a shift towards more acidic pH values of the medium (as detailed in Section 3.3) and a decay of ζ -potential. As mentioned in Section 3.2, the pH values for those emulsions were 6 and 7, corresponding to ζ -potential values lower than -30 mV (Fig. 4), considered the minimal value for highly stable emulsions. However, using sodium bicarbonate 0.1 M, the droplets had a higher ζ -potential (around -50 mV) at the corresponding pH of the solution after grinding, confirming this salt concentration as the optimum for the recovery of intact OBs.

3.5. Effect of washing intact COB preparations on OB storage quality

The presence of active exogenous and endogenous enzymes associated to the OB interface depends on the pH of extraction, on the number of washing steps, and on any heat treatment applied to increase the shelf life of the material (Chen et al., 2014; Zhao et al., 2016).

However, the removal of exogenous proteins during the extraction using appropriate media would eliminate the need of long holding time at high temperature for enzyme denaturation. As detailed by Millichip et al. (1996), the exposure of OBs to 9 M urea resulted in the progressive removal of contaminating proteins, leaving only the integral ones.

This section examined whether it would be possible to reach the same purity of the urea-WOB from crude extract of intact COB (extracted using a 0.1 M sodium bicarbonate solution, as detailed in Section 3.2), using the same solution as a washing medium, and its effect on the storage stability. If sodium bicarbonate were to be used in an industrial process, then it may be necessary to carry out a final water wash to remove the salt to meet recommended levels of sodium in food and ingredients. For this reason, the COB preparations were washed in urea (9 M) or 0.1 M sodium bicarbonate (pH 9.5) and subsequently rinsed in water to remove traces of the washing solution. The sequential washing in 0.1 M sodium bicarbonate and water, removed the majority of exogenous proteins from the COB preparation (final concentration of $2.69 \pm 0.21\%$ dry matter), reaching the same content of the urea WOB ($2.60 \pm 0.05\%$). The removal of exogenous protein from the 0.1 M COB extract had an effect on the surface charge of the washed droplets (Fig. 5). Compared to the COB preparation, the PSD of the washed OB in sodium bicarbonate (0.1 M) and subsequently washed in water, did not show any size increase (data not shown) and it displayed a zeta potential trend very similar to the urea-WOB preparation ($P < 0.05$).

Although the peripheral proteins could prevent the lipid oxidation of oil bodies (Karkani et al., 2013), the presence of enzymes already active during the imbibition process (Theimer & Rosnitschek, 1978), can cause the OB structure breakdown. The use of the sodium bicarbonate washing solution can remove exogenous proteins in a concentration dependent fashion, reaching a comparable purity to the urea-washed extract, considered the purest preparation. We hypothesized that a reduction in protein content extracting and washing the OBs using 0.1 M sodium bicarbonate, would correspond to the removal of active germinating enzymes from the extract, and so the extension of the droplet integrity over storage. For this reason, OB emulsions (10% lipid weight basis) with a different content in protein, were kept at room temperature (20 °C) and the particle size was evaluated over a period of four weeks. NaN₃ (0.02 mM) was added in all preparations to avoid microbial growth (Lichstein & Soule, 1944).

In Fig. 6 it is shown the $D_{4,3}$ of COB emulsion extracted using only water, rich in peripheral proteins and the urea-WOB, practically free from their presence as detailed in Section 3.3. With a lower protein content compared to the water recovered COB, the preparation extracted and washed in sodium bicarbonate represent the optimised extraction process, with a similar protein composition than the urea-WOB. Over the time course, unwashed COB samples recovered using

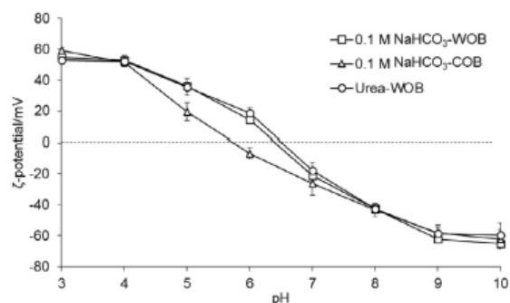


Fig. 5. Effect of washing COB using sodium bicarbonate solution on ζ -potential. COB were recovered and washed using 0.1 M NaHCO₃, pH 9.5 (□). Urea washed oil body (urea-WOB, ○) and crude oil body recovered in 0.1 M NaHCO₃ (0.1 M COB, Δ) are shown as references, representing the unwashed cream and the purest preparation, respectively.

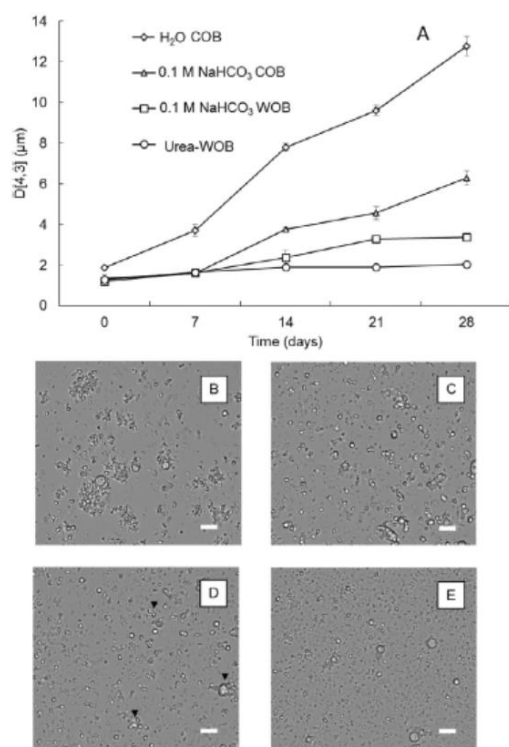


Fig. 6. A) Mean volume diameter ($D_{4,3}$) over four weeks of COB extracted with H_2O (○) and 0.1 M $NaHCO_3$ (□), and WOB using 0.1 M $NaHCO_3$ (△) or Urea 9 M (◇). Optical images after 30 days of storage of COB extracted using H_2O (B), sodium bicarbonate (0.1 M, panel C), and WOB extracted in 0.1 M $NaHCO_3$ and washed using 0.1 M $NaHCO_3$ (D) or urea 9 M (E). Scale bar represent 10 μm .

water were the least stable, with clear membrane disruption and reduced number of droplets after four weeks (Fig. 6B). Significant improvement in the physical stability of OB on storage were achieved by grinding seed in 0.1 M sodium bicarbonate, and even more on washing this material with the same solution. The most stable samples were the ones washed in urea (9 M) without showing altered droplet structure (Fig. 6E) nor a considerable size increase. Despite the small difference in $D_{4,3}$ between the washed emulsions, the one recovered and washed in sodium bicarbonate showed a loss of oil body structure (Fig. 6D, indicated by arrows), suggesting a slight enzymatic activity over the storage trial.

Different enzymes such as lipase (Lin, Moreau, & Huang, 1982; Lin, Wimer, & Huang, 1983), phospholipase (May, Preisig-Müller, Höhne, Gnau, & Kindl, 1998), protease (Sadeghipour & Bhatla, 2002), have been localised in the proximity of the OB over the germination process. The presence of those enzymes in protein-rich extracts, leads to the rapid destabilization of the OB structure over time. Purifying the OB using the correct combination of pH and buffer, limits the carry-over of active germinating enzymes, leading to a better stability of the emulsion over time (Fig. 6).

4. Conclusion

The findings of this work demonstrate that different methodologies adopted in grinding affect the integrity and the characteristics of the

recovered oil body emulsion, such as particle size, protein content and ζ -potential. This study shows that the use of a medium with a high alkalinity overcomes the buffering capacity of the seed material in solution; using a low solid-to-media load (1:7 w/w), and a concentration of 0.1 M of the sodium bicarbonate, the system appears to maintain the integrity of OB droplets by creating enough repulsion forces to avoid coalescence. The differences in the purity of the OB preparations is a result of the ability of the alkaline medium to interfere with the bonds that connect exogenous proteins with the surface of OBs. However, the exposure of OBs to high pH values and salt concentration, may promote coalescence due to deprotonation of N- and C-terminus of oleosin, decreasing the number of salt bridges with the phospholipid. Over the 90% of OBs recovered after soaking the seeds for 16 h (then grinding using 0.1 M sodium bicarbonate at a ratio of 1:7 (w/w), and washed in the same solution) showed an equal protein composition and surface potential as the purest OB preparation (urea-WOB). Moreover, the removal of exogenous proteins retained the physical stability of oil bodies probably due to the removal of hydrolytic enzymes, which may lead to a reduction of time and temperature required for biochemical stabilization. These results offer an important contribution on the route for the development of a method to extract intact and pure OBs from oil-seed rape seed suitable for the food industry.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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