

**THE IMPACT OF UPSTREAM AND DOWNSTREAM PROCESSING ON
THE QUALITY OF OIL BODIES RECOVERED FROM PARTIALLY DE-
HULLED SUNFLOWER SEEDS**

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Thesis submitted to the University of Nottingham for the Degree of Doctor of
Philosophy

August, 2016

Abstract

Few publications on oil bodies or oleosomes seem concerned about their quality (chemical and physical) *ex-vivo*. This work attempts to identify the main factors (processing and pre-processing) that affect the quality/integrity of sunflower seed oil bodies recovered through a wet-milling process. The physical state of seeds during wet milling had a significant impact on the quality of the oil body suspension. Pre-soaking for 6 hours before wet milling and multiple washing with alkaline buffer (0.1M sodium bicarbonate) was performed to isolate high quality oil body suspensions. It was evident from different physical measurements such as particle size, ζ -potential and light microscopy that pre-soaking had a positive influence on the quality of oil body suspensions with no significant signs of aggregation or coalescence. It was also observed that the resultant washed oil body suspensions were highly surface charged (-28.4 ± 1.2 mV) indicating very stable suspension phase behavior. Washing oil bodies not only removes non-integral, extraneous proteins (derived from the seed matrix) but enriches the lipid content including Tocopherol (α -tocopherol: 491.6 mg/kg of washed oil bodies compared with 252.6 mg/kg crude oil bodies). Changes in the composition of oil bodies after washing have been observed before, but this research also monitored the size of oil bodies after washing, and our results indicate that certain factors can shift the distribution of droplet size. It is believed that any change in average size of droplets indicate the presence of disrupted oil bodies whose surface chemistry has changed enough to compromise their integrity on washing. The retention of droplet size on washing may, therefore, be diagnostic for the recovery of intact oil bodies. An assessment of the integrity of oil bodies recovered from sunflower seeds after accelerated aging (5 months) was carried out. Free fatty acid was more pronounced in oil rather than oil bodies, this could be due to the elimination of some of the free acid bound to oil body during washing. Although some minor variation was observed during seed aging, however, the oil bodies remained stable in the final suspension. The results indicate that oil body membrane was extremely robust under extreme conditions and the integrity of oil bodies was preserved. In

addition, oil bodies obtained in this study were resistant to oxidation due to the presence of naturally occurring antioxidants (including vitamin E) associated with them.. The results indicate that the physical barrier of surface membrane protein (oelolin) protect oil bodies against pro-oxidants.

ACKNOWLEDGEMENTS

This thesis is could not have been completed if not for the involvement and support of the following people, to whom I am deeply indebted. First of all, grateful to Allah for guiding me through this path. Thank you to my supervisor, Associate Professor. David Gray, for his patience, guidance, a great mentor and unwavering support. To my co-supervisor Dr. Ian Fisk, thank you very much. I also would like to thanks Prof. Foster, Prof Hill for his guidance and supporting during the study. To Dr. Channell, Dr. Linforth, Emily Fong, all staff in Food Sciences thank you very much for your kindness and support in every aspect during my studies. It was a great pleasure to meet all of you. To my colleagues and friends, thank you very much for your kind support, I really appreciated every suggestion and help from all of you. To Dr. Kartini, Dr. Nuzul, Dr. Norazalina, Dr. Ruben, Dr. Heng Hui, Dr. Deepa, Dr. Candy, Nizaha, thank you for being such a good friends. Thank you for your support especially to Dr. Briars, Dr. Abson, Dr. Uswatun Hasanah, Gedi, Pujuan and Rafidah.

I am also indebted to my wonderful family for their endless support throughout this journey. To my children Aina Yasmine, Ammar Zariff and Amni Syuhada mama would like to thank all of you for the love and support. To my beloved husband, many thanks for your sacrifice and unwavering support. Your endless support during thick and thin, love and understanding is much appreciated. Last but not least to my family back home, your endless support as always, eased my journey. Finally, financial support from Islamic Development Bank is gratefully acknowledged. Last but not least appreciations to Universiti Teknologi Malaysia for study leave and Institute Bioproduct Development (IBD).

ABBREVIATION

Alkali-WOB	Alkali washed oil bodies
ANOVA	Analysis of Variance
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
COB	Crude oil bodies
Cryo-EM	Cryo Electron Microscopy
ER	Endoplasmic reticulum
FFA	Free fatty acid
GC-MS	Gas Chromatography Mass Spectrometry
PL	Phospholipids
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PV	Peroxide value
SEM	Scanning Electron Microscope
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide

SDS	Sodium dodecyl sulphate
SSAA	Salt saturated accelerated aging
TAG	Triacylglycerol
TEM	Transmission Electron Microscope
WOB	Washed oil bodies

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CHAPTER 1

INTRODUCTION

1.1 Background

Recently, there has been considerable interest in utilising plant lipid materials to replace animal derived ingredients in food products. Driven by healthy lifestyle choices and a 'wellness' approach, the demand for vegetable oil is increasing especially for consumers who want food products that are safe, cheap and provide health promotion. The ability to process oils and fats is vital in order to make them acceptable for food applications. The major vegetable oils by production are soybean (54%) followed by rapeseed oils (14%) and sunflower (8.5%). The remainder comes primarily from, cottonseed, peanut, palm kernel and copra. To date, the annual worldwide oil production achieved approximately 170 metric tonnes (Market Report DOI 10.1002/lite.201400071).

Most oil is extracted through pressing with the residual cake being treated with a solvent to remove traces of oil left behind. Conventionally, oilseed production requires extraction with solvents such as hexane to produce a high yield of oil. Solvent extraction is widely used in the oil and fats industry. Despite having good oil production (high yield), the use of hexane causes of issues including the risk of explosion in factories plus workers being unhealthy due to prolonged exposure to this toxic solvent. Additionally, large volumes of solvent are required to extract oil from the seed thus creating environmental issues such as pollution. These disadvantages facilitate the need for a new platform to produce

oil derived from plant materials. Moreover, the industry is not only restricted to food products for human consumption hence, oil recovery is also important in the development of biofuel and the need for environmentally-friendly approaches for production.

Alternatives to solvent extraction processing have been developed and this is an expanding area of research. Interest in producing oil which has a lower impact on the environment is increasing and development of oil using high technology is in demand. There are a number of studies that have investigated new methods of extracting oil from oilseed for human consumption. Among the processing methods is solvent-solvent extraction, membrane filtration, aqueous or enzyme-assisted aqueous extraction. The aqueous extraction process is considered to be a green approach. However, this aqueous processing produces relatively low oil yields and a high content of oil in the residue has discouraged its commercial application. To overcome this issue, the use of hydrolytic enzymes to increase the oil yield was reported (Dominguez *et al.*, 1994, Rosenthal *et al.*, 1996).

Currently, plant species that can be used as biomaterials for incorporation into food products are becoming more popular. For example, soybean lecithin is widely used in food products as an emulsifier for confectionaries. One of the most important uses of plant material is for its lipids. Lipids in plant materials are worth exploring and have many advantages. Plant lipid can be used in non-edible industries such as in biofuel. For instance, vegetable oils such as palm, rapeseed, sunflower and soybean have the potential for biofuel application (Demirbras, 2008). Interestingly, plant lipids are also known to exist in subcellular organelle called oleosomes or oil bodies. These organelles are tiny subcellular spherical structures that provide energy during germination or post-germination. The structure of oil bodies are mainly composed of a triacylglycerol (TAG) in the core, surrounded by monolayer of phospholipids (PL) and the surface of the oil body is

covered with a protein known as oleosin along with minor proteins such as caleosin and steroleosin.

Oil bodies have been extensively studied during the last decades (Garcia *et al.*, 1988; Murphy and Cummins, 1989; Tzen and Huang 1992; Huang, 1996), in different plant species such as maize germ (Nikiforidis *et al.*, 2013), sunflower seeds (Beisson *et al.*, 1996; White *et al.*, 2006; Fisk *et al.*, 2006); rice bran (Nantiyakul *et al.*, 2012), soybean (Iwanaga *et al.*, 2008); rape seed (Tzen *et al.*, 1993; Katavic *et al.*, 2006; Jolivet *et al.*, 2011; Jolivet *et al.*, 2009 and *Echium plantagenum* (Gray *et al.*, 2010). However, the effect of recovery regimes and post-recovery (downstream) processing on oil bodies has not been extensively reported.

It is suspected that the quality of recovered oil bodies depends on the various conditions such as pH, grinding medium composition, seed pre-treatment and others. Obtaining good quality oil bodies is important for consistent functional performance *ex vivo*. Oleosins are alkaline proteins with a molecular weight in the range of 16-25kDa; they confer a net negative charge on the surface of oil bodies at neutral pH (Tzen and Huang, 1992; Tzen *et al.*, 1990). Oleosin is synthesised during seed development and maturation; so they play an important role in physically stabilising these organelles *in vivo*. Due to these properties, oil bodies have received attention as natural oil droplets that can be recovered from seeds through wet-milling and potentially used in a range of food and non-food applications such as pharmaceutical and cosmetic (Iwanaga *et al.*, 2007). Furthermore, these natural emulsions offer versatility as an ingredient; for example oil bodies from sunflower seed have been used to create High Internal Phase Emulsions (HIPEs) for gel networks in a colloidal system (Nikiforidis and Scholten, 2015).

In food manufacture, oil bodies could be used to replace egg yolk and oil in mayonnaise making, or in salad dressing preparations. Since the entire surface of oil bodies is covered with oleosin (protein) and phospholipids embedded with protein, it can protect emulsions from aggregation or coalescence. For example, researchers have attempt to enhance the stability of the oil body-based emulsions using polysaccharides such as pectin (Iwanaga *et al.*, 2008) or xanthan gum (Nikiforidis & Keosseoglou, 2010) and carrageenan (Wu *et al.*, 2012). This property could also useful in non-food applications such as cosmetic and pharmaceutical purposes.

Oil bodies can be simply extracted by a flotation–centrifugation method (Murphy and Cummins, 1999). In this current work, seeds were homogenised simply using a kitchen blender, filtered with cheese cloth before a flotation-centrifugation step. The final buoyant cream fat pad was obtained and is referred to as crude oil bodies.

Extraction of oil bodies plus purification has advantages to obtain pure intact oil bodies. Crude oil bodies were repeatedly washed with buffer or washing solution to get pure oil bodies without extraneous protein. Though much has been studied with the respect to the properties of oil bodies, very little is known and published with regards to obtaining oil bodies of good quality. Good quality described in this study refers to the stability of oil bodies on their physical and chemical states. When oilseeds are crushed in the presence of water, a milky emulsion is generated, but is this emulsion composed of intact oil bodies or of lipid droplets formed through the release of oil from damaged oil bodies, oil that is then stabilised by a mix of natural surface-active materials present in the seed. The hypothesis tested in this work was that wet-milling hard oilseeds results in the breakage of oil bodies and the *in-situ* formation of artefact oil droplets with a physical stability and surface properties distinct from intact oil bodies;

furthermore, that intact oil bodies can be recovered if the seeds are suitably plasticised through pre-soaking the seed in an aqueous medium. The quality of extracted oil bodies may change with seed pre-treatment such as storage conditions or time but this has not been studied. The seed storage conditions and duration of storage are recognised to have an impact on the oil quality, however; in-depth studies into the impact of seed storage on oil body quality have not reported.

Incorporation of ionic solutes (NaCl), detergent (Tween 20) and chaotropic agents for purification of oil bodies such as 9M urea have been shown to successfully increase the integrity of oil bodies during the recovery process (Tzen *et al.*, 1997). In 1998, Milichip, discussed the effect of chaotropic agents and concluded that 9M urea is a suitable washing solution. Lacey and her team (1998) also agreed that 9M urea did not denature the (oleosin) protein. However, the evidence for this relationship is still inconclusive; therefore further investigation needed to be carried out to examine nature of the changes in oil body quality caused by washing regimes.

The thesis intends to determine the extent to which high quality oil bodies can be isolated *ex vivo* and whether the intact oil body structure is preserved. In order to achieve this, factors affecting the quality of oil bodies were investigated. Firstly, the quality of seeds was determined before processing. It is important for audit trail purposes if oil bodies obtained in the future are shown to come from the right source. Several analyses were carried out to guarantee oil bodies obtained in this study achieved the main goal. The following scope is to optimise the processing conditions to isolate intact and stable oil bodies. Stable and intact oil bodies are deemed to be 'good quality' (physically and chemically). The foundation idea of this work is to investigate the impact of upstream and

downstream factors on the properties of oil bodies from sunflower seeds particularly on partially de-hulled sunflower seeds.

Sunflower seeds are widely planted in European countries and in other parts of the world. This research may contribute to the designing of better processing conditions for producing high quality oil bodies especially from sunflower seeds- these organelles could be used directly in products, or further destabilised to yield bulk oil for frying and other applications. The understanding of oil bodies in terms of the physical state as well as chemical state is useful for other relevant research on different plant materials such as oil seed rape (OSR). This study offers some important insights into the understanding of oil body surfaces. The creation of a food emulsion generally requires the generation of kinetically stable oil-water interfaces. For instance, a food formulator could easily profit from the pre-existing natural emulsion formed from the dispersion of intact oil bodies in their products for example mimics sauces, salad dressing, or desserts. Because oil bodies can be used as an intermediate product, the potential for application in relevant food products is promising.

1.2 Aim

The overall aim of this research was to test a range of selected factors that may affect the quality oil bodies recovered from partially de-hulled sunflower seeds. In order to achieve this aim, a several objectives were undertaken to:

1. Study the seed pre-treatment on the quality of oil bodies including dehulling process and cold storage.
2. Optimise pre-soaking time on the morphology of oil bodies *ex vivo*.
3. Determine the location of oil bodies in sunflower seeds by imaging

4. Develop a method to recover oil bodies from partially de-hulled sunflower seeds
5. Establish a washing regime on the physical properties of oil bodies
6. Investigate insight on other seeds such as oil seed rape (OSR)
7. Examine the impact of storage conditions on the quality of oil bodies *ex vivo*.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The focus of this section was to summarise the current knowledge and understanding of oilseed processing. The mechanisms of emulsion instabilities are also highlighted. Overview on oil bodies and its relevance to the current study are discussed.

2.2 Oilseed Processing

Worldwide oilseed production will increase dramatically over the years driven by many factors such as higher consumption of edible oil, the development of the biofuel industry and the needs for green chemistry (Jolivet *et al.*, 2013). Moreover, oilseeds such as soybean, rape seeds and sunflower seeds are vital ingredients for the food and animal feed industries. Table 2.1 shows the processing of oilseeds in European Countries.

Table 2:1 Processing of oilseeds crop in European Countries (Source: DG Agriculture and Rural Development European Commission AGRI C4)

	Crushing (%)	Meal (%)	Oils (%)
Rapeseed	96.5	57.0	41.0
Sunflower seed	88.0	55.0	42.0
Soybean	92.5	79.0	20.0

Solvent extraction is the conventional oilseed processing method which uses organic solvents such as hexane to produce high yields of oil; unfortunately it also affects health and the environment. An efficient processing method that does not have a detrimental effect on the quality of the oil is required to replace the conventionally used solvents. It is desirable that this new process is environmentally-friendly and non-toxic. With regards to the production of oil, the oilseed obtained from aqueous methods are typically less efficient and producing a lower yield of oil.

In the case of oil extraction processes that do not use hexane type solvents, several applications of enzymes in oil and fats have been reviewed. The enzyme treatment assessed offers higher yields of oil and produce high quality meal (Dominguez *et al.*, 1994). The cell walls have to be degraded for oilseed extraction and also to enhance the extractability of oil from oilseeds. A number of enzymes have been used in oilseeds production such as amylase, glucanase, protease, pectinase and also cellulolytic and hemicellulolytic enzymes (Dominguez *et al.*, 1994; Fullbrook., 1984).

Since aqueous extraction has been accepted, there are a number of research initiatives conducted that combine aqueous extraction and manipulate enzyme to extract oil from oilseed crops (Rosenthal *et al.*, 1996, Kapchie *et al.*, 2010). Aqueous extraction with or without enzymes produce oil with similar or better quality than conventional extraction such as direct hexane preparation or prepress solvent extraction. The physical and chemical refining in edible oil processing is presented in Figure 2.1.

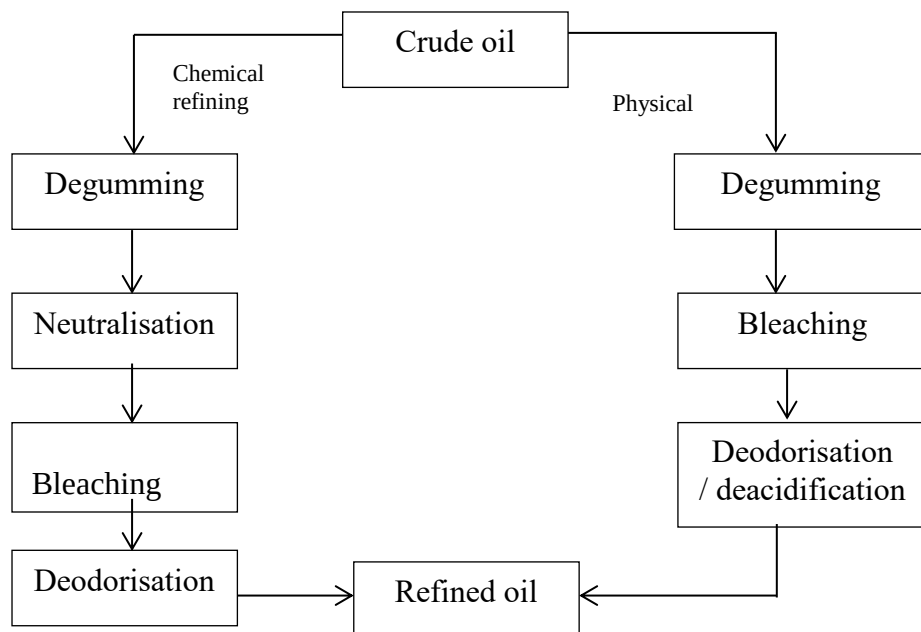


Figure 2:1 General overview of a physical and chemical refining in edible oil processing (Adapted from de Greyt and Kellens, 2000 in Hamm and Hamilton, 2000)

2.3 Preparation of Seed for Oil Extraction

2.3.1 Cleaning

This is one of the first preliminary operations that must be done to remove contaminants from the raw materials. It is important to be able to detect the presence of metal fragments and remove them from raw material, to prevent damage to the food processing equipment and contamination of the final products. In general, simple vibrating screens with variable aspiration are used for fine cleaning.

2.3.2 Seed Drying

After cleaning, the seeds are dried to reduce the moisture content and to control the water activity in the seeds. If the seeds are high in water activity, there is the potential for increased microorganism activity such as mould, and the presence of mite, parasite and others. These external factors might decrease the quality of seeds, physically and chemically. For instance, sunflower seeds ideally require a moisture content of at least 4-6 %, and rape seeds around 8% moisture. This stage is very important to monitor the drying of the seeds in the proper way.

2.3.3 De-hulling

Most seeds can be processed either complete with their outer shell or with a proportion of it removed. One of the reasons for removing the hulls might be to increase the protein content of the cake or meal and decrease the volume to be extracted. First of all, the de-hulling step breaks the hulls. Once the hulls are

broken the separation process includes screening and aspiration, the separation process normally performed with two or more separate steps. Since the hull provide issues of waste disposal, the final hull content will be adjusted to maintain the desired protein content in the product while not removing excessive quantities of hulls. An industrial scale de-hulling machine was is shown in Figure 2.2



Figure 2:2 Industrial scale of de-hulling machine to separate the sunflower hull with kernel. Source <http://www.asia.ru/en/ProductInfo/421697.html>

2.3.4 Flaking

Generally seeds must be flaked. Flaking ruptures the cell structure in preparation for pressing. However, it is important to monitor the temperature and moisture of the seed prior to flaking to prevent the seeds from shattering rather than flaking. If shattering occurs then this will cause a problem during the later processing steps. The seed generally go through smooth rollers to produce a flake which is typically 0.3-0.4mm in thickness. The rollers are usually hydraulically loaded and are typically 600- 700 mm in diameter and 1000- 1500 mm long.

2.4 Sunflower seed

Sunflower seeds are widely used in the oil and fat industry as source of edible oils. The genus name for sunflower is from its Greek name *helios* meaning sun and *anthos*, meaning flower. The cultivated sunflower (*Helianthus annuus*) is one of the 67 species of *Helianthus*. It is a dicotyledonous plant and a member of the Compositae family (*Asteracea*) and have composite flower. The height of the plant at maturity can reach typically 1 to 4.5 meters.

The fruit of the sunflower plant recognised as seeds that are compressed, flattish; oblong with a truncated top. Additionally, the sunflower seed consists of a pericarp or hull, and a kernel with two cotyledons and an embryo. Typically, sunflowers are in bloom from July through September, but this depends on the region and climate in which it is grown.

Historically, sunflower seeds were grown by American Indian tribes throughout North America. The plant was brought to Europe by Spanish travellers in 1569. Later Tsar Peter the Great brought himself the plant from Europe to Russia. At first, the plant was grown as an ornamental but after that it has since been used as food and for medicinal purposes.

Figure 2.3 shows an example of sunflower seeds. Nowadays, there are two types of sunflower seeds 1) oilseed type (for oil production) and 2) non-oilseed type or known as confectionary type. The planting of sunflower seeds around the world was displayed in Figure 2.4. Oil processed from sunflower seeds is widely used for cooking and comparable with other commercial edible oil in the market.



Figure 2:3 Photographs of sunflower seeds (*Helianthus annuus*) and its kernel.

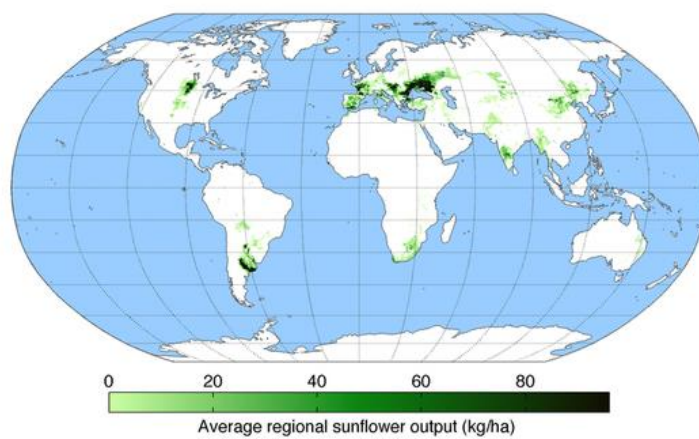


Figure 2:4 A world map of sunflower plantation (Source: <http://fall11ethnobotany.providence.wikispaces.net/Sunflowers>)

2.4.1 Sunflower Seed Composition

Most seeds contain storage oils in the form of triacylglycerol, which comprise 5-50 % of the total seed dry weight. Table 2.2 represents chemical composition of sunflower seeds. It is noted that sunflower seed have high crude protein content (21.75%). The amount of crude fibre is 15.51 %. The nutrient composition of whole sunflower seeds is summarized in Table 2.3.

Table 2:2 Chemical composition of sunflower seeds (in original matter) (Tsuzuki *et al.*, 2003)

Nutrients	Values
Dry matter	93.10
Metabolisable energy (kcal/kg)	4,925
Crude protein (%)	21.75
Calcium (%)	0.33
Total phosphorus (%)	0.72
Crude fibre (%)	15.51

Table 2:3: Average composition of sunflower seeds (Rosa *et al.*, 2009)

Component	Sunflower seeds % (by weight)
Lipid	50-60
Protein	10-20
Carbohydrate	18
Fatty acids	
Palmitic (C16:0)	5.90
Stearic (C18:0)	3.53
Oleic (C18:1)	25.77
Linoleic (C18:2)	63.59

Table 2.3 shows the average composition of sunflower seeds. Sunflower seeds contain insoluble carbohydrates, or crude fibre, which is mainly composed of cellulose, hemicellulose, lignin, small amount of pectin, gums and mucilage and indigestible starch. Lipid is the main component in sunflower seeds comprise about 50-60 % of weight. Sunflower seeds typically consist of lipid and 10-20 % protein.

2.4.2 Processing of Sunflower Oil

Sunflower seed is one of the four major oilseeds produced globally. World production of vegetable oil is represented in Figure 2.5. Total annual production of sunflower oil in 2013/14 was 15.59 million metric tons (USDA, Statistica 2015). The processing of sunflower oil typically requires a two-step process involving mechanical pressing followed by solvent extraction. These two process combined recover a high quality and quantity of oil. The exact compositions of sunflower seeds vary according to their origin. Sunflower seeds generally are dried to a moisture content of 7-8 %, which is suitable for storage and for their later processing. Without separating the shell, de-oiled meal consist of a protein content of approximately 26-28% and a fibre content of approximately 24-28%. Due to this composition, sunflower meal is also suitable for feeding ruminants, poultry and monogastric animals (Tsuzuki *et al.*, 2003).

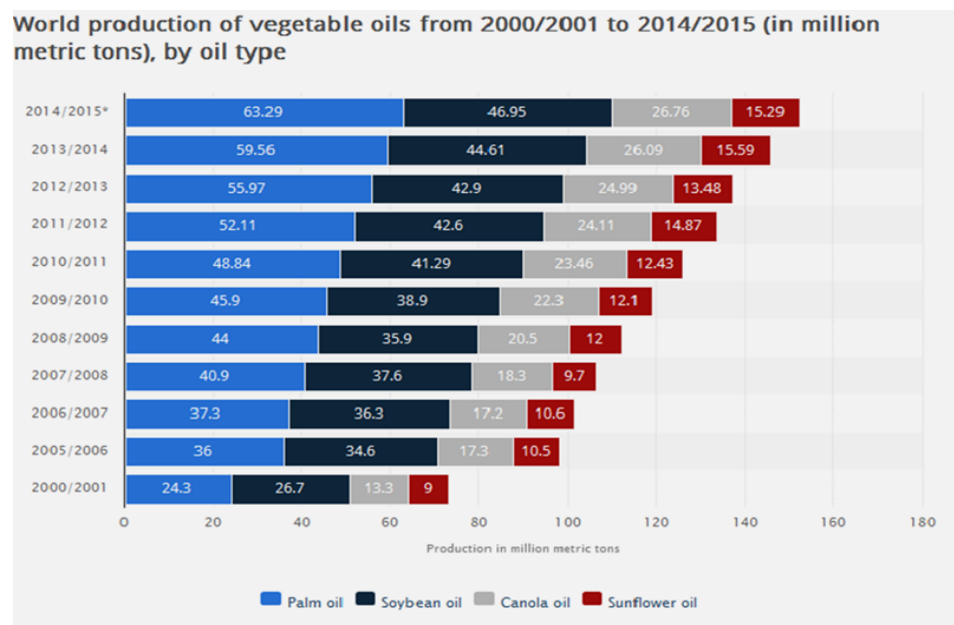


Figure 2:5: World production of vegetable oils from 2000/2001 to 2014/2015 (in million metric tons, by oil type (Source: US Department of Agriculture, USDA Foreign Agriculture Service, Statistica 2015, <http://www.statista.com/statistics/263933/production-of-vegetable-oils-worldwide-since-2000/>)

In order to increase the protein content of meal, the de-hulling process was introduced. The de-hulling process normally involves the opening of the seeds by impact then separating the kernel by screening and aspiration. In the oil and fat industry, the two-step de-hulling process is generally used to obtain high protein content. Usually after de-hulling, the kernel is flaked, cooked and mechanically pressed to achieve 18-22% oil, then sent to the solvent extractor.

2.5 Oil bodies

2.5.1 Oil Body Biogenesis

Oil body formation has been discussed previously (Murphy and Vance, 1999; Huang, 1996; He and Wu., 2009). Formation of oil bodies from seed sources have been suggested to bud from endoplasmic reticulum (ER) by process of vesiculation. Oil bodies are synthesised during seed maturation.

Oleosin, a protein is located on the surface of oil bodies and are synthesised on ribosomes attached to the ER. Furthermore, oleosin is either directly inserted or localised in the budding membranes. Fatty acids are used for the synthesis TAG. When the TAG is completely surrounded by monolayer of PL and oleosin, then it will release into the cytosol as an oil body (Huang, 1996). Oil bodies are degraded during seed germination, and the triacylglycerols are converted to sugars to support the growth of the seedling (Huang *et al.*, 1992).

2.5.2 Oil Body Degradation

TAGs are mobilized to provide a carbon source of energy during seed germination and post germination. The model of an oil body during synthesise and degradation is displayed in Figure 2.6. The specialised peroxisome involved during this process is known as glyoxysome. TAGs which are stored in oil bodies need to be hydrolyzed into fatty acids before they can be used for metabolic activity. Hydrolysis of TAGs occurs to produce fatty acids which are then further broken down via β -oxidation. The hydrolysis products (acetyl-CoA) may be converted to carbohydrates from the glyoxylate cycle and gluconeogenesis (Buchanan *et al.*, 2000).

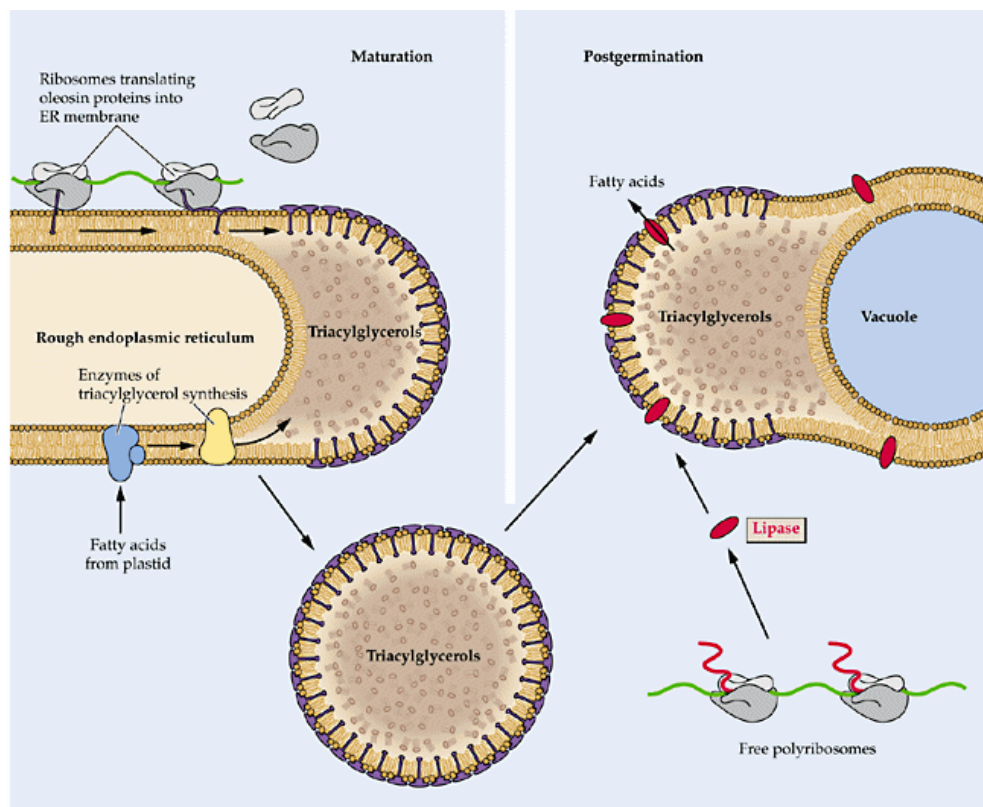


Figure 2:6: Model of an oil body in maize embryo synthesis and degradation during seed maturation and post-germination (Huang *et al.*, 1993).

2.5.3 Oil Body Composition

The complete oil body structure consists of TAGs, PL and oleosins, as shown in Figure 2.7. Oil bodies are synthesised in the endoplasmic reticulum (ER) as the seed matures and then play an important role in storing energy. The energy store is then used during germination and post-germination. Oleosins assist in the stabilisation of the oil bodies to chemical and physical environment stresses such as extreme temperature, moisture or presence of oxidative agents. In mature seeds, oil bodies are stable and do not coalesce even after prolonged storage (Huang *et al.*, 1993). Physical stability of oil bodies depends on two factors:

- i. Surface strength prevents closely associated or aggregated oil bodies from coalescence *in vitro*. Surface strength *in vivo* allows oil bodies to be highly compressed against each other without coalescence and remain as individual entities against physical forces during seed desiccation (White *et al.*, 2008).
- ii. Electrostatic repulsion provided by the negatively charged surface of the organelle. Oleosin orient the molecule in the way that negative charges are exposed to cytosol, while the positive charges face the interior to the TAG matrix of the oil bodies. These positively charged residues also interact with the negatively charged PLs (phosphatidylserine and phosphatidylinositol) and FFAs. These make overall charges of the oil body surface negative at pH 7.0 and prevent oil bodies from aggregating and coalescing (Huang, 1992).

The sizes of oil body vary between different seeds and are approximately 0.2-2.5µm in diameter (Huang, 1992). Oil bodies isolated from oilseeds consist of approximately 94-98% neutral lipid, 0.5-2% phospholipids and 0.5-3.5% proteins

(Huang, 1994). The composition of the neutral lipid contained in the oil body varies in different plant species (Tzen *et al.*, 1993).

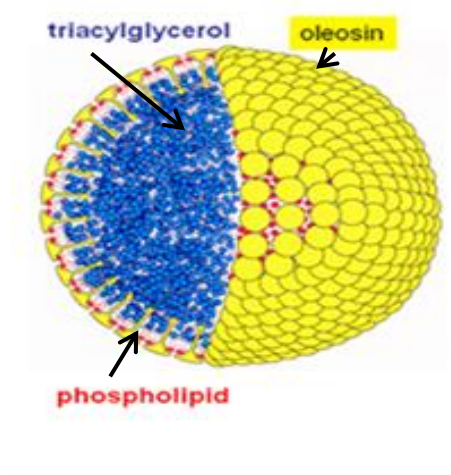


Figure 2:7: Model of oil body representing triacylglycerol (TAG) in the centre, a monolayer of phospholipids and a surrounding layer of oleosin on the surface (Huang, 1992).

2.5.3.1 Oil Bodies Protein

Oleosin in oil bodies are associated with protein that covers the oil body surface. More than 90% of the proteins are represented by two alkaline proteins of 16-30 kDa, dependent on species and molecular mass. Oil bodies only consists 0.5-3.5% protein, of this 80-90% is oleosin with the majority of remainder consisting of protein caleosin (calcium binding) and steroleosin (sterol binding). They are also found in oil bodies but in lower amounts compared to oleosin.

Figure 2.8 displays the basic structural domains of oil bodies. There are 1) an amphipathic N-terminal domain (40-60 amino acids), 2) a central hydrophobic

domain with proline knot consisting of three proline and one serine residue (68-74 amino acids) and 3) a C-terminal amphiphatic domain (33-40 amino acids) (Vance and Huang, 1987). The hydrophobic portion of the oleosin molecule penetrates the PLs layer and TAG matrix. The amphipathic portion resides on PLs layer to exterior. The sequence of oleosin consisting of 181 amino acids is illustrated in Figure 2.9. The position of the hydrophobic region is underlined from position 39 to 110.

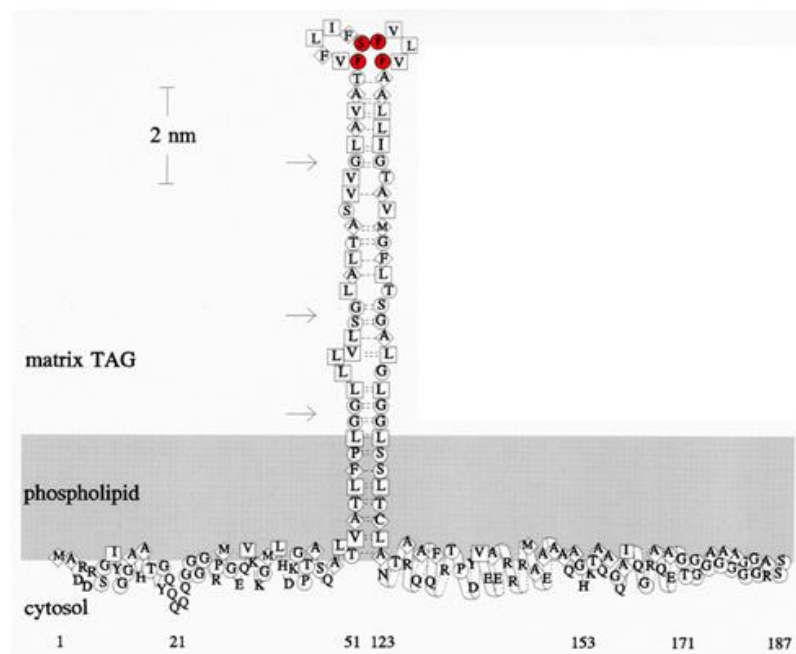


Figure 2:8: Structure of oleosin consists of a N-terminal domain, a central hydrophobic domain and a C-terminal amphiphatic domain (Huang, 1992).

10	20	30	40	50
TTTTYDRHFT	TTQPHYRQDD	RSRYDQQTHS	QSTSRTLAI	ALLPVGGILL
60	70	80	90	100
GLAALTFIGT	LIGLALATPL	FVIFSPIIVP	AVLTIGLAVT	GFLASGTFGL
110	120	130	140	150
TGLSSLSYLF	NMVRQTAGSV	PESLDYVKGT	LQDAGEYAGQ	KTKDFGQKIQ
160	170	180		
STAHMGDQG	QGVVHAQVGG	GKEGRKSGDR	T	

Figure 2:9: Amino acid sequence of common sunflower seeds (*Helianthus annuus*) oleosin consisting of 181 amino acids. Highlighted in yellow are polar (position 1-38), and turquoise are hydrophobic (position 39-110) amino acids (P29529, UniProtKB, ExPASy).

Oleosins play a major role to prevent the oil bodies from coalescing and thereby maintain the oil bodies as discrete and small organelles. However, oil bodies coalescence is prevented by oleosin in normal intracellular conditions. Furthermore, this contributes sufficient surface in order for lipase enzyme anchorage during germination (Huang, 1992).

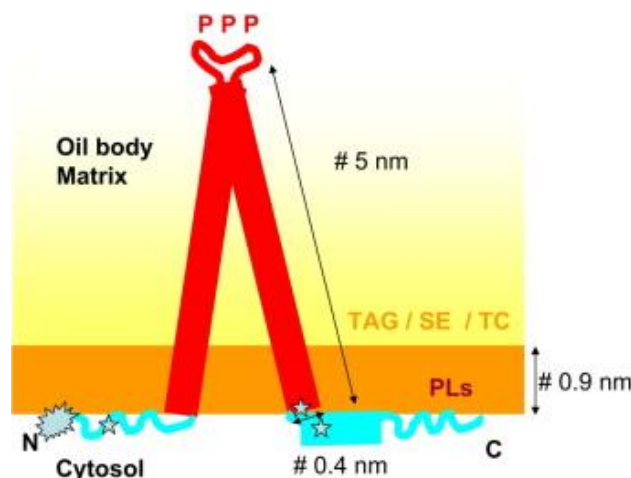


Figure 2:10: Proposed model for oleosin conformation within lipid body interface. Region enriched with positively charged residues found between central region and N and C extensions are presumably involved in interaction with charged phospholipids (Purkrtova *et al.*, 2008).

Purkrtova *et al.*, (2008) proposed a model for oleosin structure and is presented in Figure 2.10. The presence of caleosins constitutes a minor group of integral in oil bodies. Caleosins possess a half-calcium binding site with oleosin and was divided into three-domain structure. In general, the molecular weight of caleosin between 25 and 35kDa.

2.5.3.2 Oil Bodies Phospholipids

Phospholipids in plant tissues are known to be synthesized in the endoplasmic reticulum. Presumably, oil body phospholipids are also synthesized in the endoplasmic reticulum during seed maturation. Phospholipids are an integral part of seed oil bodies, composed of a monolayer around the TAG matrix and provide an environment for protein to locate. Phospholipids in oil bodies consist of phosphatidylcholine (PC), phosphatidylserine (PS),

phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Tzen and Huang, 1992). The phospholipids composition varies with the plant species but the major phospholipids constituent is phosphatidylcholine (41-64.1%), and minor phospholipids fractions present in the sample include phosphatidylserine (18.3-33.1%), phosphatidylinositol (6.9%) and phosphatidylethanolamine (2.8-15.8%) (Tzen *et al.*, 1996).

The protein and phospholipid molecules interact due to the charge and polarity within the molecules (Vance and Huang, 1987). Oil bodies phospholipids develop during oil body biogenesis and the phospholipids content varies between plant species. In reconstituted oil bodies, the presence of neutral lipids, oleosin and phospholipids are very important conferring stability to an oil body but without either oleosin or phospholipids, coalescence occurs and leads to instability of oil body (Tzen *et al.*, 1992).

2.5.4 The Suitability of Oil Bodies to Form Emulsions

One of the key function requirements of several food systems, pharmaceutical and cosmetic products is the ability to form an emulsion. An emulsion is defined as a dispersion or suspension of two immiscible liquids such as oil in water or water in oil.

- i. Oil in water (o/w): An emulsion consisting of oil droplets dispersed in an aqueous phase. Milk and mayonnaise are examples emulsion of oil-in-water emulsions.

- ii. Water in oil (w/o): An emulsion that consists of water droplets dispersed in an oil phase. Butter and margarine are good examples of water-in-oil emulsions.

The stability of an emulsion depends on the balance of forces associated with the interface of the oil and water. When the droplets of the disperse phase approach each other they are attracted by Van der Waals' forces. Consequently, if the attractive forces are not stabilised by repulsive forces the droplets fuse and coalescence occurs.

2.5.4.1 Emulsion Instability

Emulsions are thermodynamically unstable and the instability is related to the changes occurring within the emulsion. The changes occur that affect the emulsion stability possibly due to creaming, flocculation, coalescence and Ostwald ripening in the emulsion system. Creaming or sedimentation in food emulsions is a mechanism where separation occurs by gravitational motion of the particles in the emulsion. Flocculation arises when two or more droplets stick together and form aggregates but they still remain as separated droplets. However, coalescence occurs when droplets merge together and form a larger droplet but are not retained as individual identities. Coalescence is irreversible process. Droplets coalescence can be controlled by ensuring that there is strong repulsive interaction between droplets. Ostwald ripening is a process where larger droplets grow at the expense of smaller droplets due to mass transport of dispersed phase material through the continuous phase. The schematic diagram in Figure 2.11 represents the mechanism that influences instability in food emulsions (McClement, 2007)

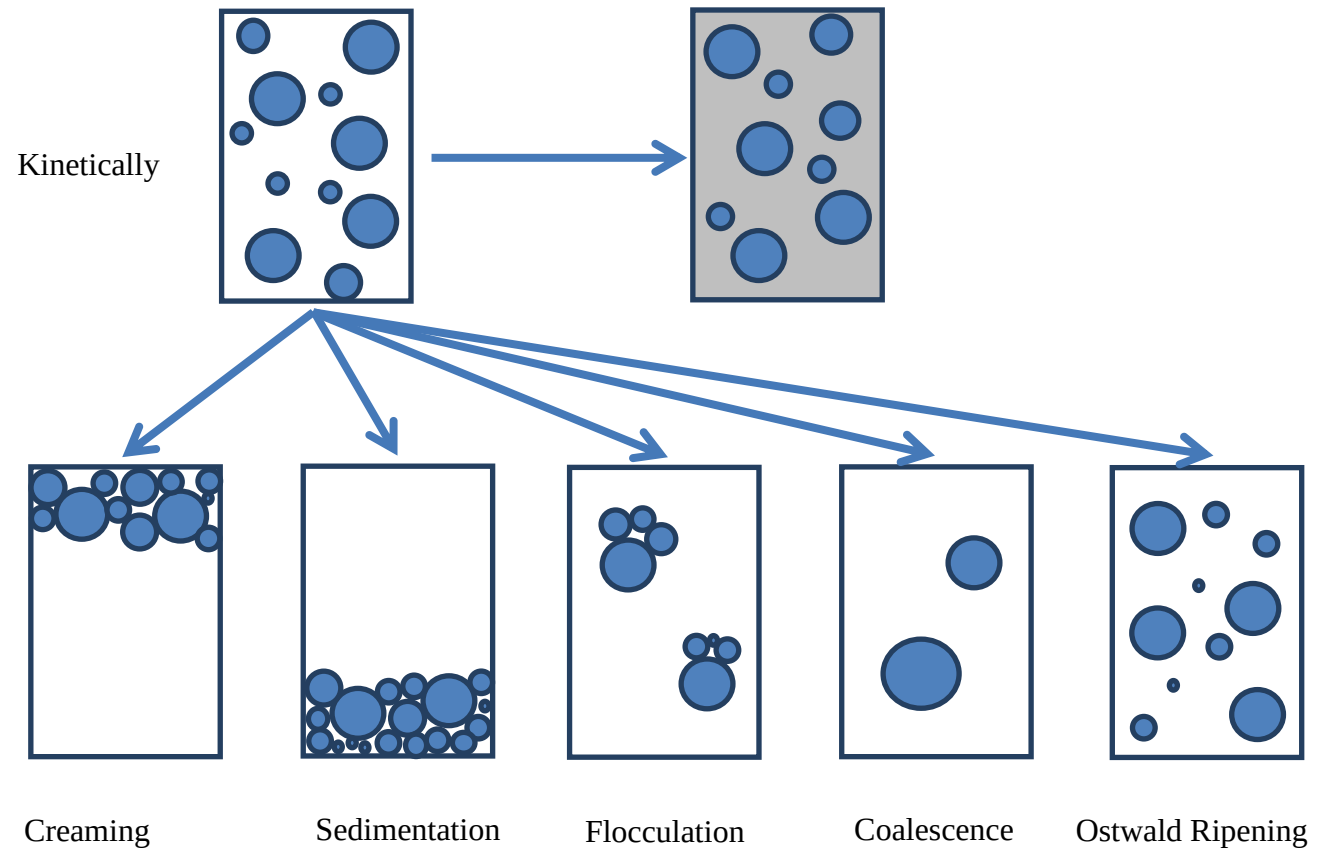


Figure 2:11: Schematic diagram representing the instability mechanism in an emulsion system (McClement, 2007).

2.5.5 Oil Body Suspension

Oil bodies can be recovered using an aqueous extraction method. This method is safe and non-toxic. The hydrophobic properties of surface oil bodies make it easier to incorporate in food formulation. Oil bodies in suspension can be analysed by particle size, zeta potential and creaming or turbidity measurements. An oil body is sensitive to pH (Nantiyakul *et al.*, 2012) and has a negative charge at neutral pH (Tzen *et al.*, 1993). The surface charge of the droplets is very sensitive to pH as a change in pH can alter the charge of the droplet. When the charge is high enough the droplets will repel each other preventing aggregation due to the electrostatic repulsion between them. When the pH is far from the isoelectric point, the oil body suspension is stable against creaming. However, at pH near isoelectric point, the suspension is not stable against aggregation. The electrostatic and steric stabilisation is illustrated in Figure 2.12.

The size of droplets in a suspension or emulsion plays an important role, because it determines the stability, appearance of the final product. The distribution of particle size is represented by a plot of droplet frequency (volume), against droplet size (diameter) (McClement, 2005, 2014). An oil body suspension has an electrical charge which strongly influences their stability. A charge generated on the droplet surface, effectively provides a barrier which prevents droplets from coming together to aggregates.

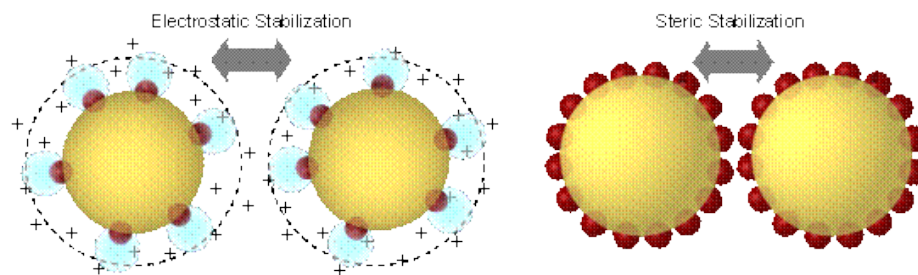


Figure 2:12: Schematic stabilisation of the emulsion. The adsorption of charged particles creates a repulsive interaction between droplets of similar surface charge. Steric stabilisation creates elastic shell around the oil preventing from coalescence. (Source:<https://acswebcontent.acs.org/prfar/2009/reports/P9891.html>).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

Sunflower seeds (oilseed type) were purchased from fresh harvested (October 2013) season in Belgium (Cargill, United Kingdom). The viable (proven by the germination tests described below) black-seeded oil seed rape (*Brassica napus* L. code PR46W21) were drilled on 3rd Sep 2012 and harvested on 14th Aug 2013. The standard chemicals was purchased from Sigma Aldrich United Kingdom. All the chemicals were analytical grade or higher, and sourced from Fisher UK (Loughborough, UK) unless otherwise stated.

3.2 Sunflower Seed De-Hulling

De-hulled sunflower cotyledons were obtained by manually feeding whole seeds into a rotating Glen Creston impact mill (London, UK) at setting 4 (~ 180 rpm) at a feed rate of approximately 10 seeds/s. The rotating hammer was sufficient to crack and thus separate the majority of cotyledons from the hulls. This mixture of sunflower was subjected to pneumatic separation using a Kason shaking sieve air separator (Stoke on Trent, UK). The sunflower husks were pulled off in the rotating air stream and collected in a cloth filter. The remaining intact and de-hulled seeds would remain above the sheet and the cotyledons falling

through for collection. Whole seeds, which failed to separate, were re-fed through the Glen Creston and the process was repeated until all the husks were removed.

3.3 Germination Test

Germination tests were conducted on both fresh and old seeds stored under accelerated aging condition for five months. ‘Between paper’ method (International_Seed_Testing_Association, 1985) was performed on 20 seeds in triplicate.

3.4 Salt Saturation Accelerated Aging (SSAA)

Fresh sunflower seeds (200g) were stored in a sealed box under 40° C with 60% relative humidity (RH) for five months. The 60% RH was achieved using a saturated sodium bromide solution (Greenspan, 1997).

3.5 Measurement of Textural Characteristics

The cracking force, average rupture force and average rupture energy was measured using Texture Analyser (T.A HD plus Stable Micro System, United Kingdom). Twenty five replicates were used for each textural measurement. The samples was selected prior to testing and any visible cracks on the hull and de-hulled were rejected. The set up for the texture analyser are as follows: Pre-test speed, 1.5 mm/s; Test speed, 0.5mm/s; Post-test speed, 10mm; Test distance, 1.5 mm; Trigger type, Auto; Trigger force, 0.20N; Acquisition rate, 200PPS; Load

cell; 100kg; Probe SMS P/1S. A single seed was placed over a central point of the test surface and the graph was drawn based on force (N) and time. Initial peak is considered as initial cracking material and force applied is initial cracking force.

3.6 Imaging

3.6.1 Transmission Electron Microscopy (TEM)

Electron micrographs were prepared as described previously (White *et al.*, 2006). Selected areas of sunflower seeds (approximate 1 mm thick) were fixed in 2.5 % (v/v) glutaraldehyde in cacodylate buffer (0.05 M, pH 7.4) and washed with cacodylate buffer. The samples were then post-fixed in 2 % (w/v) osmium tetroxide for 3h before dehydration in increasing concentration of ethanol (50, 70, 90 and 100 % (v/v)), embedding in Spurr resin and polymerization. Thin sections (0.5 μm) were cut and stained with toluidine blue for light microscopy before ultra-thin sections (80 nm) were selected, cut and mounted on copper grids and stained using uranyl acetate and lead citrate for electron microscopy (JEOL 1010 TEM; JOEL Ltd., Herts, UK).

3.6.2 Cryo-Electron Microscopy

Dry sunflower seeds were glued on to a brass sample holder with carbon glue and subsequently frozen in liquid nitrogen. The sample was transfer into a cryogenic sample holder. The sample was held at -135°C and sputter coated with Platinum for 60 seconds. The sample was then transferred to the SEM chamber and maintained at -135°C . The sample was imaged at an accelerating voltage of 5-15 kV using the SEM column of a FEI Quanta 3D 200 microscope.

3.6.3 Light Microscopy

The microstructures of suspension were determined using optical microscopy (Nikon microscope Eclipse E400, Nikon Corporation, Japan). A drop of the emulsion was placed on a glass slide and covered with a cover slip. The prepared glass slide was observed under the microscope at difference magnification of (40 x, 20 x).

3.7 Oil Body Recovery and Washing

3.7.1 Isolation of Oil Bodies from Sunflower Seeds

Sunflower seeds (100g) were soaked in deionised water under 4° C in the dark place for 2h, 6h and 12h, respectively. The sunflower seeds were homogenised in deionised water (1:5 w/w) using kitchen blenders (Kenwood BL315 blender, Havant, UK) for 2 minutes. Two drops of sodium azide (0.02M) were added to avoid the growth of microorganism. The slurry was then filtered using three layers of cheesecloth under vacuum. The filtered was centrifuged at 7500 rpm (Beckman J2-21 centrifuge, fixed rotor JA-10) for 20 min, and at 4°C. The creamed oil body layer floating on the top of the homogenate was carefully collected and called crude oil bodies (COB). For washing, crude oil bodies were re-suspended in distilled water or 9 M urea (pH 9) at a ratio of 1:4, (w/v) (oil body : washing solution), vortexed vigorously and centrifuged at 4000 rpm (Thermo Jouan CR3i Multifunction, UK) for 20 min at 4 °C before the recovery of the washed oil body pad. The crude oil bodies were further purified by washing with deionised water and labelled as water washed (WWOB). For urea washed, the crude cream pad was washed once with 9M urea (pH 9) and repeatedly rinse with deionised water; labelled as urea washed (UWOB). The crude oil bodies were washed with 0.1M sodium bicarbonate adapted from Beisson *et al.*, (2001) and

labelled as alkali washed (Alkali-WOB). The oil bodies was centrifuged at 4000 rpm for 20 mins at 4°C. The oil bodies were stored at 4 °C for a maximum of 2 days prior to use. Non-soaked seeds (100g) were homogenized straight in 500 ml of buffer solution, and oil bodies recovered as per soaked seed protocol.

3.7.2 Isolation of Oil Bodies from Oil Seed Rape (OSR)

Fresh rape seeds (100 grams) were soaked in deionised water at 4° C in dark place for 2h, 6h, and 12h respectively. Two drops of sodium azide (0.02M) were added to avoid the growth of microorganism. The soaked rape seeds from each soaking time were sieved from soaked water and homogenized (Kenwood BL315 blender, Havant, UK) in 500ml of buffer solution (0.1M Tris-HCl, pH 8.0, two drops of sodium azide (0.02M) for 2min. The slurry was filtered through three layers of cheesecloth; the remaining filter cake was pressed. The filtrate was centrifuged at 7500 rpm (Beckman J2-21 centrifuge, fixed rotor JA-10) for 20min at 4° C. The resulting creamy pad on the top of the mixture, which was crude oil bodies (COB), was collected carefully. The crude oil bodies were re-suspended in 9M urea (1:4 w/w), vortexed for 2min and centrifuged at 9000 rpm (Beckman J2-21 centrifuge, fixed rotor JA-10) for 20min at 4° C. This creamy pad was then rinsed and centrifuged with deionised water for three more times to remove urea. Non-soaked seeds (100g) were homogenized straight in 500ml of buffer solution, and oil bodies recovered as per soaked seed protocol.

3.8 Compositional Analysis

3.8.1 Moisture Content

The moisture content was determined by drying samples (~200 mg) in opened screw-cap microtubes (2 ml) to constant weight for at least 48 h in a vacuum oven (Gallenkamp, UK) at -900 mBar and 40 °C.

3.8.2 Lipid Extraction

3.8.2.1 Bligh and Dyer Lipid Extraction

Lipids were recovered and purified by a modified procedure (Bligh and Dyer, 1959). Samples (0.25 g) in microtubes were extracted with solvent mixture consisting of chloroform:methanol:water (1:2:0.8 v/v) and vortexed for 1 min. Then, chloroform (0.25 ml) was added, vortexed for 1 min and followed by water (0.25 ml) (chloroform: methanol: water = 2: 2: 1.8) and vortexes for 1 min. The mixture was centrifuged at 4000 rpm for 10 min at room temperature to give a two-phase system. Organic bottom phase containing the lipids was recovered by using a needle syringe, evaporated under nitrogen stream to dryness and determined gravimetrically. Samples were stored under nitrogen (-20 °C) in a small volume of chloroform for further analysis.

3.8.2.2 Mini Beadbeater (MBB) Lipid Extraction

The total lipid content of the dried sample (~200 mg) was determined gravimetrically by repeated extraction with isooctane (500 µl) and steel beads (1.0

mm diameter, Biospec, OK, USA) using a Mini-Beadbeater-16 (MBB) (Biospec, OK, USA) for 30 sec at the maximum shaking speed (3,450 oscillations/min). The beaten samples were centrifuged at 4000 rpm for 5 min. Supernatant (450 µl) was collected in a glass Bijou bottle (7 ml). The extraction was repeated twice. The pool extracts were evaporated under nitrogen stream to dryness. The lipid content was then calculated based on dry weight basis. The samples were stored under nitrogen (-20 °C) in a small volume of chloroform for further analysis.

3.8.3 Protein Measurement

3.8.3.1 Protein Content

The protein content of the defatted sample (as described in section 2.5.4) was measured using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). The samples were dried at 60°C for 30 min and then solubilized with 2 % sodium dodecyl sulphate (SDS) solution (1 ml) at 60 °C for 30 min. Samples were vortexed (1 min) and centrifuged (4000 rpm for 2 min). Supernatant was collected and diluted (to within the range of the standard curve) with 2 % (w/v) SDS solution. 50 µl of the diluted samples were then mixed with 1 ml of standard working reagent (10 ml BCA solution and 200 µl 4 % cupric sulphate), incubated at 60 °C for 30 min and measured absorbance at 562 nm against a blank containing everything but the sample. The protein standard curve was prepared using 50 - 400 µg/ml bovine serum albumins (BSA) (Sigma, USA).

3.8.3.2 Protein Separation by SDS- PAGE

Protein concentration was determined using the bicinchoninic acid method (section 3.8.3.1). Proteins in dried oil body samples were then diluted to equal protein concentration with 2 % (w/v) sodium dodecyl sulphate (SDS) solution. Protein samples (20 µl) were mixed with 20 µl of sample buffer (Laemmli sample buffer (Bio-Rad, UK) and 5% (v/v) β-mercaptoethanol), heated at 95 °C for 5 min and cooled on ice. Proteins were characterized by gel electrophoresis (SDS-PAGE) using 15 % resolving gel in 4 % stacking gel (Bio-Rad Laboratories, Hertfordshire, UK). The gel was positioned in SE 600 separation unit and suspended in running buffer (100 mM Tris, pH 8.3, 100 mM Tricine and 0.1 % SDS, (Bio-Rad, UK). Electrophoresis was run at 120 V until the dye front was at about 1 cm from the bottom of the gel. After the electrophoresis, the gel were fixed and stained with Coomassie blue (R-250) (Bio-Rad, UK) and destained with excess pure water. Images were processed using PDQuest Quantity-One 4.6.5 (Bio-Rad, Hercules, USA).

3.8.4 Fatty Acid Composition

The lipid fraction (~0.01g) was dried and then solubilized in chloroform (1ml). The lipid sample was placed into amber HPLC vials (Chromacol, Hertfordshire) and converted to fatty acid methyl esters (FAMES) by the addition of trimethyl sulphonium hydroxide (200 µl). The solution was allowed to derivatise for 10 min at room temperature. A Trace GC Ultra gas chromatograph equipped with a DSQ mass spectrometer (Thermo Scientific, Loughborough, UK) was employed, with a flame ionization detector (FID) and auto injection system (CTC Analytics PAL system auto sampler, Essex UK) and operated in the split mode. One microliter of sample was injected into a capillary column (Phenomenex Zebron ZB-FFAP, California, USA) 30 m × 0.25 mm I.D. coated

with nitroterephthalic acid modified polyethylene glycol (0.25 μm film thickness). The injection temperature was 200 $^{\circ}\text{C}$. The oven temperature was initially held at 120 $^{\circ}\text{C}$ for 1 min and increased to 250 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C}/\text{min}$. The final temperature of 250 $^{\circ}\text{C}$ was held for 2 minutes. The carrier gas was helium (30 ml/min). Retention times of standards (Supelco 37 Component FAME Mix, Supelco, PA, USA) were used to identify detected FAMEs. Methyl pentadecanoate (250 $\mu\text{g}/\text{ml}$ in chloroform) was used as an internal standard. Identification was also verified by comparing mass spectrum with standard library through Thermo Scientific Xcalibur V1.4 software program.

3.8.5 Tocopherol

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 μl through a Security Guard guard-column and separated on a Zorbax RX-C18 5 μm (250 $\times$ 4.6 mm) column with the column oven set at 20 $^{\circ}\text{C}$. 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 of α -tocopherol, at approximately 23.2 minutes, was achieved at excitation and emission wavelengths of 298 and 328 nm respectively and quantitation achieved using the linear formula produced from a calibration curve of α -tocopherol standards in methanol containing 1% BHT

3.8.6 Oxidative Stability

3.8.6.1 Lipid Hydroperoxides

The lipid hydroperoxides were measured according to a method of Shantha and Decker (1994) and modified by Nuchi *et al.* (2001). Lipid extracts (200 μ l) were reacted with 2.8 ml of methanol/1-butanol (2:1, v/v), 15 μ l of 3.94 M ammonium thiocyanate and 15 μ l of 0.072 M iron (II) solution (consisting of the equal volume of 0.132 M BaCl₂ in 0.4 M HCl; and 0.144 M FeSO₄·7H₂O). Samples were left at room temperature for 20 min before measuring absorbance at 510 nm against a blank containing everything but the sample. Cumene hydroperoxide was used as a standard (0.01 - 0.5mM) and results were expressed as mmole per kg of lipid in material based on a dry weight basis.

3.8.6.2 Free Fatty Acid Determination

The non-esterified fatty acids were determined as described previously (Walde and Nastruzzi, 1991). Three stock solutions were prepared: solution A, 0.1 M Tris/HCl buffer (pH 9.0); solution B, 2 mM phenol red in 0.1 M Tris/HCl buffer pH 9.0; and solution C, 50 mM bis (2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane. The assay solution D was then prepared by mixing solution A (0.375 ml), solution B (0.125 ml) and solution C (50 ml) together. Sample solutions were prepared from isopropanol extracts. Wet samples of oil bodies (0.3 g) were weighed into centrifuge tubes and 1 ml of isopropanol was added. The tubes were shaken using a vortex mixer for 1 min. Then, 1 ml of isopropanol was added and the tubes were mixed for another min. The tubes were centrifuged at 2,800rcf for 10 min. Supernatant was collected, evaporated under nitrogen stream and then, re-suspended in 1 ml isopropanol. The measurements were performed by adding solution D into 1.8 ml cuvette and the absorbance at 560 nm were

recorded. Afterwards, 30 μ l of isopropanol extracts were added and mixed. The absorbance was recorded again. From each reading, the change in absorbance at 560 nm caused by 30 μ l of the extracts could be determined and calculated as percentage of free fatty acids in total lipid extracts. Standard curve was made by using standard oleic acid 2.54 - 25.4 mM (≥ 99 % (GC), Sigma Ltd., Gillingham, UK).

3.8.7 Oil Body Physical Analysis

3.8.7.1 Preparation of Oil Body Suspension

Oil body suspension was prepared by mixing one gram of oil body cream (crude oil body or washed oil body) in deionised water (9ml) to make up of 10% of the suspension. A uniform dispersion was achieved by passing the mixture for 10 times using Potter Elvehjem Homogeniser (Wheaton, USA) at 700 rpm.

3.8.7.2 Particle Size

The droplet diameters of oil body suspension were determined by using a laser diffraction particle size analyser equipped with universal liquid module (LS 13 320 Beckman Coulter, High Wycombe, UK) and deionised water as fluid. Fraunhofer theories of light scattering using the polarization intensity differential scattering technology (PIDS) covering a size range from 0.04 - 2,000 μ m, obscuration 4 with no sonication and a pump speed of 40 %. The mean values were reported as the volume mean diameter D (4, 3) or surface mean diameter, D (3, 2). Each individual particle size measurement was determined from the average of three readings made per sample.

3.8.7.3 Zeta Potential Measurements

Oil body suspensions (30 μ l) were diluted in same grinding medium. Diluted emulsions were then injected into the measurement chamber of a particle electrophoresis instrument (Delsa Nano C Particle Analyzer, Beckman Coulter, Inc., USA). Settings used were: Temperature 25°C; refractive index of dispersant = 1.330; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm. The zeta potential (ζ -potential) was then determined by measuring the direction and velocity of the droplets in an applied electric field from which ζ -potential was calculated using Beckman Software. Each ζ -potential measurement was reported as the average of three readings made per sample.

3.9 Statistical Analysis

3.9.1 Statistical Analysis

All experiments were performed in triplicate, unless otherwise specified. Data and graphs were processed using Microsoft Excel (2011) including averages and standard deviations. Statistical analysis was performed in Minitab version 16.2.2 software. Significant differences were identified by Post-Hoc Tukey's test ($P < 0.05$). Principal component analysis (PCA) was performed using XLStat version 2015.1.01.

CHAPTER 4

EFFECT OF SEED PRE-TREATMENT ON THE QUALITY OF RECOVERED OIL BODIES

Despite the stable nature of mature sunflower seeds (they can be stored for extended periods of time and still be viable through germination), it is recognised that the quality of extracted oil can change due to the seed pre-treatment used; such as with storage conditions or time (Moncaleano-Escandon *et al.*, 2013) and retention of seed coat or hull during oil extraction. Most work published on oil bodies pay little, if any, attention to the history of seeds and how they have been stored or pre-treated. Work was therefore undertaken to establish the impact of seed storage and pre-treatment on the quality of oil bodies.

4.1 Hulled and De-Hulled Sunflower Seeds

Hulling is the process of removing hulls from the oilseed to obtain high quality edible oil by the processing of kernels (Gamea, 2013). In order to determine the suitability and the quality of oil bodies produced in future experiments (see section 5.2 to section 5.3.4.1 for details), the selection of optimum raw material is vital. The hull or outer coating of the seed, is typically 20-30 % of the seed by weight, depending on the variety, and contains mostly crude fibre with insignificant amount of fat (Sharma *et al.*, 2009).

Various types of de-hulling equipment are available. The most popular in the processing industry are based on impact methods. Industrially, sunflower seeds are expressed directly from whole seeds for their oils but the process suffers from rapid wear and tear of machinery. Comparisons were made between samples which were de-hulled or not prior to extraction of oil bodies to determine which material is best to use for further studies.



Figure 4:1: Image of sunflower seeds (confectionary type) a) whole seed b) de-hulled seeds (kernels)

Figure 4.1 shows the difference in appearance of hulled and de-hulled sunflower seeds. To determine whether this physical difference in seeds leads to differences in the extracted oil bodies, further tests were completed by analysing the composition of the sunflower seeds. Firstly proximate composition was tested such as lipid and protein contents.

Table 4:1: The lipid and protein contents (% dry weight basis) of hulled and de-hulled sunflower seeds by Bligh & Dyer (1959) technique.

Samples	Lipid content (%)	Protein content (%)
Hulled	45.07 ± 2.03^b	22.47 ± 3.89^d
De-hulled	54.28 ± 2.90^a	32.50 ± 2.87^c

Note: ^{a-d} Means that do not share a letter are significantly different ($P < 0.05$)

The results shown in Table 4.1 indicate that there is a significant difference in the lipid content of hulled and de-hulled sunflower seed samples on a dry weight basis, as analysed by the Bligh & Dyer (1959) technique, with the de-hulled seeds containing more lipid. The protein content also increased significantly on removal of the seed hull or coat; this is due to removal of fibre-rich solids. One of the advantages of de-hulling is the increase in the total yield of oil (Gupta and Das, 1999; Sharma *et al.*, 2009; Gamea 2013).

It was hypothesised that the quality of raw material will affect the quality of recovered oil bodies. To test this hypothesis, physical measurements of the oil body cream were carried out. Figure 4.2 shows images of oil body cream produced from hulled and de-hulled sunflower seeds.

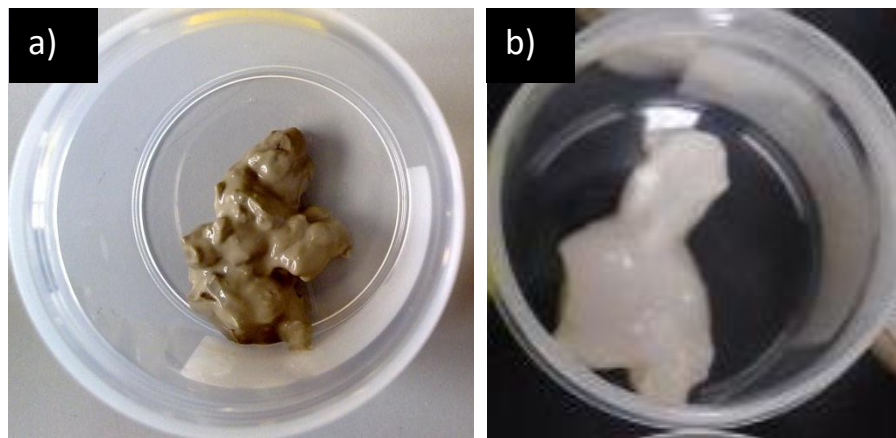


Figure 4.2: Oil body cream of a) hulled b) de-hulled sunflower seeds.

Oil bodies produced from de-hulled sample material had a pleasant beige colour whereas, in contrast, an oil body cream extracted from hulled sunflower seed was darker (greyish). This was most likely due to transfer of pigment from the hull to the oil bodies, creating another drawback for using hulled seed, as the hull gets mixed with the cream. Therefore, the application of oil body cream from hulled seeds may be limited in food products; because de-hulled material had increased protein and oil content and has a better physical appearance, thus it is clearly advantageous to select de-hulled seed as the raw material for future study.

4.2 Mechanical Force

As demonstrated above, sunflower seeds are de-hulled (fully or partially) before conventional processing to increase the protein content and reduce the crude fibre of the 'remnant meal' after oil extraction. Initial experiments also suggested that de-hulled seed gave a higher yield of oil body material compared with the hulled seeds. The oil body material of de-hulled seed was also shown to have a pleasant physical appearance. Therefore, de-hulled material appears to be the best choice.

However, it is unknown if the process of de-hulling affected the quality of the seeds or led to damage of oil bodies housed within the cytoplasm as damaged seeds may potentially reduce the oil yield and quality (Gunasekaran *et al.*, 1988; Parde *et al.*, 2002). De-hulling of sunflower seeds involves a high level of impact created by a hammer mill (Glen Creston), followed by separation of the kernel and seed coat, and removal of hulls by air flow. Therefore, to ascertain whether the process of de-hulling affected the quality of oil bodies, the resistance of the sunflower seed under compressive loading was measured. This information, on the mechanical or textural properties of sunflower seeds and their kernels, could be

useful to reduce the physical damage sustained during mechanical processes which are commonly based on impact crushing methods.

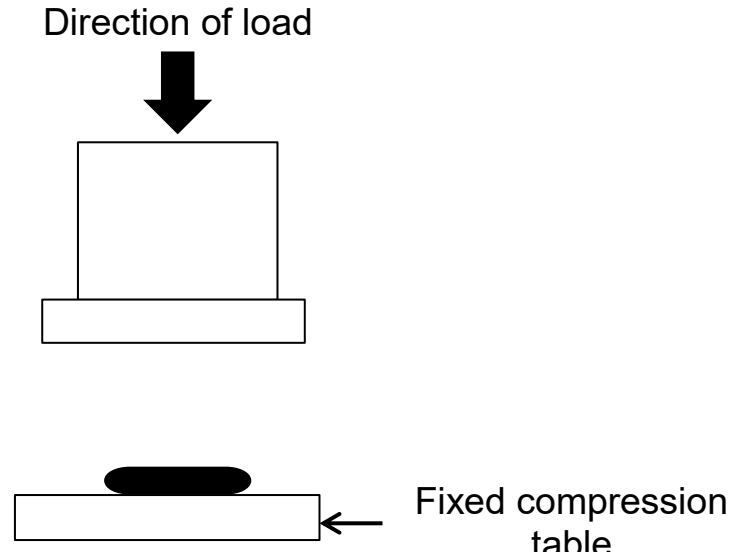


Figure 4.3: Compression test of sunflower seeds under horizontal loading.

Mechanical damage of plant crops has been extensively studied in soybean (Parde *et al.*, 2002; Gunasekaran *et al.*, 1988), sunflower seeds (Hernandez and Belles, 2007; Gupta and Das, 2000; Sharma *et al.*, 2009), karingda seeds (Suthar and Das, 1997) and melon seeds (Sharma *et al.*, 2009). This current work used the texture analyser to test the initial cracking force and rupture energy, and correlated their effect on mechanical impact during the de-hulling process. The schematic diagram of the compression test is illustrated in Figure 4.3.

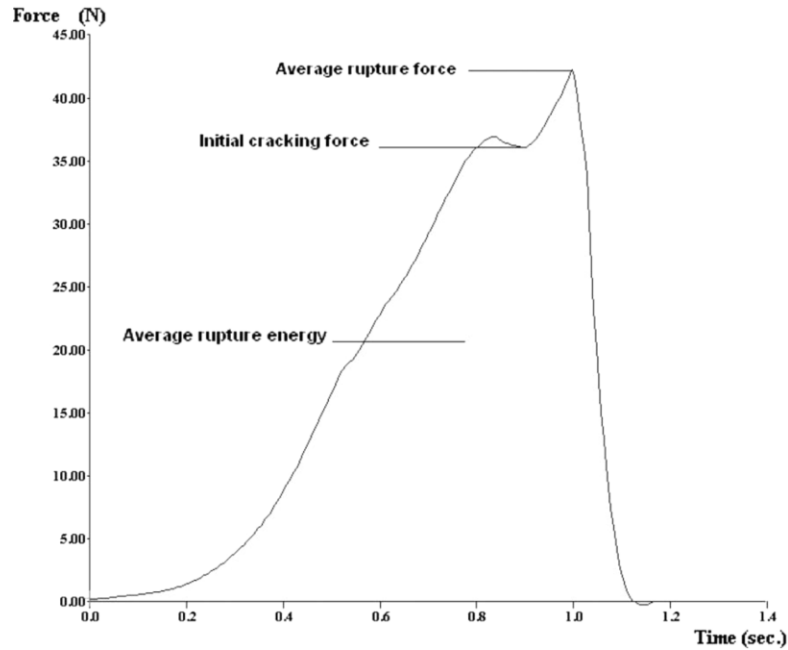


Figure 4:4: Typical force-deformation curve showing textural characteristics (Sharma *et al.*, 2009).

A typical force-deformation curve of seed is presented in Figure 4.4. The initial peak is considered as the initial cracking of the test material and so the force related to this initial peak position is considered to be the initial cracking force. Initial cracking force may be responsible for the de-hulling of seeds while the rupture force (shown by the second peak) and rupture energy contributes towards the oil removal from the oil bearing material (Sharma *et al.*, 2009). The average impact of mechanical force for the hulled and de-hulled sunflower seeds studied in this thesis is displayed in Figure 4.5.

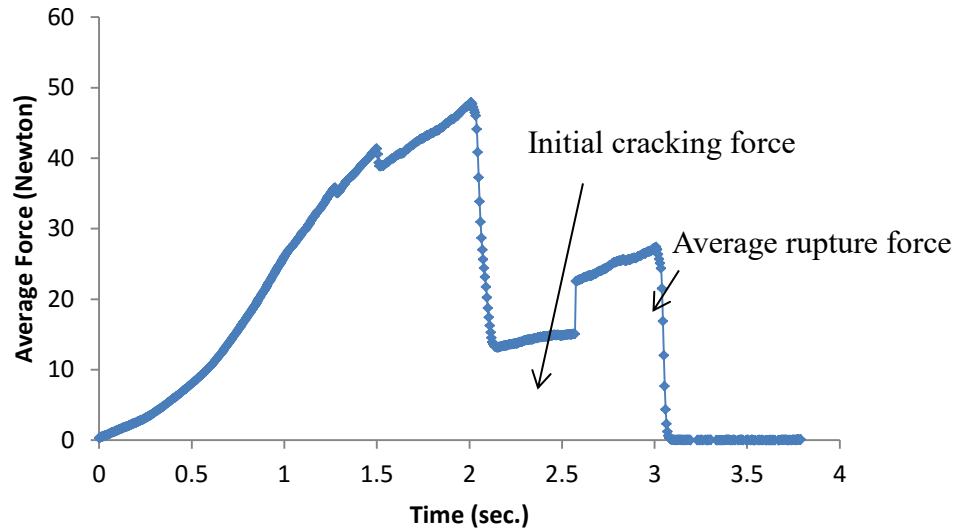


Figure 4:5: Average impact of mechanical forces over time on hulled sunflower seeds (n=25).

The surfaces of sunflower seeds used in this experiment are irregular in shape; the contact area between the samples was expected to change. The graph shows that the initial cracking peak was detected in hulled samples at 2.0 seconds at 14.5 N of compression force. This level of force (14.5 N) in this study is lower than initial cracking force (25 N, 6% moisture content) of sunflower seeds of *Morden* variety (Sharma *et al.*, 2009). Therefore, the results shown in this study might be attributed to the toughness of the hull shielding the kernel from damage. If less force (< 14.5 N) was applied, the hull is difficult to rupture and decreases the amount of non-recovered kernel which would result in a decreased de-hulling efficiency. Increasing the force during the testing of hulled samples from 14.5 N to 26.3 N contributed to the total damage of the seeds.

In this work, sunflower seeds were de-hulled prior to oil body extraction. Using the hammer mill should break up the seed coat into two separate cotyledons

without producing too many broken seeds or dehulled seeds (Gupta and Das, 1999). The results indicated that sunflower seeds were not damaged at 14.5 N during compression however, this force was sufficient to break up the seed coat. In this way, the de-hulling process retained the sunflower kernels and as a result preserved the oil bodies in the cytoplasm. Therefore the mechanical forces of de-hulling have been shown to not overly damage the kernels and therefore de-hulled material is still the best choice for future studies.

Obtaining consistent and reliable yields of oil bodies was very important in this study. Despite the de-hulled confectionary type (black and white stripe) being deemed to be the best for future study, a supply issue meant that it was not possible source another batch of this particular seed. Therefore a new batch of sunflower seeds was purchased; these seeds were they oil seed type (black hull) and were from a freshly harvested season in Belgium (October 2013). These sunflower seeds were used as a new raw material to continue the remainder of the study.

4.3 Germination Test

In order to determine the quality and viability of these seeds, a germination test was carried out using ‘between paper methods’ as mentioned in Chapter 3 (section 3.3). The sigmoid graph displayed in Figure 4.6 shows the germination rate of sunflower seed over time.

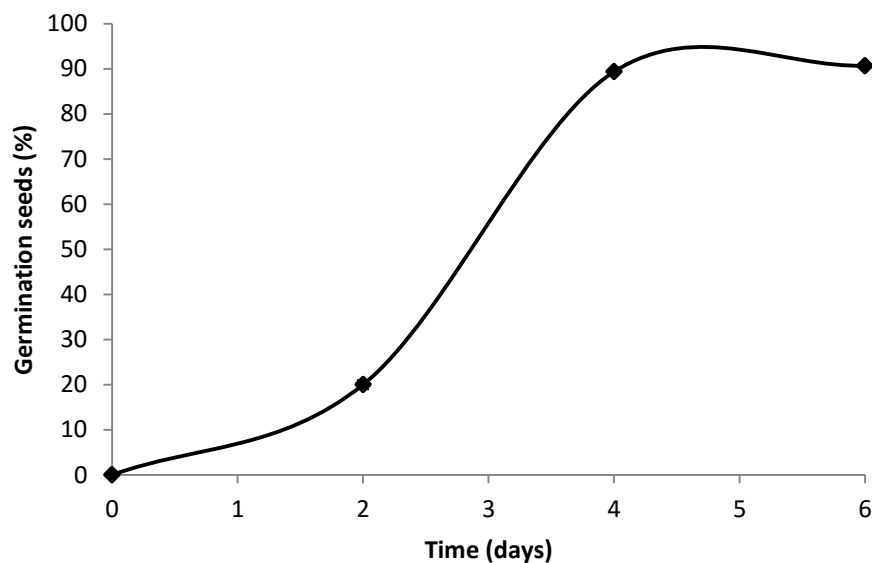


Figure 4:6: Germination of sunflower seeds over time using “between paper method” (ISTA, 1985).

As can be seen, the seeds start to germinate after day 1 and the germination rate achieved lag phase after day 4. It was found that the germination rate for sunflower seeds from this oil seed type sunflower seeds cultivated in October 2013 was 95%. The result indicates that the newly purchased sunflower seeds used in this work is of high quality and therefore suitable for this study.

4.4 De-Hulling Sunflower Seeds

In order to obtain de-hulled seeds (which were shown to be the most ideal in section 4.1) from the second batch of seeds a de-hulling process had to be conducted in house, as suggested in section 4.1, prior to oil body extraction. Figure 4.7 demonstrates how sunflower seeds (oil seed type) were treated during this de-hulling process.



Figure 4:7: De-hulling process (step 1-6) of sunflower seeds in Sutton Bonington Campus, University of Nottingham. The whole sunflower seed (oil seed type) was crushed in a Glen Creston mill then followed by screening to separate the seed coat.

The de-hulling process was not fully successful in this work due to the incomplete de-hulling of all the sunflower seeds. The reason for this inconsistency was probably due to the seed's coat having the same dimension as the kernel, thus the process of crushing the hull was challenging. Another explanation for the difficulties experienced in the de-hulling process could be due to the plastic consistency of the hull which makes it adhere strongly to the kernel. Since there was a limitation in equipment availability and time constraints, selection of the best, available raw material was necessary. Figure 4.8 shows the selection of sunflower seeds which have been discussed so far.

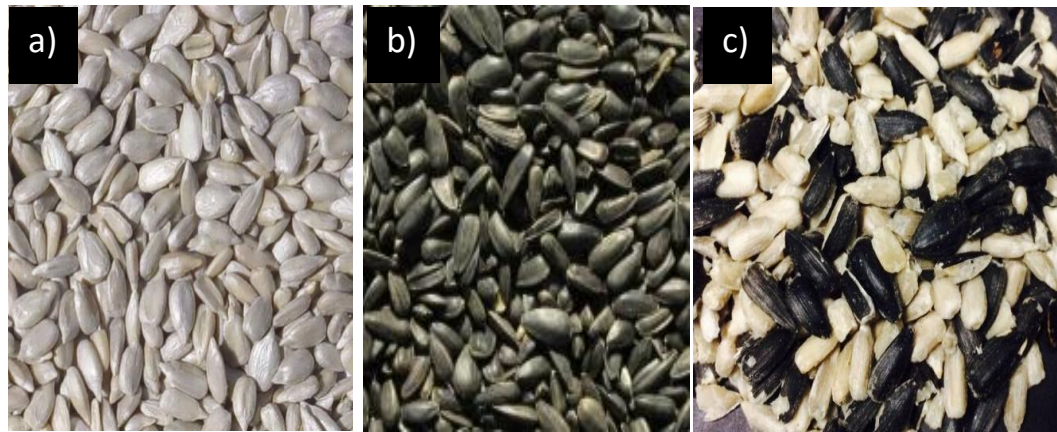


Figure 4:8 Image of sunflower seeds represent in this work. a) De-hulled sunflower seeds b) Hulled sunflower seeds c) Partially de-hulled sunflower seeds.

Due to the de-hulled confectionary seed being unavailable it was proposed that the partially de-hulled seed, prepared in house from the Belgian oil type seed, be used instead. However, to determine whether the partially de-hulled seed was a suitable alternative, an initial test was carried out to recover oil bodies from the partially de-hulled sunflower seeds and basic measurements were undertaken such as basic composition (lipid and protein) and physical observation. Firstly the

composition was assessed and compared to the previously studied seeds (section 4.1) as shown in Table 4.2.

Table 4.2: The lipid and protein content (% dry weight basis) of hulled, partially de-hulled and de-hulled sunflower seeds by the Bligh & Dyer (1959) method.

Samples	Lipid content (%)	Protein content (%)
*Hulled	45.0 ± 3.24 ^b	22.47 ± 1.23 ^b
Partially de-hulled	55.2 ± 4.46 ^a	30.53 ± 1.27 ^a
*De-hulled	54.3 ± 2.90 ^a	32.51 ± 2.65 ^a

Note: ^{a-b} Means that do not share a letter are significantly different (P<0.05)

*Samples extracted from confectionary type of sunflower seeds.

Table 4.1 represents the comparison of lipid and protein contents, on a dry weight basis, of the different types of sunflower seeds. Based on results shown in Table 4.1, the lipid and protein content extracted from partially de-hulled sunflower seeds was not statistically different (P>0.05) to de-hulled sunflower seeds. Therefore, the results indicate that in terms of composition partially de-hulled appears to be a suitable alternative to be used as a raw material for the remainder of the study.

Physical observation of oil body cream extracted from de-hulled and partially de-hulled sunflower seeds was also compared and is shown below in Figure 4.9.

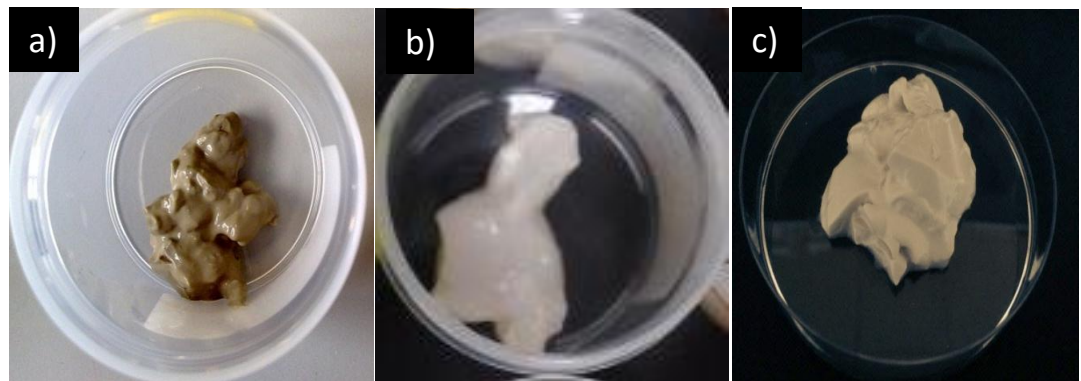


Figure 4.9: Comparison of oil body cream extracted from a) hulled sunflower seeds b) de-hulled sunflower seeds c) partially de-hulled sunflower seeds.

Based on the results obtained, the physical appearance of oil body cream extracted from partially de-hulled was compared with oil bodies cream extracted from de-hulled sunflower seeds. Oil body cream extracted from partially de-hulled are beige in colour which is better than the grey colour of hulled but not quite as good as the white of de-hulled. Oil body cream extracted from partially de-hulled sunflower seeds was more favourable than hulled sunflower seeds (see Figure 4.2). Therefore, to overcome the limitation in sourcing the optimum seeds, partially de-hulled seeds were shown to be a comparable and suitable substitute and so were selected as the best raw material used for the remainder of this study.

4.5 Effect of Chilling Seed on Oil Body Quality

With the selection of the best raw material, it was important to establish the optimum method of storing the seed to maintain the best quality of extracted oil bodies. Temperature management was crucial in order to maintain the quality of harvested crop. Information concerning the physiological changes in seeds during storage is little documented and details of the ideal storage conditions for sunflower seeds is still scarce. Therefore, knowledge of the effect of different storage conditions on this raw material is required. Ideal storage conditions for this raw material are crucial to maintain its freshness and quality of the seeds. All seed may deteriorate with time but the rate of deterioration can be controlled by storing them under favourable storage conditions.

A reduction in temperature may affect the metabolic process and lead to natural denaturation of the plant materials thus, might lower the quality of this agricultural product. Some commodities which experience temperature reductions immediately after harvest, to a non-freezing temperature, may have a shortened life and experience chilling injury.

However, contrary to this, Harrington's Thumb Rules reveals that a 6°C reduction in storage temperature may double the life span of seeds. This is because; maintaining the relative humidity (RH) and temperature are important factors for longevity. It is recommended to store products such as seeds, vegetables or fruits at refrigeration temperatures (of approximately 4°C) to enhance their life span and minimise the spoilage caused by microorganisms. Santana (2012) investigated the effect of time and storage temperature on the chemical and physical characteristics of soybean pods and concluded that storage at 7°C for 6 days maintained the starch content and colour. However, it is

currently unknown if storage of sunflower seeds at low temperatures will also preserve the oil bodies.

Additionally, in an industrial processing system, to store sunflower seeds at ambient temperature would require less energy compared to storing seeds at lower temperatures (4°C). Therefore, to determine whether there were any major differences to the quality of oil bodies upon storing chilled or at ambient temperatures, partially de-hulled sunflower seeds were stored for 7 days at both temperatures. Then a simple test was carried out to observe if chilling had an effect on oil body quality. This would enable the optimum storage conditions to be determined.

After storage at 4°C the oil bodies were recovered as described in section 3.2.1 (Material and Methods). The average particle size and distribution was measured, and qualitative assessment by microscopy was performed. The results were reported, and compared with seed stored at ambient temperatures, in the following sections.

4.5.1 Particle Size

The particle size of the oil bodies was determined after seed storage at lower temperature (4°C). In figure 4.10 the volume mean diameter (4, 3) of crude and washed oil bodies are presented. Crude oil bodies displayed relatively large oil body droplets ($16.4 \pm 1.2 \mu\text{m}$) however; washing the oil bodies slightly reduced the diameter to $10.2 \pm 0.8 \mu\text{m}$. The larger particle droplets suggest that the suspensions were aggregated and that perhaps some coalescence had occurred.

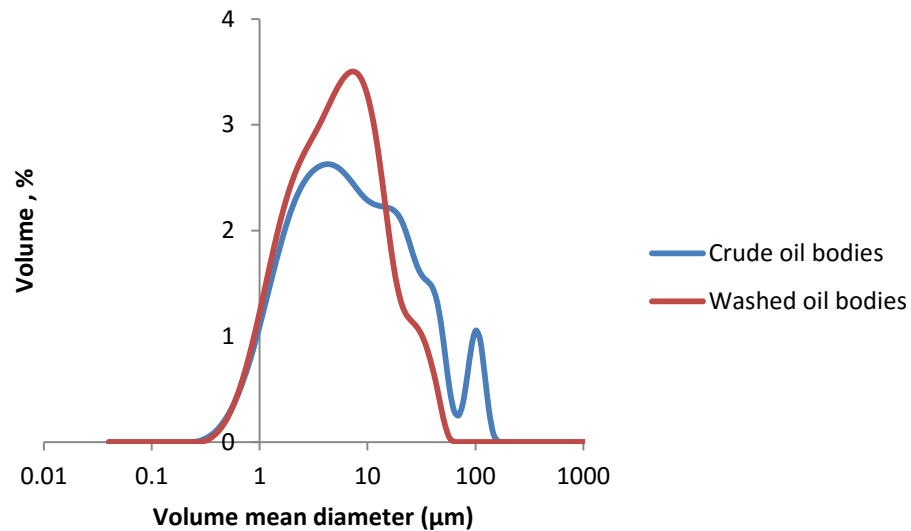


Figure 4:10: Particle size distribution of oil body suspension extracted from partially de-hulled sunflower seed under chilled storage conditions (4° C).

4.5.2 Imaging of Oil Bodies

To support the particle size data, images of oil bodies under chilled storage were obtained using a microscope and assessed. The raw materials were left for 7 days at 4°C. Comparison of oil body morphology after recovering from seed stored under different temperatures is demonstrated in Figure 4.11. Based on the results obtained in this work, signs of aggregation and coalescence were detected in the crude oil body preparation from both storage conditions (ambient and chilled). Interestingly, oil bodies extracted from seeds stored at low temperature (4°C) showed larger particle size after the washing process. The significant size increase after washing was due to damage of oil bodies during chilling and resulted in formation of hybrids of oil body droplets. The results indicate that the suspension was unstable and the oil bodies were damaged, attributed to the fact the raw materials were stored under chilled conditions (4°C).

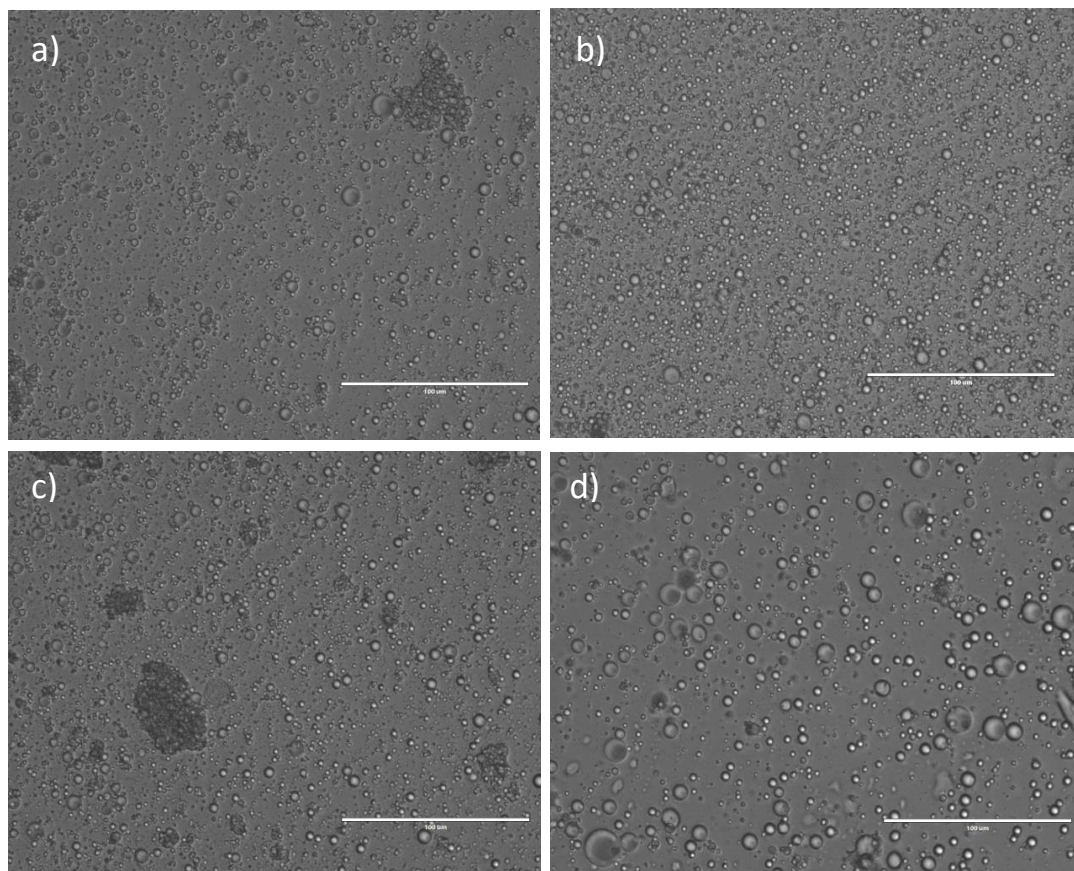


Figure 4:11: Images of oil bodies extracted from seed stored at low (chilled) temperature (4°C) and ambient temperature (20°C). **(a)(c)** represent crude oil bodies (COB), pH 7.32 while **(b)(d)** represent washed oil bodies (UWOB), pH 7.65 **(a)(b)** Seed storage under ambient temperature (20°C) and **(c)(d)** store under chilled temperature 4°C. Oil bodies was washed with 9M urea once and repeatedly was with deionised water to remove urea residue.

Lyons (1973) had established the term 'chilling injury' referring to any plant species that were killed during chilling or above freezing point. Bedi and Basra (1993) highlighted that the sensitivity of chilling injury is based on several factors such as 1) species and cultivar involved 2) initial water content of seeds 3) the temperature to which seed is exposed 4) the duration of the exposure 5) the period during the course of germination when the seed is exposure takes place.

However, the results obtained in this work, confirm that when the seeds are exposed to low temperature (4°C); chilling injury is likely to affect the properties of oil bodies. This low quality of seeds is very likely to increase the chance of getting low quality oil bodies. It contradicts Harrington's Thumb Rule, where lower temperatures preserve the quality of products. Czaikoski (2012) evaluated the effect of storage and temperature on vegetable-type soybean grain and recommended to store at 5°C for three days to retain the Vitamin C level. Partially de-hulled sunflower seeds used in this work showed significant changes with regards to the quality of oil bodies. It is not therefore the ideal temperature (4°C) to store the raw materials.

The information gathered in this work is useful to select suitable conditions for oilseed material. Sunflower seeds used in this study were kept at ambient temperatures (20°C) in the Dry Materials Room in the Biomaterials Building within the Sutton Bonington Campus, University of Nottingham prior to use.

4.6 Summary of Results

To establish the impact of seed storage and pre-treatment on the quality of oil bodies, the selection of raw material is crucial. High quality seeds were obtained by partially de-hulling the seeds, a process which increased the protein content and produced a more favourable oil body cream compared to hulled seed. In fact, the forces involved in the de-hulling process did not damage the oil bodies but were sufficient to break up the seed hull prior to oil extraction. The seeds used in this work achieved a 95 % germination rate, an indication of the freshness of the sunflower seeds as determined by the germination test. Due to limitations during de-hulling process, partially de-hulled sunflower seeds were chosen as a

raw material throughout the study as it had similar characteristics to the fully dehulled sample. Sunflower seeds ideally can be stored at ambient temperature and definitely not chilled as it may lead to the damage of oil bodies.

CHAPTER 5

IMPACT OF PROCESSING ON OIL BODY QUALITY

5.1 Imaging of Oil Bodies *In Vivo*

It was reported that oleosin plays a major role in maintaining the size of oil bodies (Huang, 1992). Figures 5.1 and 5.2, shows the features of oil bodies when viewed using a Transmission Electron microscope. Oil bodies are spherical and densely packed within the cotyledon tissue. Transmission Electron Microscopy (TEM) and Cryo- Electron Microscopy were used to observe oil bodies *in vivo*. During TEM preparation, the seed tissue was cut into sections approximately 1 mm in thickness, fixed with 2.5 % glutaraldehyde, and post-fixed with 2 % osmium tetroxide before ultra- thin (80nm) sections were selected. Oil bodies are formed in eukaryote cells at the surfaces of vesicles in plant cells (Murphy, 1989). The oil bodies are primarily located near the cell wall, as shown in Figure 5.1. It can be seen that oil bodies were tightly packed within the cell however, they still remained as individual organelles. The shape of oil bodies is irregular and compaction occurred within the cytoplasm and cell wall. However, oil bodies reform once released in the surrounding media *ex vivo*

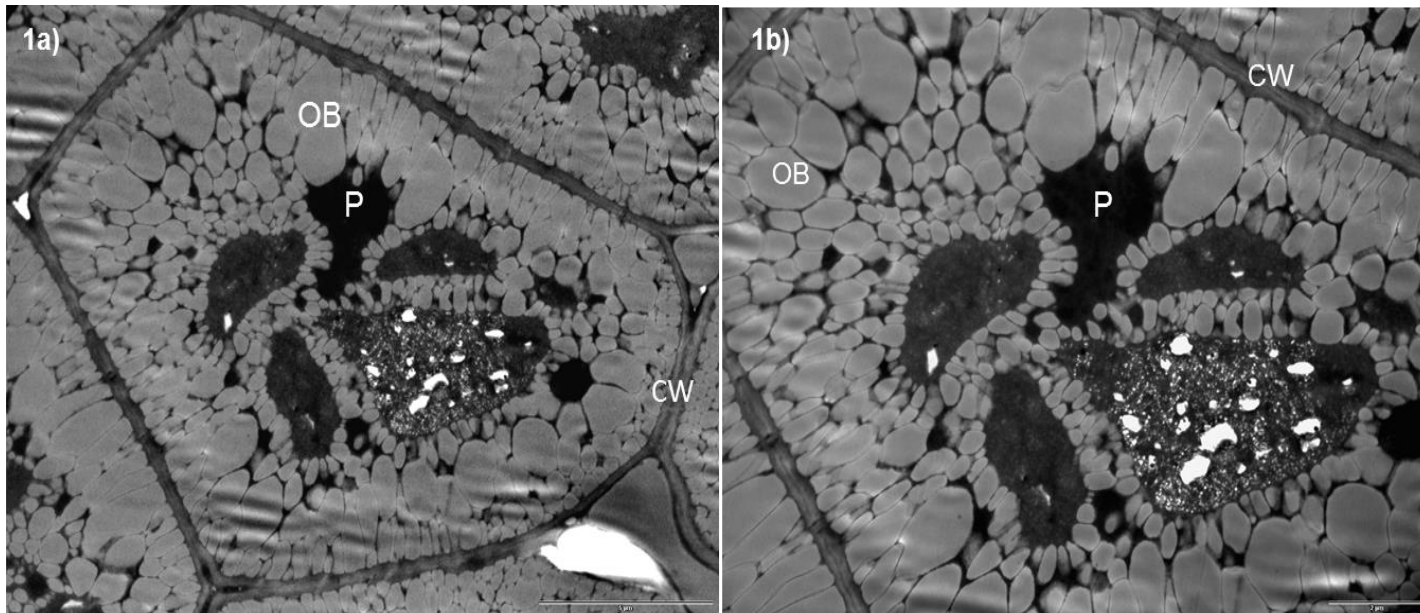


Figure 5:1 The structure of oil bodies appear as light grey irregular shape in sunflower seeds observed under Transmission Electron Microscopy (TEM). OB = oil bodies, CW = cell wall P= plastid. Scale bars represent 1a) 5 μm 1b) 2 μm . Oil bodies were compressed within the cell however, they remained as individual organelle.

An advanced microscopy technique was used in this study to observe the oil bodies. Figure 5.2 shows a micrograph of the structure of the oil bodies within the sunflower seeds. Cryo- Electron Microscopy required the sample to be frozen at -93°C for 23 minutes prior to visualisation to resolve molecular features below 1nm (Northrop and Connor, 2009). Contrary to TEM preparations, Cryo-EM preserved the sample structure to its native form without chemical fixation and embedding step (Northrop and Connor, 2009). It is noted that oil bodies have very strong surfaces that prevent them from coalescing *in vivo* which allows them to stay as single entities. Within the micrograph, individual cells and some of their main features were evident. It can be seen that oil bodies occupy most of the space inside the cotyledon cells.

Unlike the protein and lipid bodies, the walls which surround the cell are primarily composed of cellulose, hemicellulose and lignin. Therefore, the images of oil bodies are easily recognisable observed under Cryo-EM rather than TEM as bright shining small spherical organelles. However, image of oil bodies was flattened under TEM resulting from dehydrated during sample preparations (Cabra and Samso, 2015).

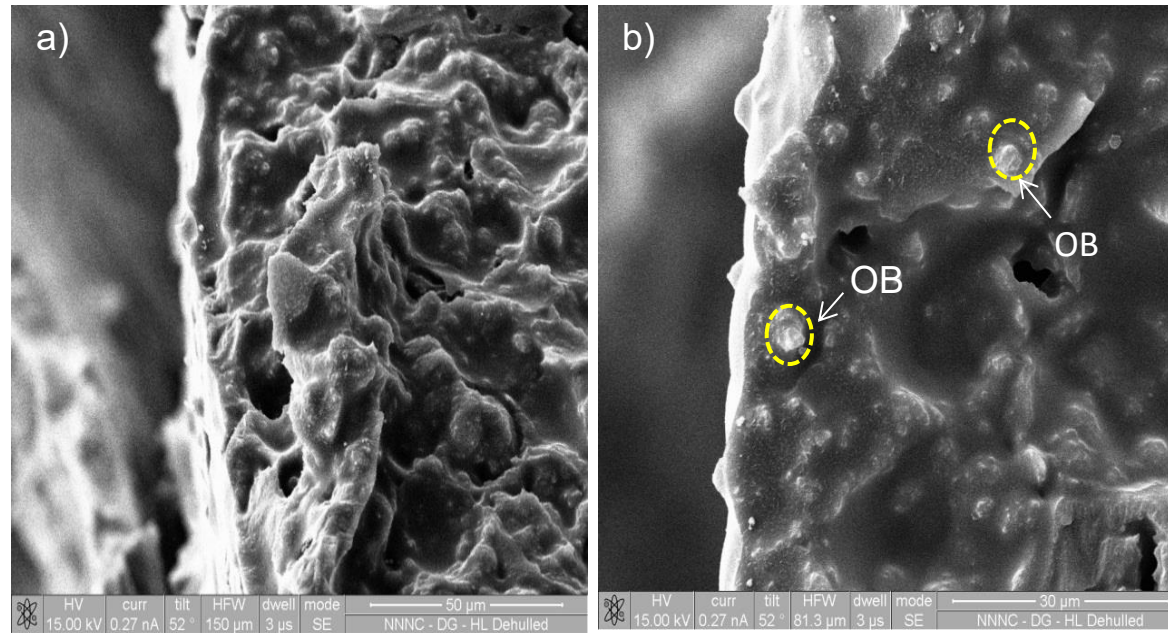


Figure 5:2 Images of oil bodies appeared brightly shining granules observed under Cryo-EM at different magnification. Scale bars represent a) 50 µm, b) 30 µm. Oil bodies can be observed as tiny spherical structures and occupy most of the space within the cytoplasm. Cryo-EM preserved the sample structures to its native form

5.2 Isolation of Oil Bodies

Oils in plant material can be found in small-sized organelles called oil bodies or olesomes (Tzen and Huang, 1992). These oil bodies are mainly composed of triacylglycerol (TAG), phospholipids (PL) and a protein called oleosin (Tzen *et al*, 1993). Oleosin prevents the coalescence of oil bodies in the cytosol of oilseed cells (Tzen *et al*, 1992; Tzen and Huang, 1992). Oil bodies can be recovered from oilseed and display physical and chemical stability *ex vivo*. The physicochemical characteristics of oil body dispersions depend on the method applied for oil body recovery, the presence of exogenous proteins, the pH value and NaCl concentration (Karkani *et al*, 2013; Iwanaga *et al.*, 2007; Nikiforidis & Kiosseoglou., 2010; White *et al.*, 2006). At neutral pH they have net negative charge which prevents coalescence *ex vivo* especially when oil bodies are dispersed in a suspension.

It is possible to recover these intact and spherical subcellular structures using different techniques and methods. Oil bodies technically can be produced by homogenising the raw material, filtering and centrifuging to obtain the buoyant fat pad. The aim of this work was to recover high quality oil bodies from plant seed materials by aqueous extraction. Aqueous extraction involves the use of water as an extraction medium, following homogenisation allowing the release of oil into the bulk liquid phase. A series of experiments was performed to identify the processing conditions required in order to achieve high quality oil bodies which includes several measurements.

5.2.1 Processing Parameters for Recovery of Oil Bodies.

In general, the cell wall of plant cells needs to be disrupted before extraction of the lipid can occur. In order to reach the aim, the work closely depends on the reliability of the isolation procedures. Disrupting the tissue mechanically helps to remove its cytoplasmic content. Additionally, insoluble wall material can be treated either with combinations of aqueous buffer solutions or organic solvent or detergent to remove soluble contaminants and lipids. There are several reported procedures used to isolate the oil bodies by the disruption of the cell walls. The impact of processing conditions, upstream and downstream on the quality of recovered oil bodies, was systematically studied.

The size of oil bodies are species dependent and likely affected by nutritional and environmental factors (Huang, 1992). The native size of oil bodies is around 0.2-3.0 μm . Isolation of oil bodies is very crucial in order to release the oil bodies by disrupting the cell walls and plasma membrane. There are numerous studies on extraction of oil bodies from different type of plant seed materials (Huang, 1992; Milichip *et al*, 1996; Tzen, 1997). Oil bodies isolated via aqueous extraction appear to be stable physically and also stable against oxidation based on recent studies (Chen *et al.*, 2012; Fisk *et al.*, 2008; Gray *et al.*, 2010). Depending on the recovery process, destabilisation may result in an increase in droplet size during storage due to oil droplet coalescence (Nikiforidis and Kiosseoglou, 2012; Nikiforidis *et al.*, 2011). Therefore, it was important to select the appropriate solution to obtain reliable oil bodies so that they would not coalesce during the process.

Sunflower seeds was homogenised by using kitchen blender with the grinding medium at certain forces and shear, followed by filtration and centrifugation processes (Beisson *et al.*, 2001). It is noted that different kitchen

blenders and food processors have different shear and force. The raw material was subjected to chopping by the blades to disrupt the seeds into smaller particles. Since the kitchen blender was sufficient to obtain lipids and is an easy process to do, thus it is suitable to be used as the preparation method of choice in a laboratory. These approaches have a number of advantages such as suitability for preparation of small quantities of raw material and the seeds are completely crushed into small particles.

The grinding medium used in this work was cold deionised water (4° C). There is a limitation if sunflower seeds are used as the raw material; where rapid reaction of chlorogenic acid occurs once sunflower seeds have contact with alkaline solution. Chlorogenic acids are tannin-like compounds which are from the polyphenol group that functions as part of the oxidase system in plants (Taha and Nockrasy, 1981). During alkaline extraction, chlorogenic acid is oxidised by polyphenol oxidase enzymes to a highly reactive o-quinone intermediate that interacts with the NH₂ group of lysine, the SH group of cysteine, the SH₂ group of methionine and the indole ring of tryptophan (Weisz *et al.*, 2010; Pedrosa and others, 2000). As a consequence, discolouration occurred where the oil body's milk and cream turned green (Figure 5.3)



Figure 5:3: Photo showing (a) oil body's milk, (b) oil body's cream, (c) oil body's milk (alkaline grinding medium) (d) oil body cream (alkaline grinding medium). Sunflower seeds were ground with sodium bicarbonate, filtrated and centrifuged before the buoyant cream was obtained. The milk and cream turns to the distinctive green colour due to oxidation when sunflower seeds react with alkaline solution which was used as the grinding medium.

The formation of green pigment is well described by Yabuta *et al.*, (2001). They found out that the green colour is a novel benzacridine trihydroxy derivative which is formed by the condensation reaction of two molecules of oxidised chlorogenic acid with one molecule of a primary amino compound under aeration in an alkaline condition (Figure 5.4). Under these limitations, it is suggest that deionised water rather than the solution of bicarbonate works best as a grinding medium throughout the remaining study.

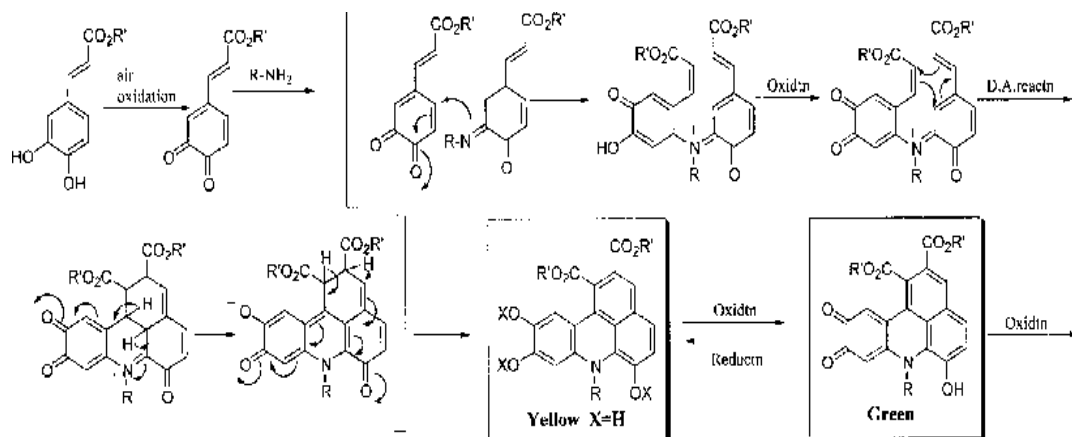


Figure 5:4: Mechanism of green pigment formation from chlorogenic acid and amino compound (Yabuta *et al.*, 2001).

Method of recovery of oil bodies has significant influences on the size of oil bodies which also varies between plant species. Preliminary results showed that the diameter of oil bodies increased in volume mean diameter ($D_{4,3}$) size during isolation process especially after washing treatment.

Recent evidence suggests that the diameter of oil bodies increased dramatically from their nature size (0.5-3.0 μm) (Huang, 1992) to 28.6 μm . Volume mean diameter is very sensitive to detect larger particle size compared to volume surface diameter ($D_{3,2}$) which commonly detects small particle oil droplet. The size of oil body droplets still remained larger as shown in Figure 5.5 and this indicated that coalescing of the oil droplets was suspected to form during the washing step.

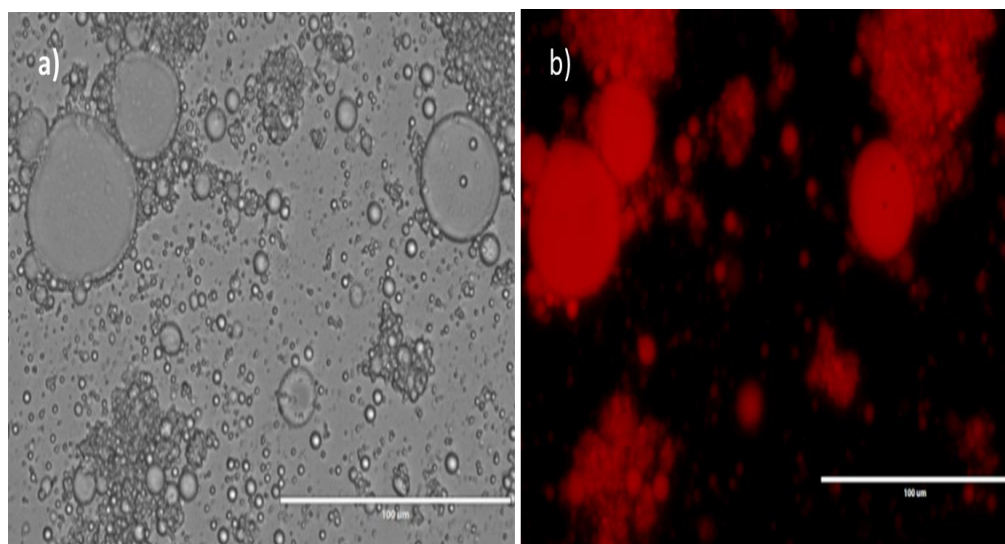


Figure 5:5: Microstructure of oil bodies isolated with kitchen blender using deionised water as a grinding medium and washed with 9M urea. Oil bodies suspension was stained with Nile Red. Aggregation and coalescence of oil droplets were observed under Fluorescence Microscopy. Both images were captured at the same time and same suspension preparation. The red stain is a lipid droplet and the black area represents the continuous phase. Scale bars represent 100 μ m.

In the protocols developed here to isolate oil bodies, deionised water was used as a grinding medium and 9M urea as a washing buffer. However, it appears that the size of oil bodies has dramatically increased specially during the washing step. These rapid changes had a serious effect on the size of oil bodies which was probably due to coalescence (Nikiforidis and Kiosseoglou, 2010; Nikiforidis *et al.*, 2011). Besides, it is also evident that the oil bodies produced were accompanied by an oiling off (data do not shown). Previous research has already drawn attention to the effect of pre-soaking on different plant species linked to oil body recovery. In theory, oil bodies are remarkably stable against aggregation or coalescence; however the results obtained in this work suggest otherwise, unless

the droplet recovered are enriched in damaged/ hybrid oil bodies. The process for optimising oil bodies was optimised. To limit damage to oil bodies during recovery, we decided to test the impact of softening/ plasticising the seed tissue by imposing a seed pre-soaking regime

5.2.2 Pre-Soaking

It could be advantageous to pre-soak seeds for a period of time prior to grinding. During pre-soaking, water molecules penetrate into the cell molecules and hydrate the cell. Previous researchers have extracted oil bodies from a variety of species. However, they were not consistent and standard methods varied. For instance, some researchers used non-soaked seeds (Fisk & Gray, 2011; White *et al.*, 2008; Tzen & Huang, 1992; Tzen *et al.*, 1993). Some of researchers reported soaking soybeans overnight (Iwanaga *et al.*, 2007) or for 1 hour (Tzen *et al.*, 1990) or at least 20 hours (Waschatko *et al.*, 2012). Researchers also commenced pre-soaking of maize germ seeds for 24 hours (Nikiforidis *et al.*, 2013; Nikiforidis & Kiosseoglou, 2010). As well as other plant species jojoba, teosinte and wheat were pre-soaked for 24 hours prior to wet milling (Tzen *et al.*, 1990). Previous research has already drawn attention on pre-soaking of different kinds of plant species linked to oil bodies recovery, however; specific information has not been published (Tzen *et al.*, 1993; Tzen *et al.*, 1990).

The best value of the performance can be obtained by optimising process condition. Seeds were pre-soaked for 2, 6 or 12 hours prior to aqueous extraction at 4°C. The pH of seed tissues were measured before and after soaking. Two grams of soaked seeds were ground with mortar and pestle in 3 ml of distilled water before measuring the pH. The pH of the tissues did not show any sign of reduction, which could possibly indicate that no enzymatic reaction occurred.

However, if fatty acids generated through the action of enzyme such as lipase which, were adsorbed to the seed matrix, no reduction in pH would be recorded.

To confirm that no lipolytic enzymes catalysed-reactions had occurred, a simple diagnostic test was conducted. It is concern that pre-soaking might cause enzyme activity during lipolysis which potentially weakens the oil bodies. For instance, some amounts of oleic acid (internal control) were added on the seed matrix and pH changes were recorded. If TAG could hydrolyse to free fatty acid this would cause a reduction in the pH value (i.e. increase the acidity), then pre-soaking would not have been selected as a pre-treatment process. In general, each oleic acid that diffuses across the plasma membrane (adsorption and desorption) would release one H^+ in the cytosol but removed one H^+ when oleic acid was esterified, as consequence pH was reduced (Kamp *et al.*, 2003).

Table 5:1: Diagnostic test during seed pre-soaking. The measurement of pH was the mean of three replicates.

Sample	pH
Deionised water	6.79
Deionised water + oleic acid	5.57
Seed + Deionised water	6.75
Seed + Deionised water + mortar & pestle	6.70
Seed + Deionised water + 15 μ l oleic acid	6.67
Seed + Deionised water + 15 μ l oleic acid + mortar & pestle	6.63

Table 5.1 shows the results of the diagnostic test carried out during the seed pre-soaking step. In theory pH affects an enzyme's activity such as lipase. Lipase is a water-soluble enzyme that acts at the oil-water interface to catalyse the hydrolysis of ester bonds in lipid substrates into free fatty acids and glycerol. Lipase activity is lower in acidic pH. However, if there is increased lipase activity it is probably due to metabolic activity initiated by growth of embryo. The pH of the seed obtained during the diagnostic test demonstrates no reduction of pH and therefore; minimised the enzyme reaction. However, this test does not prove that lipolysis was not occurring.

The changes of pH before and after soaking followed by mortaring were presented in Table 5.2. Non-soaked seeds or seeds soaked for the shortest times (0h, 2h) revealed some changes in the diameter of oil bodies when observed under microscopy. With the increasing in time of soaking (6h and 12h), a significant decrease in the diameter of oil bodies were observed (f, h) under microscopy presented in Figure 5.6 especially after the washing step. Some of the non-soluble pigment was clearly seen in all crude samples (a, c, e, and g) however, these were clean after washing treatment.

Table 5:2: pH before and after soaking the seeds and pH of the mixture after grinding.

Time (hours)	pH of the soaking water	pH after mortar
0 (Non-soak)	ND	6.72
2	6.68	6.70
6	6.64	6.68
12	6.60	6.58

Deionised water = pH 6.8

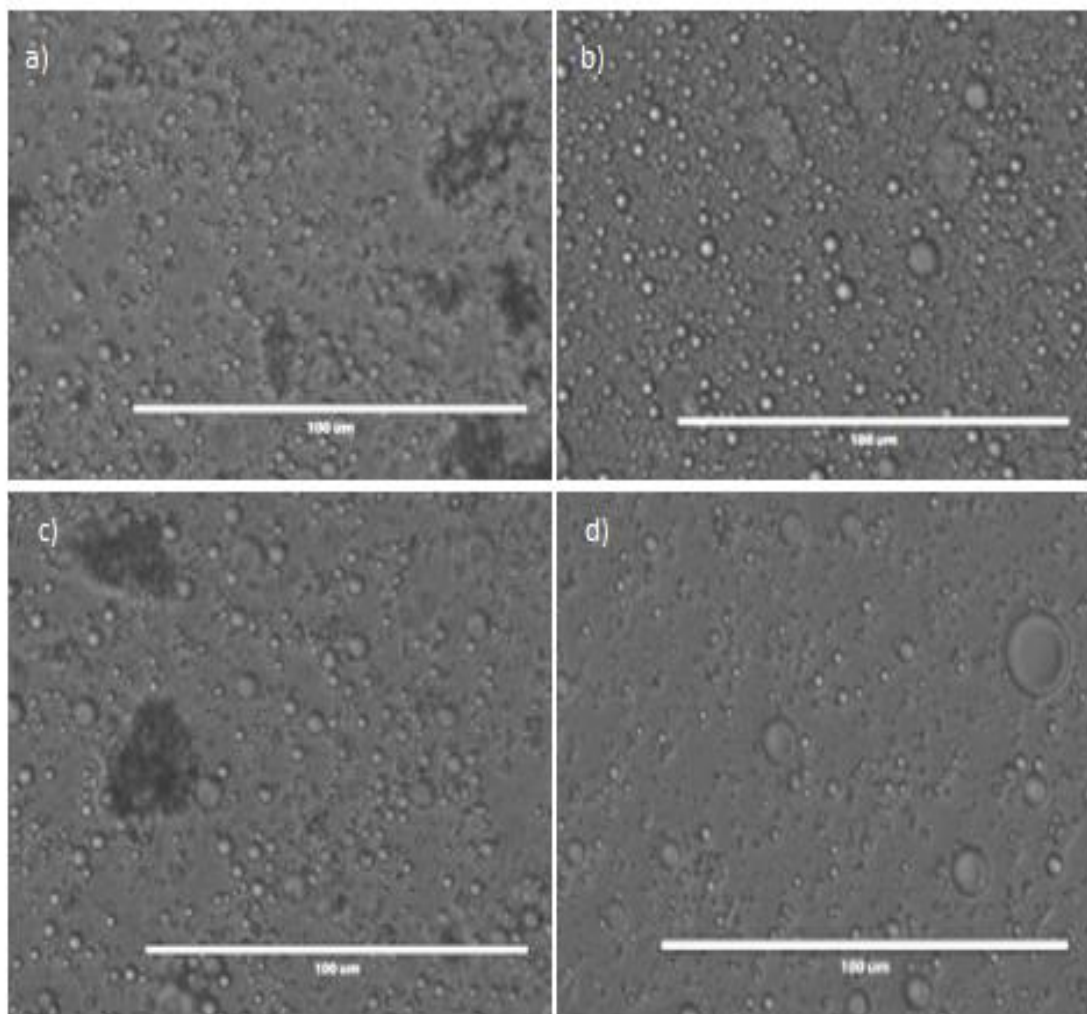


Figure 5:6 : Image of oil body suspensions from sunflower seeds during optimisation of seed pre-soaking. **(a)(c)** crude oil bodies **(b)(d)**washed oil bodies. **(a)(b)** non-soaking**(c)(d)** pre-soaked for 2 hours. Scale bars represent 100 μm .

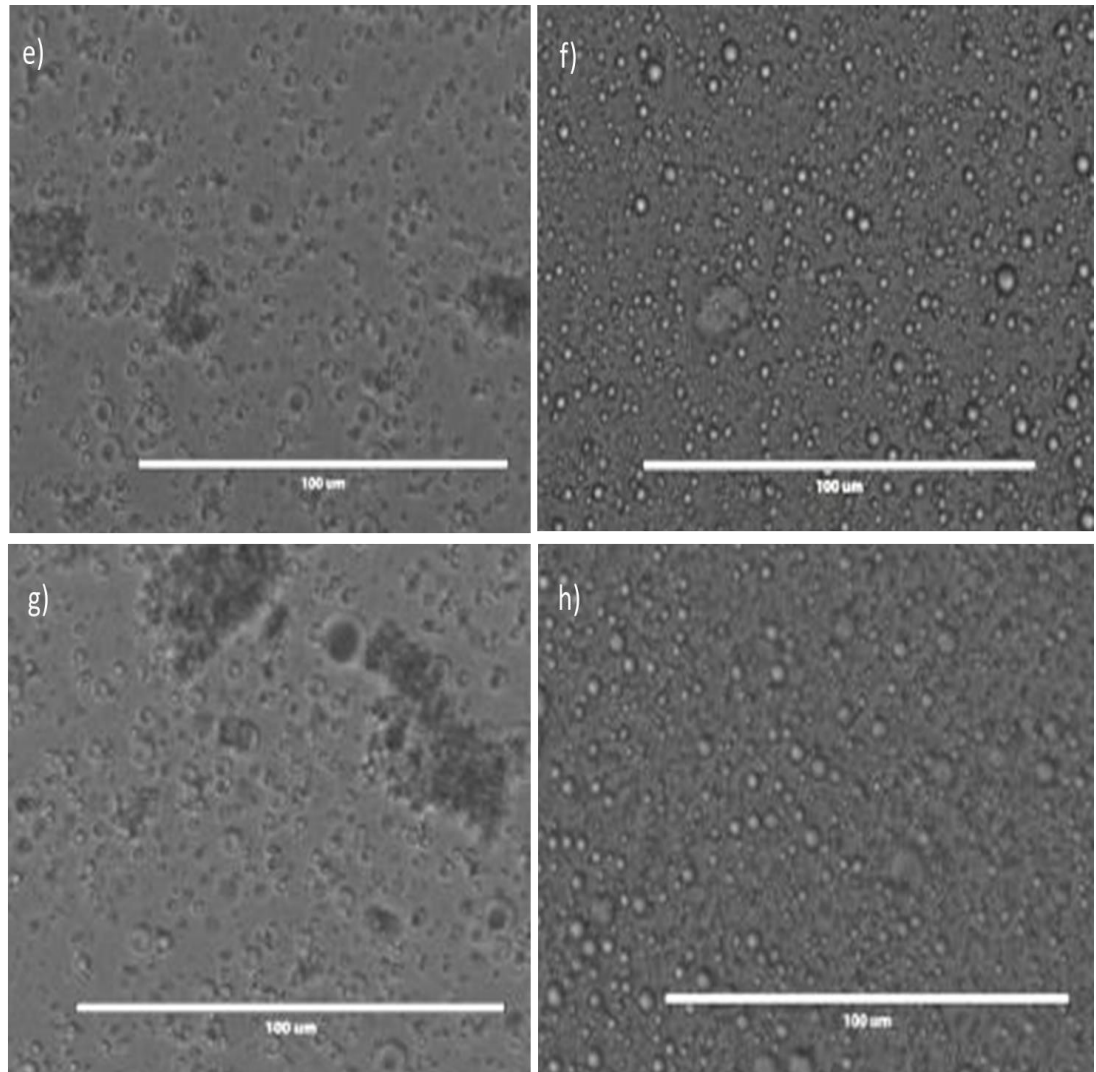


Figure 5:7 : Image of oil body suspensions from sunflower seeds during optimisation of seed pre-soaking. **(e)(g)** crude oil bodies **(f)(h)** washed oil bodies. **(e)(f)** pre-soaked for 6 hours **(g)(h)** pre-soaked for 12 hours. Scale bars represent 100 µm.

Most of the pre-soaked seed samples showed slight changes in pH after grinding with the mortar and pestle but the results are negligible. The results from the diagnostic test and pH were consistent. Table 5.3 shows the diameter of crude and washed oil bodies extracted from seeds that had been soaked for different lengths of time. It shows that the size of oil bodies decreased after soaking for 6 hours and 12 hours. Since 6 hours is sufficient to allow water molecules to penetrate the cell matrix therefore, it is selected as optimum pre-soaking time to be undertaken prior to the extraction process.

Bradford (1990) pointed out that imbibition is considered as a triphasic process, with rapid initial of water uptake (Phase I) followed by a plateau phase with little change in water content (Phase II) and subsequent increase in water content corresponding with growth of radicle (Phase III). During phase II, seed undergoes many processes necessary for germination and also known for enzyme activation such as lipolytic and proteolytic (Copeland and McDonald, 1995). Pattern of water uptake in partially de-hulled sunflower seeds is shown in Figure 5.8.

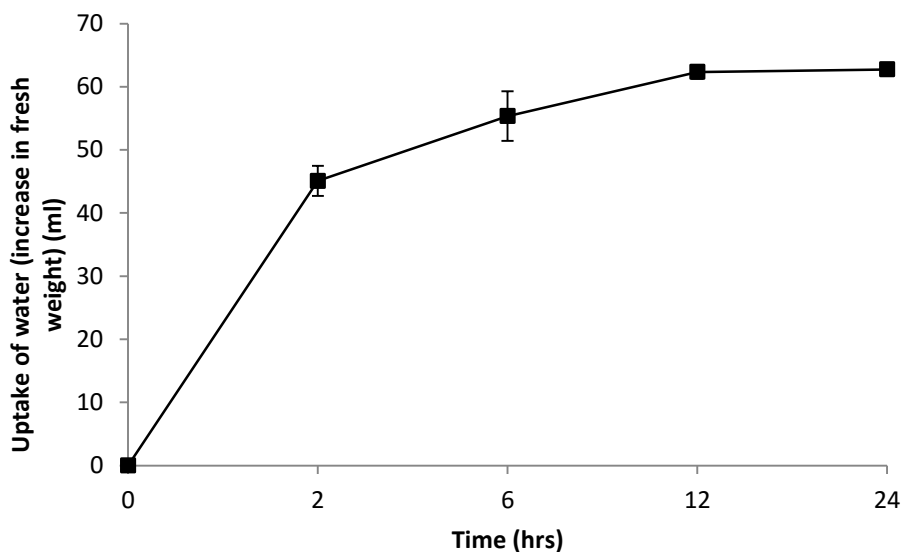


Figure 5:8: Water uptake of partially de-hulled sunflower seeds by increase in fresh seed weight.

Based on triphasic process, pre-soaked seed for 6 hours was considered as Phase I however, the rate and enzyme activity was highly dependent on environment factors in the seed such as temperature, oxygen, pH and others. The action of enzyme on the oil body membrane can affect the surface chemistry and oil body integrity (Hoppe and Theimer, 1997). Leprince *et al.*, (1998) highlighted the important of oleosins to prevent coalescence during seed imbibition of water, therefore negating the effect of enzyme on immediate oil body collapse.

Table 5:3: Effect of seed soaking time on the Volume mean diameter of oil bodies.

Seed soaking time (h)	Crude oil bodies (μm)	Washed oil bodies (μm)
0	11.06 ± 0.5^a	5.08 ± 0.3^c
2	11.20 ± 0.2^a	5.12 ± 0.4^c
6	9.08 ± 0.6^b	3.13 ± 0.0^d
12	9.01 ± 0.6^b	3.15 ± 0.0^d

Note: ^{a-d} Means that do not share a letter are significantly different. Tukey 95% Simultaneous Confidence Intervals

The volume mean diameter of oil bodies measured after the pre-soaking are presented in Table 5.3. All of the washed oil bodies were decreased in size compared to their respective crude preparations. It can be seen that, the size of non-soaked seed and seeds soaked for 2 hours follow a similar trend. The size of crude oil bodies in samples pre-soaked for 6 hours was slightly bigger ($9.08 \pm 0.6 \mu\text{m}$). However, this was reduced by three times after washing to approximately $3.13 \pm 0.0 \mu\text{m}$. The size of the crude sample was slightly bigger due to carry-over of cell contaminants or any interference of non-solubilise pigments or aggregation. Since 12 hours of soaking showed similar trend to 6 hours thus, it is suggested that 6 hours for seed pre-soaking is an optimum time.

With regards to the results obtained in this work, Specific Surface Area (SSA) was determined in order to ensure the oil bodies were tiny organelles and the graph is displayed in Figure 5.8. It is noted when the surface area is large it indicates the particle size obtained is small, however conversely if the surface area is small it is expected the size of oil bodies is large. The results obtained in this

work recorded that a high surface area was gained after 6 and 12 hours of soaking. The surface area for unsoaked seed or seeds soaked for a short time (2 hours) have less than 16000 cm²/ml. Both non-soaked seed and seeds soaked for 2 hours have low surface area values, indicating that the size of oil bodies is slightly bigger.

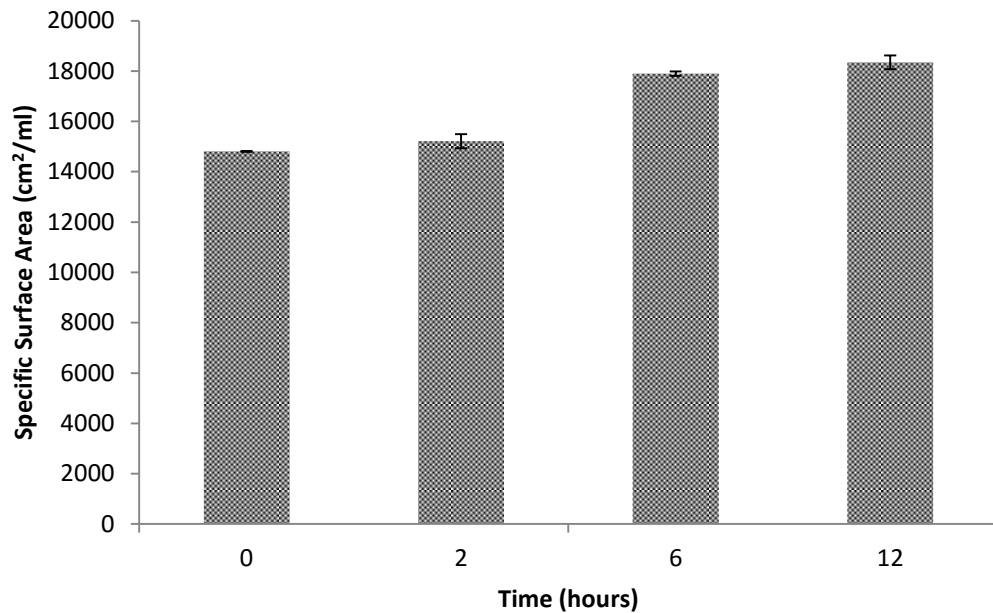


Figure 5:9 Specific Surface Area (SSA) of oil bodies at different pre-soaking times. Error bar represent standard deviation.

It has been shown that partially de-hulled sunflower seeds required 6 hours to imbibe water. However, it is unknown whether 6 hours is specific to partially de-hulled sunflower seeds or if it is suitable for any variation of sunflower seeds. To confirm this, a different type of sunflower seed was purchased and pre-soaking time was determined according to the previous protocols. The seeds were soaked in deionised water at 4° C for 2h, 6h or 12 hours. Images of the oil bodies were observed at respective pre-soaking times and are presented in Figure 5.10.

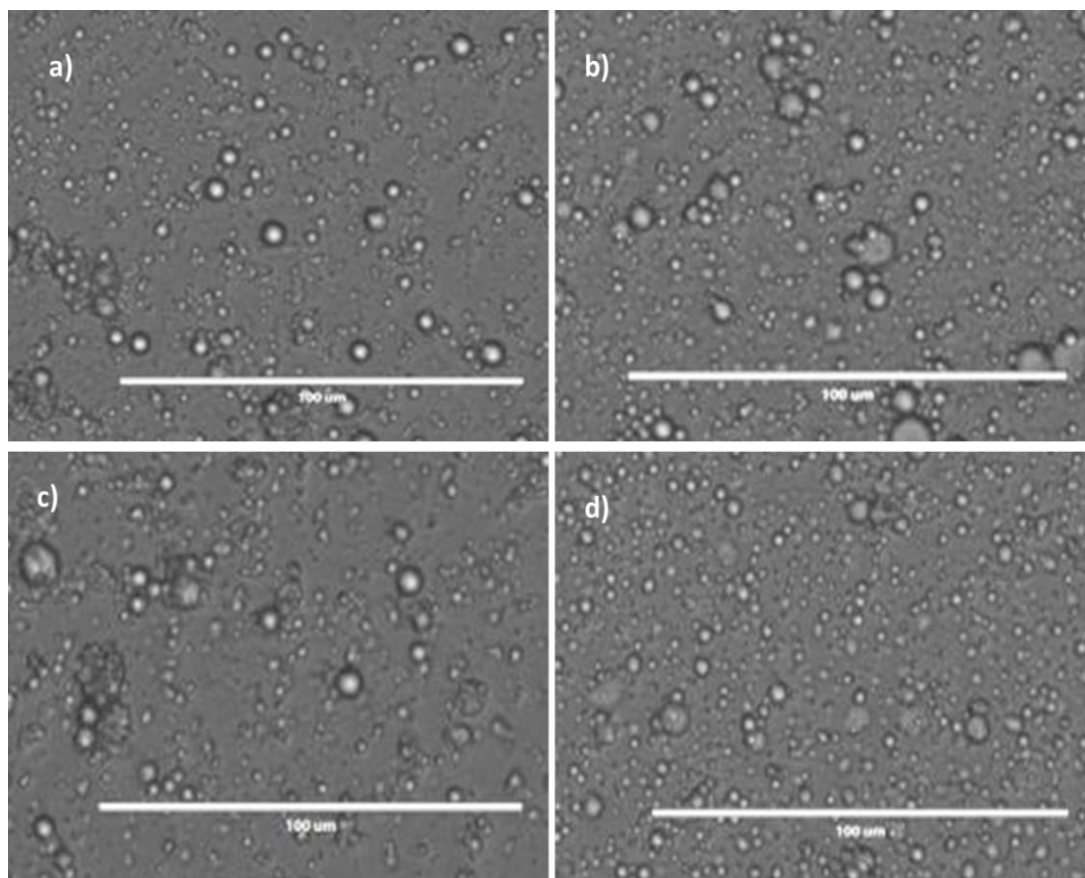


Figure 5:10: Microscopy images of oil body suspensions from a different variety of sunflower seed during seed pre-soaking. **(a)(c)** crude oil bodies **(b)(d)** washed oil bodies. **(a)(b)** non-soaking**(c)(d)** pre-soaked for 2 hours. Scale bars represent 100 µm.

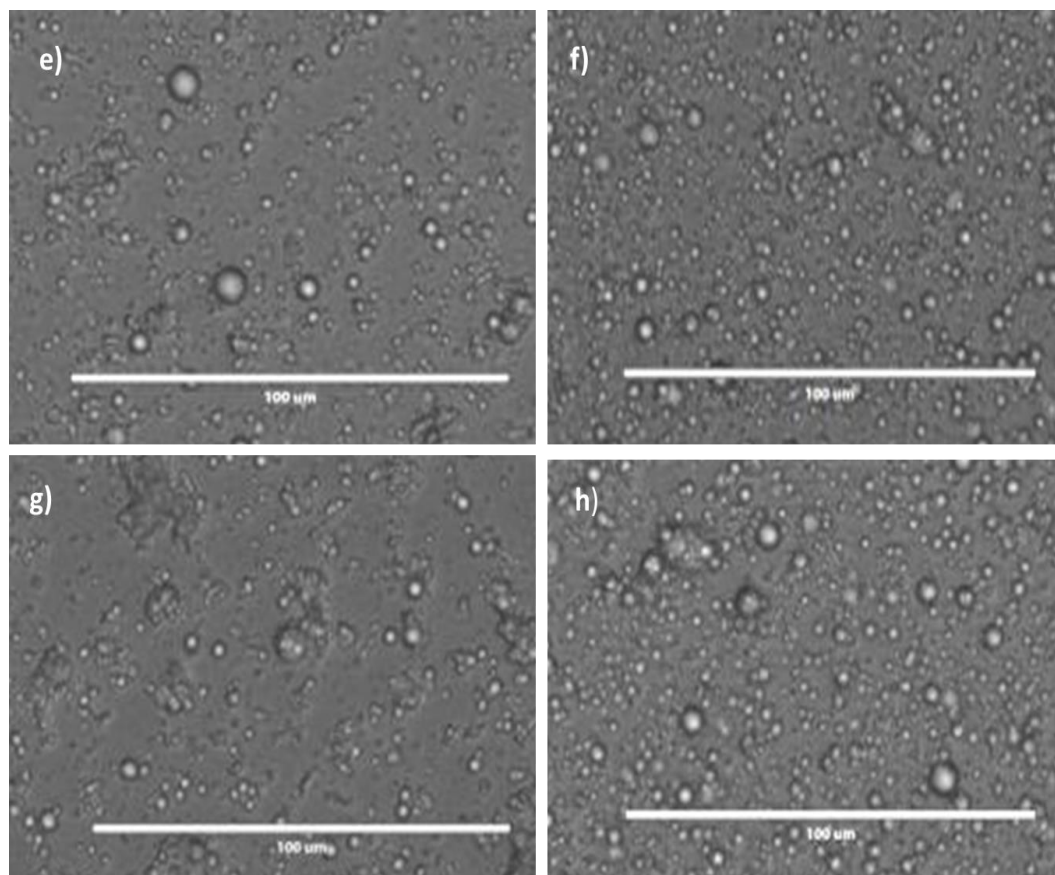


Figure 5:11: Microscopy images of oil body suspensions from a different variety of sunflower seed during seed pre-soaking. **(e)(g)** crude oil bodies **(f)(h)** washed oil bodies. **(e)(f)** pre-soaked for 6 hours **(g)(h)** pre-soaked for 12 hours. Scale bars represent 100 μm .

It should be noted that sunflower seeds used in this test had been de-hulled. The crude oil body samples aggregated in non-soaked seeds and after 2 hours of soaking. Although non-soaked seeds or seeds soaked for short time revealed slightly larger sized oil bodies, however small and intact oil bodies were observed after 6 hours and 12 hours of soaking.

Table 5.4 shows the pH values of oil body suspensions after different pre-soaking times. It was noted that the pH of oil body suspension at different pre-soaking time ranged from approximately pH 7.09- 7.16 for crude oil body samples and washed oil body preparations. The results indicate that there is no change in pH during the measurement. Figure 5.11 shows a photo of sunflower seeds during pre-soaking.

Table 5:4: pH of the oil body suspension at different seed pre-soaking times.

Time (h) / Sample	Crude oil body	Washed oil body
0	7.12	7.13
2	7.09	7.10
6	7.11	7.16
12	7.08	7.12

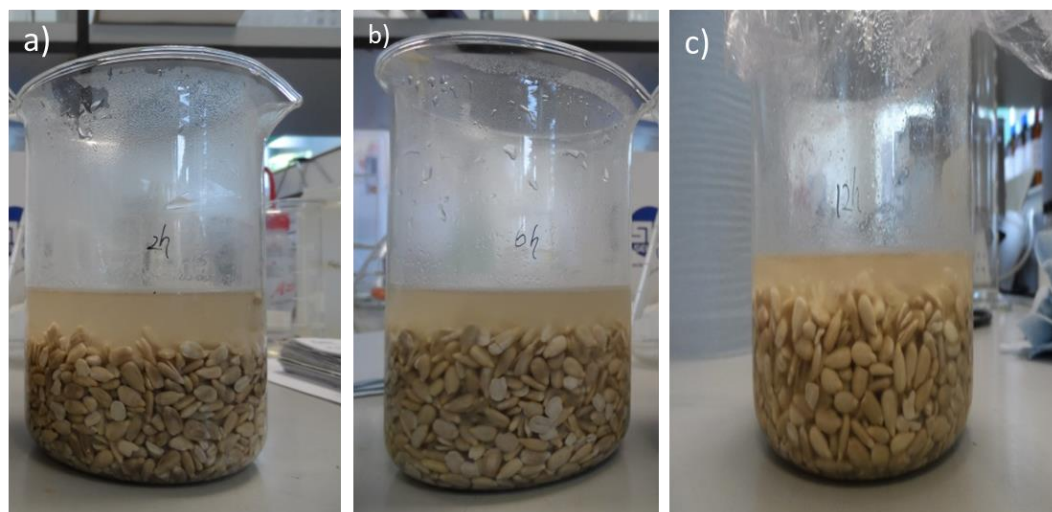


Figure 5:12 Photo of de-hulled sunflower seeds during pre-soaking at 4° C a) Soaking for 2 hours b) soaking for 6 hours c) soaking for 12 hours. The seeds were pre-soaked with deionised water for 12 hours prior to isolation of the oil bodies. The soaking water is slightly darker after 12 hours of soaking.

For housekeeping, raw material used in this study (partially de-hulled seeds) was compared with de-hulled. As a result, the optimum pre-soaking time for sunflower seed is 6 hours. On the other hand, it was important to investigate the physical state of the oil bodies during purification by selection of washing regime. In order to select the most appropriate washing solution, a set of experiments were carried out and the results are presented in the following section. A comparison was made accordingly with different washing regime and the changes of oil bodies were recorded respectively.

5.2.3 Effect of Washing Regime on the Quality of Oil Bodies

Oil bodies from partially de-hulled sunflower seeds can be extracted using an aqueous extraction method, followed by filtration and centrifugation to produce a buoyant crude oil body after carried out pre-soaking treatment. Pre-soaking before wet milling and multiple washings with various medium is undertaken to isolate high quality oil bodies. So far, this is the first attempt to report how to extract high quality oil bodies in sunflower seeds. In fact oil bodies can be easily extracted via floatation (Murphy and Cummins, 1989) by liquid-liquid phase separation as oil bodies tend to float upon centrifugation (Decker *et al.*, 2000). One should note that the size and the shape of oil bodies should be preserved even after the isolation process (Murphy and Cummins, 1989; Jolivet *et al.*, 2013). Similar size of oil bodies (0.5 μm – 2.5 μm) was reported in a range of plant species including rapeseed, cotton, maize and peanut (Tzen *et al.*, 1993).

Despite being approximately the same diameter as the oil bodies extracted in the current research they differed in their lipid contents. The study of purified oil bodies from various plant materials have been extensively reviewed (Tzen *et al.*, (1996); Jolivet *et al.*, (2006) and Lacey *et al.*, 1998). However, recent literature has emerged that offers contradictory results in oil body recovery (Nikiforidis *et al.*, 2013; Karkani *et al.*, 2010). Therefore, there is an opportunity and interest in this area which offers further investigation for understanding the structure of oil body from plant seeds materials. The need to improve the existing method is vital not only for its food functionality but also to optimise the processing conditions. We hypothesise that the washing regime will affect the surface chemistry of oil body and alter the physical stability in the final suspensions.

During the purification process, native and pure oil bodies can be obtained by using a suitable washing regime. Purification through washing treatments is

expected to remove cell debris, extraneous protein and other contaminants. However, the degree of purification is dependent on the aggressiveness of the treatment. If washing can improve the quality of oil bodies chemically and physically, then potentially it can be applied to various food products such as sauces, dressing or as an emulsifier for food products (Waschatko *et al.*, 2012; Iwanaga *et al.*, 2007). Oil bodies are pre-emulsified within the seeds therefore, health care and cosmetic industry can also benefit from this finding (Decker *et al.*, 2003).

Because the washing treatment is important to purify and clean the oil body preparation prior to analysis, it is vital to select an appropriate washing solution. A set of experiments was conducted to obtain data to address this gap in knowledge. The compositional and physical characteristics of washing regimes were compared and discussed further in the following section. During washing, any cell contaminant such as cell tissues, debris (such as carry-over during dehulling process), and extraneous protein or defected can be removed, thus purifying the oil body suspension. To illustrate the type of contaminant, Figure 5.12 shows a microscope image of cell debris that was removed during a washing treatment upon centrifugation.

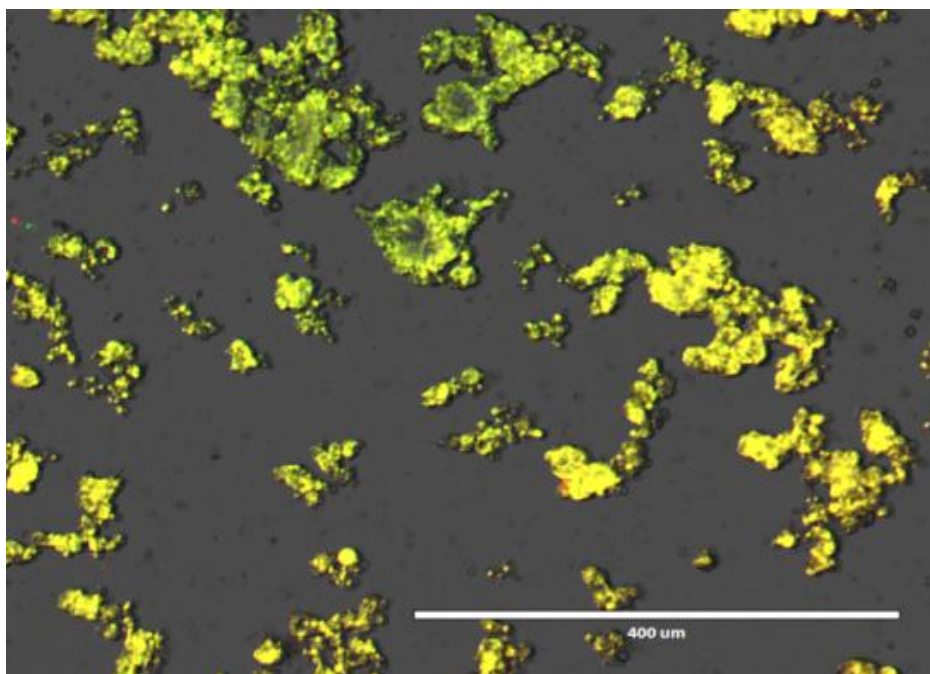


Figure 5:13: Image of cell debris (overlay) during washing treatment observed by fluorescence microscopy. Scale bar represents 400 μm . The black area represents the washing media and yellow fragments are cell contaminants. The samples were collected from the debris embedded in the centrifugation tube during centrifugation steps. The oil bodies floated upon centrifugation.

5.3 Influence of Sunflower Seed Pre-Soaking and Oil Body Washing Regimes on the Quality of Final Oil Body Suspension

The quality of oil bodies is important and reflects the stability in the final suspensions. Seed pre-soaking and oil body washing regime is one of the main contribution for obtaining high quality of oil bodies.

5.3.1 Basic Composition of Oil Bodies

Sunflower seeds were pre-soaked for 6 hour prior to isolation with deionised water before washing with selected medium prepared. It was noted that crude oil bodies contain cell debris and residual extraneous protein plus insoluble materials. The mean moisture content of oil bodies in this study was 45.7 ± 1.3 %. Oil bodies were composed of approximately 94-98 % w/w neutral lipids and 0.5-3.5 % protein. Composition of oil bodies from crude and washed oil bodies are presented in Table 5.5.

Table 5:5: Lipid and protein content (dry basis) of oil bodies prepared using different washing regimes

Sample	Lipid content (%)	Protein content (%)
Crude	87.0 ± 0.0^b	8.06 ± 0.1^a
Water washed	84.9 ± 0.8^c	5.16 ± 0.0^b
Urea washed	96.3 ± 1.2^a	1.42 ± 0.0^d
Alkali-washed	94.4 ± 0.7^a	1.96 ± 0.1^c

Note: ^{a-d} Means that do not share a letter are significantly different. Tukey 95% Simultaneous Confidence Intervals

The higher protein content of the crude oil bodies was mainly due to the large extraneous proteins that cover the oil body together with cell debris which technically originates from the de-hulling process. The washing treatment helps to remove the cell contaminants as well as extraneous protein. After repeatedly washing with the washing medium, the oil bodies obtained were considered to be purified and clean in preparation prior to analysis. The loose protein was washed away and remaining integral protein is gained (Tzen *et al.*, 1997).

Oil bodies washed with 9M urea had a high lipid content which was around 96.3 % and low in protein content (1.42 %), compared to crude samples which had approximately 87.0 % lipid and 8.06 % protein. Further washing the crude oil body material with alkali yielded oil bodies with around 94.4% lipid content and 1.96% protein. The results indicate that 9M urea was effective to remove cell debris and extraneous protein from oil body prepared from partially-dehulled sunflower seeds. However, the lipids content was not significantly different ($P>0.05$) from lipids washed with alkali. The protein content decreased significantly ($P<0.05$) in all washing treatments. To summarise, washing treatment successfully purified the oil bodies by removing protein.

Previous work has demonstrated the efficiency of washing crude oil bodies by applying 9M urea in their protocols to obtain discrete organelles from sunflower seeds (White *et al.*, 1998; Milichip *et al.*, 1996; Lacey *et al.*, 1998). The results indicated in this work confirm that more protein was removed after the urea wash. It is clear evidence that once the washing treatment was applied the amount of protein content was decreased and higher lipid content was achieved.

The total protein content of oil body preparations was measured after different washing regimes. Figure 5.13 shows the reduction of protein content

when oil body samples were repeatedly washed either in water, urea or sodium bicarbonate. It is clearly seen that the protein content progressively decreased after the first and second washes but any subsequent washing did not show any protein reduction in any of the samples. Lower protein contents were observed after application of a urea-wash or alkali-wash than protein content for water-washed.

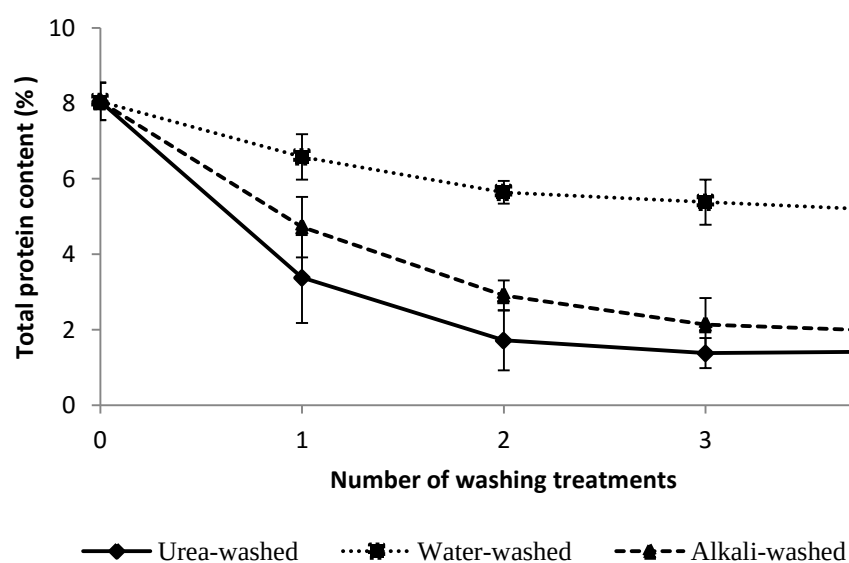


Figure 5:14: The effect of number and type of washing treatment on the protein content (%) of oil body preparations. Increasing the number of washings decreased the protein content from multiple washing regime preparation. The progressive protein reduction occurred mainly on the first and second washes and subsequent repeated washings did not show any protein reduction. Error bars represent standard deviation.

The protein compositions from different washing regimes were compared by SDS-PAGE as shown in Figure 5.14 and Figure 5.15. It is shown that after washing some of the extraneous protein has been removed. Several bands were observed suggesting that washing did not remove all the contaminant protein in

water-washed and alkali-washed oil body suspensions. Urea-washing removed most of cell contaminants as compared to alkali-washed. This is suggesting that urea-washing is more aggressive than alkali-washing.

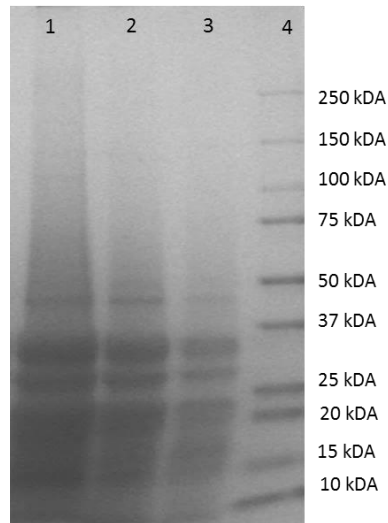


Figure 5:15: SDS-PAGE profile of oil bodies treated with different washing regimes. Lane 1, crude oil bodies; Lane 2 water-washed oil bodies; Lane 3, alkali-washed oil bodies; Lane 4, protein standard marker;

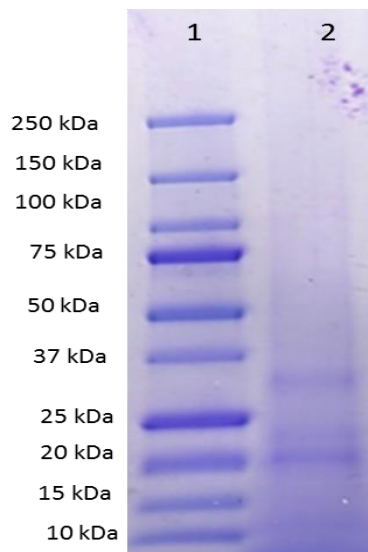


Figure 5:16: SDS-PAGE profile of oil bodies after urea-washed.

Lane 1, crude oil bodies; Lane 2 urea-washed oil bodies

It is well documented that storage proteins in seeds commonly have a high percentage of glutamine which provides a source of nitrogen for seedling growth. Sunflower seeds are classified as a dicots. Most dicot seeds contain two major protein classes, albumins (water soluble) and globulins (salt soluble) which can be distinguished by their solubility (Baudet and Mosse, 1977). In sunflower seed, 2S albumins are a major component of storage proteins (Burnett *et al.*, 2002) while, helianthinin is the major for 11S globulins present in sunflower seeds protein (Schwenke *et al.*, 1974, Durante *et al.*, 1989). Albumin has molecular weights around 10kDa to 18kDa (Thoyts *et al.*, 1996) and the molecular weight of 11S globulin is approximately 300kDa (Schwenke *et al.*, 1978; Durante *et al.*, 1989).

The protein bands of higher molecular than oleosin (20-50kDa) could be albumin and globulin since they are abundant in sunflower seeds and are extracted under the conditions of recovery in this study. Since albumin is water soluble protein, it can be extracted with the oil bodies and possibly absorbed on oil body surface, but globulin was removed at alkaline pH. In this way, chlorogenic acid was also removed at the first centrifugation (Duranter *et al.*, 1989). Additionally, glutelin was removed during alkaline wash with sodium bicarbonate (Sammour *et al.*, 1995). The globulin fraction is characterised by low content of Lysine and sulfur containing amino acid. However, albumin components have high content of glutamine and asparagine (Kortt and Cadwell., 1990).

Oleosin is the main component that stabilises oil bodies together with the presence of phospholipids. In fact, the band obtained in the SDS-PAGE profile analysis in this work is consistent with the known composition (oleosin) of oil bodies from previous work done by Huang (1992). The major associated protein known as oleosin is approximately 18-21 kDa in size. The presence of caleosin (25-27 kDa) and steroleosin isoform (39-41 kDa) were observed in all washing

treatments. The data is in agreement with other work reported previously (Lin *et al.*, 2005; Tzen *et al.*, 1990). The result indicates that albumin and globulin may be the tentative protein of the unknown bands as shown in SDS-PAGE (Fig 5.14 and Fig 5.15). These proteins may be absorbed on oil body surface therefore attributed to the characteristic of oil body suspensions.

5.3.2 Particle Size

The particle size of oil bodies after different washing regimes was compared. Prior to wet milling the seeds were pre-soaked for 6 hours then isolated in deionised water to give the crude preparation. Crude oil bodies were then purified with different washing media to recover high quality oil bodies. In this work, crude oil bodies were repeatedly washed either with 9M urea or 0.1 M sodium bicarbonate (alkali-washed). During the urea-washing treatment, the crude oil body cream was washed once with 9M urea and repeatedly washed with deionised water to remove the residual urea. However, alkali-washed was carried out by repeatedly washing with 0.1M sodium bicarbonate. It is crucial to obtain small, intact and stable oil bodies. Therefore, it is important to select appropriate solution to obtain reliable oil bodies so that they remain intact during the process.

The particle size of oil bodies after different washing regimes is presented in Table 5.6. Crude oil bodies were further purified to produce discrete organelles which were stable against aggregation or coalescence. Volume mean diameter ($D_{4,3}$) and surface mean diameter ($D_{3,2}$) of the oil body droplets were measured.

Table 5:6: Particles size of crude oil body (COB) and washed oil body (WOB) preparations.

Sample	D (4, 3) ¹ μm	D (3, 2) ² μm
Crude oil bodies	9.00 $\pm$ 0.60	3.13 $\pm$ 0.01
Urea-Washed Oil Bodies ³	23.60 $\pm$ 0.50	7.20 $\pm$ 0.29
Water-Washed Oil Bodies ⁴	8.60 $\pm$ 0.60	3.17 $\pm$ 0.01
Alkali-Washed Oil Bodies ⁴	3.40 $\pm$ 0.30	2.20 $\pm$ 0.08

Note: ¹D (4, 3) = Volume mean diameter; 2D (3, 2) = Surface mean diameter

³Oil bodies washed once with urea and repeatedly wash with deionised water is called Urea-washed (UWOB).

⁴Water washed (WWOB) or Alkali-washed (Alkali-WOB) were repeatedly washed with the respective washing medium.

The volume-mean diameters to surface-mean diameter values were compared. Urea washed oil bodies appear to have a larger diameter (23.60 $\pm$ 0.5 μm) than crude (9.00 $\pm$ 0.6 μm) and alkali-washed (3.40 $\pm$ 0.3 μm) oil bodies, respectively. However, the mean diameter of oil bodies is smaller when measured with surface-mean diameter. $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of droplets with a diameter of d_i . The alkali-washing treatment obtained small intact oil bodies in both diameters measured. The volume means diameter can be over estimate by weighting on volume. $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of droplets of diameter d_i . In this study, surface mean diameter is sensitive to smaller particle size however; volume mean diameter is sensitive to larger particle size.

The size of oil bodies was reduced three times after removing cell debris or any extraneous protein by alkali-washing from $9.00 \pm 0.6 \mu\text{m}$ to $3.40 \pm 0.3 \mu\text{m}$. The volume mean distributions of oil body suspensions from crude, urea and alkali-washed method were demonstrated respectively, in Figure 5.16. It appears that urea-washed had a larger mean particle size of $23.60 \pm 0.5 \mu\text{m}$ compared to crude sample ($9.00 \pm 0.6 \mu\text{m}$). Since urea-washing formed poly distribution and shifting to the right side therefore, this suggests aggregation or coalescence in the final suspension had occurred. The results indicate that purification with 9M urea triggered oil body droplets to form aggregations and to coalesce. An alternative explanation could be that some of phospholipid species which were integral to oil body has been removed at high concentration (Ratnayake *et al.*, 1996).

Unlike urea-washing, alkali-washing produced what appeared to be intact oil bodies ($3.40 \pm 0.3 \mu\text{m}$) compared to $9.00 \pm 0.6 \mu\text{m}$ in the crude preparation. The decreased size was probably due to the removal of unwanted material or some damaged oil bodies during the washing process. Besides, the clean oil body was observed and had no sign of non-soluble pigment (carry - over from seed's hull) interference in the final suspension. The results indicate that alkali-washing was the most appropriate washing regime to produce small, intact and stable oil bodies. The result is agreement with Beisson *et al.* (2001) which highlights the efficient of sodium bicarbonate for removing any electrostatically bound protein.

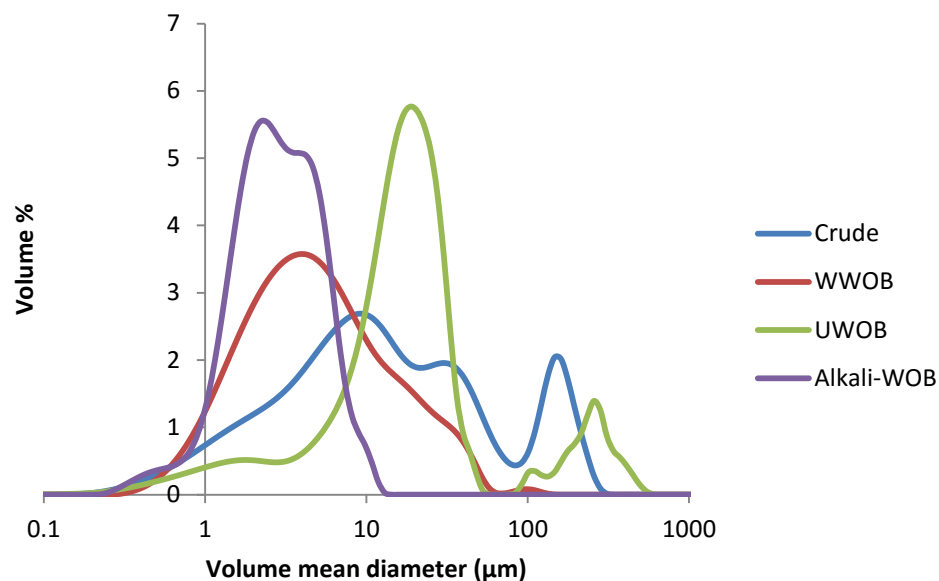


Figure 5:17: Particle size distribution of oil bodies from partially de-hulled sunflower seeds. Oil bodies washed once with urea and repeatedly wash with deionised water is called Urea-washed (UWOB). Water washed (WWOB) or Alkali-washed (Alkali-WOB) were repeatedly washed with the respective washing medium.

COB, crude oil bodies (pH 7.12)

UWOB, urea washed oil bodies (pH 7.34)

WWOB, water washed oil bodies (pH 7.25)

Alkali-WOB, alkali-washed oil bodies (pH 7.28)

In order to further investigate how the washing regimes changed the size of oil bodies, the following experiments were undertaken. Figure 5.16 displays the effect of the number of washes on the volume mean diameter. The particle size of oil body was measured by number of washing step followed by centrifugation. As number of washing steps increased, the volume mean diameter decreased progressively in alkali as well as in water- washed oil body preparations, except for urea washed. In urea-washed, the volume mean diameter increased dramatically until final washing step.

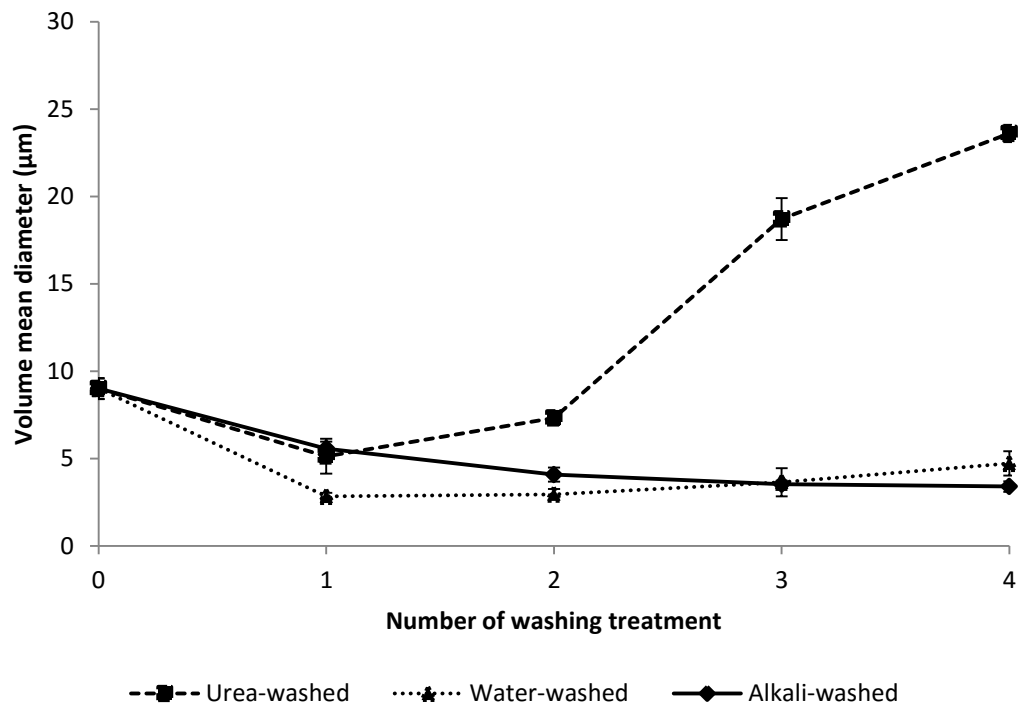


Figure 5:18: Number of washing treatments affects the Volume mean diameter (μm) with different washing regimes. Oil bodies washed once with urea and repeatedly wash with water is called Urea-washed (UWOB). Water washed (WWOB) or Alkali-washed (Alkali-WOB) were repeatedly washed with the respective washing medium.

UWOB, Urea washed (pH 7.32)

WWOB, Water washed (pH 7.20)

Alkali-WOB, Alkali washed (pH 7.26)

5.3.3 Zeta Potential

Zeta potential is the potential differences between the surface of the particle and the medium in which it dissolves or suspends. The application is widely used for the determination of magnitude of electrical charges when exposed to the oscillating voltage across a measurement cell. Any charged species will move as the voltage changes and the surface potential can be calculated from the measured velocity. Zeta potential (ζ -potential) is a key parameter to assess emulsion or suspension stability. The schematic diagram of the mechanism is demonstrated in Figure 5.18.

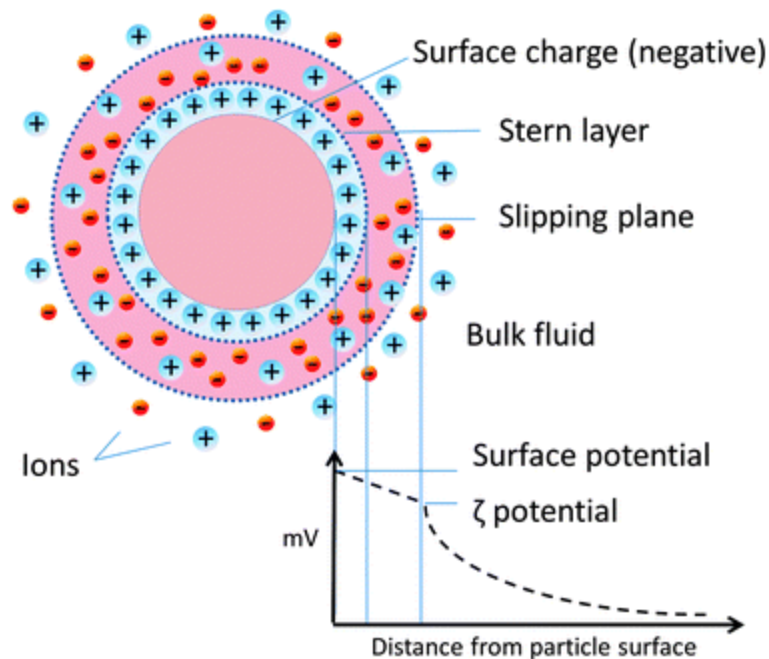


Figure 5:19: Schematic illustration of zeta potential showing zeta potential measured at slipping plane (Beckman Coulter, US).

When a charge particle is dispersed, an adsorbed double layer develops on the surface. The inner layer consists predominantly of ions or molecules with opposite charge to that of the particle (Stern layer). However, beyond the Stern layer, the electrostatic effect due to the surface charge on the particles is decreased. Zeta potential values were compared and are shown in Figure 5.18. It is noted that zeta potential values $\pm 0-10\text{mV}$ is considered as highly unstable, $\pm 10-20\text{mV}$ (relatively stable), $\pm 20-30\text{mV}$ (moderately stable) and $\pm 30\text{mV}$ are highly stable (Battacharjee, 2016). All of the prepared oil body suspensions had negative surface charge. A reduction in surface charge after washing treatment either with alkali or urea was discovered. Statistically there was no significant difference ($P>0.05$) in the surface charge between the samples measured. The surface charge values reported in this work were calculated as the average value of three replicates. The average of surface charge obtained in the crude oil body sample was -30.3 mV , water-washed was approximately -29.4 mV and alkali-washed was $-28.4 \pm 1.2\text{ mV}$ in final oil body suspensions. Zeta potential values in urea-washed were significantly ($P<0.05$) reduced from $-30.3 \pm 0.6\text{ mV}$ to $-22.4 \pm 1.9\text{ mV}$. Therefore, the results indicate the stability of oil bodies in the final suspension.

The absence of some proteins which cover the entire surface of oil bodies would contribute to the loss of surface charge after urea washing. It is noted that the negative charge is related to the protein and phospholipids function on the surface of the oil body droplet. Besides, the relatively high negative charge from crude preparations can be explained by the contribution of high protein content (albumin) which makes the oil bodies stable. In addition, the loss of surface charge may suggest the removal of some of acidic phospholipids such as phosphatidic acid or free fatty acids from the surface of the oil bodies however, this was not determined in this work.

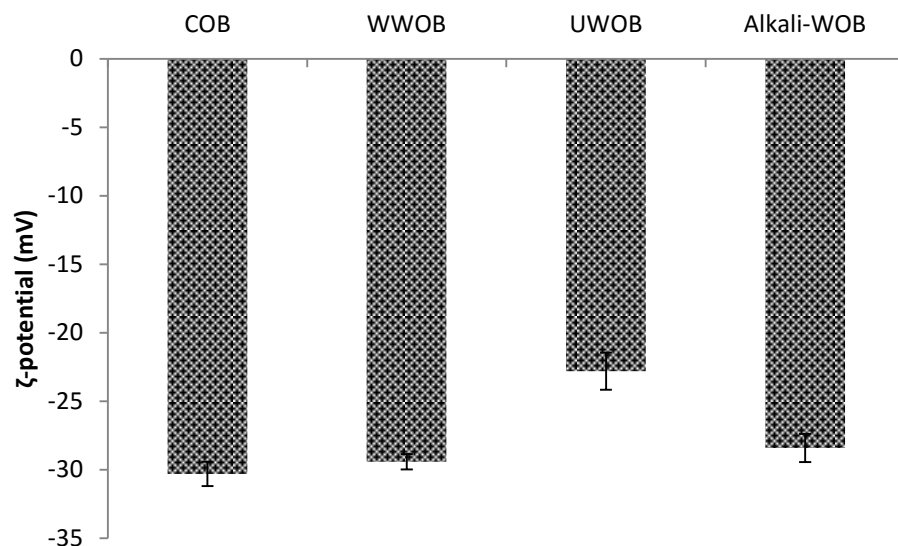


Figure 5:20: Zeta potential values of oil bodies prepared using different washing regimes. ^{a-b} Values with different letters are significantly different ($P < 0.05$). Most of the samples had a highly negative charge which is more than -25 mV except for urea washed (-22.8mV). There is a significant difference of zeta potential values in urea-washed than the other washing regimes. Oil bodies washed once with urea and repeatedly wash with water is called Urea-washed (UWOB). Water washed (WWOB) or Alkali-washed (Alkali-WOB) were repeatedly washed with the respective washing medium.

COB crude oil bodies (pH 7.12)

UWOB, urea washed oil bodies (pH 7.34)

WWOB, water washed oil bodies (pH 7.25)

Alkali-WOB, alkali-washed oil bodies (pH 7.28)

Previous research has reported on the electric charge on soybean oil bodies (Iwanaga *et al.*, 2007; Chen *et al.*, 2012). Heat-treated oil bodies (90°C), had zeta potential values around -11.7 mV compared to non-heated samples of -21.4 mV at pH 7 (Chen *et al.*, 2010). Studies on the surface charge of oil bodies have been well documented by Tzen *et al.* (1992) for various types of seeds. The results obtained from this work indicated that washing treatments may potentially alter the surface charge of oil bodies by changing the electrical magnitude. The measurement of zeta potential is useful to predict the stability of a dispersion which is one of the factors that facilitates the interactions. For instance, if the particles in suspension or emulsion have highly negative or positive charge therefore, this indicates that the dispersion is stable against aggregation. However, when there is no force to prevent particles from coming close to each other then it may suggest the instability of dispersion has occurred, as consequence generating low ζ - potential value.

5.3.3.1 Imaging of Oil Bodies

The performance of oil bodies under different washing regimes was observed using light microscopy and typical micrographs are shown in Figure 5.20. The crude oil body preparation was expected to aggregate due to larger oil body size. The situation was more pronounced after the urea-washing procedure where a population of larger droplets was observed; they were attached to each other and coalesce. Coalescence of oil droplets was also observed in the suspension. Under these circumstances, the size of the aggregates or coalescence of oil body droplet is picked-up by the particle size analyser. The distribution of large oil body droplets reflects the particle size measured in this work.

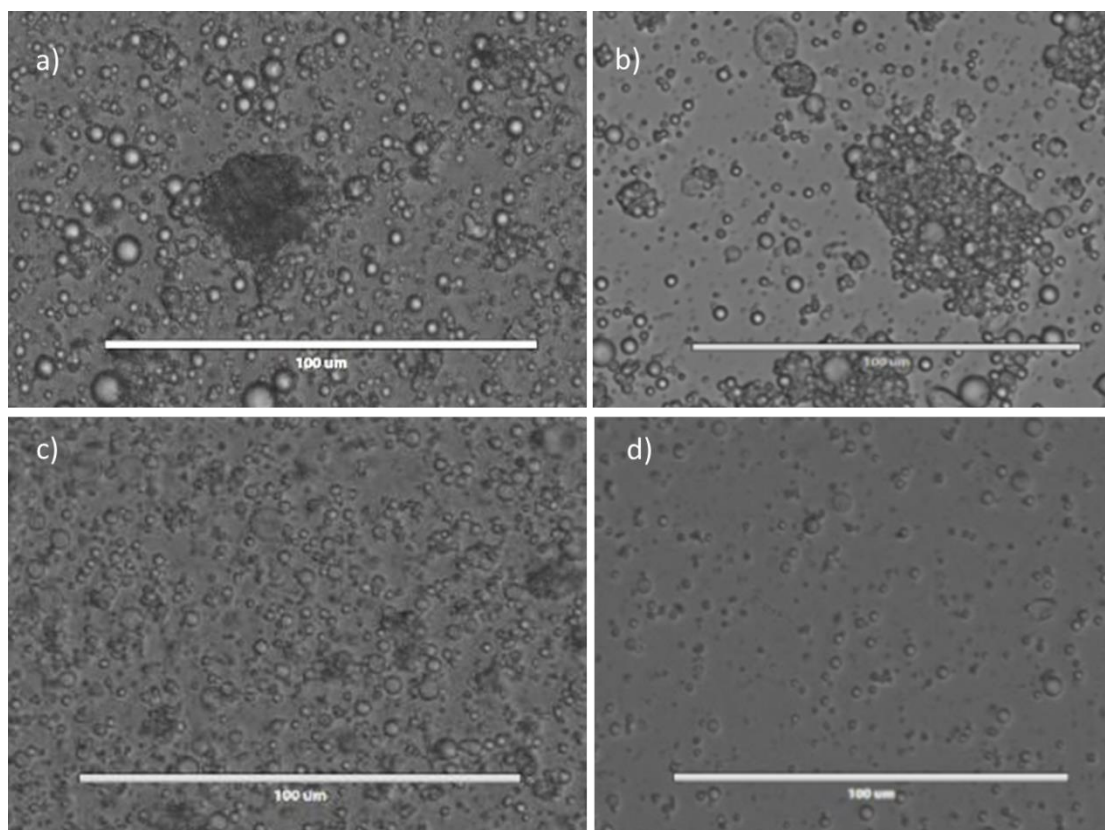


Figure 5:21: Microstructure of crude and washed oil bodies isolated from partially de-hulled sunflower seeds. Images of **a)** Crude oil bodies, **b)** 9M urea washed oil bodies, showing coalescence and leading to larger droplets **c)** oil bodies washed with deionised water **d)** alkali-washed (0.1M sodium bicarbonate) showed intact oil bodies with small droplets. Scale bars represent 100 μm . Non-soluble pigment is observed in the final suspensions of water-washed indicating that washing with water was unsuccessful to remove cell contaminants or cell debris during purification process. Oil bodies washed once with urea and repeatedly wash with water is called Urea-washed (UWOB). Water washed (WWOB) or Alkali-washed (Alkali-WOB) were repeatedly washed with the respective washing medium.

COB crude oil bodies (pH 7.12)

UWOB, urea washed oil bodies (pH 7.34)

WWOB, water washed oil bodies (pH 7.25)

Alkali-WOB, alkali-washed oil bodies (pH 7.28)

According to White *et al*, (1998) washing with urea has no effect on the stability of oil bodies recovered from sunflower seeds. However, these rapid changes are having a serious effect on the quality of oil bodies physically. Despite its long stability success (White *et al*, 1998; Milichip *et al*, 1996; Lacey *et al*, 1998) other research shows that urea induced protein denaturation leading to coalescence in sunflower seeds (Nikiforidis *et al*, 2013). It is consistent with recent works that washing with urea changed oil body behaviour. First, “oiling off” was observed from the cream surface, second the particle size of oil bodies increased dramatically, third coalescence was observed under microscopy in the final suspensions.

In nature, the stability of oil bodies relies on the phospholipids and oleosin to prevent coalescence. The evidence presented in this section suggests that destabilisation after urea-washing contributed to the loss of vital components. The close contact between the neighbouring droplets indicates that a second layer of protein has been removed during the urea wash but most likely the phospholipids have also been affected. In consequence, they are prone to come into close contact and coalesce. The stability of oil bodies is partly stabilised by extraneous protein. If it is true, then washing with urea at high concentration probably denatures the protein by disrupting the non-covalent bond (Stryer, 2000) and leads to oil leakage (Nikiforidis *et al*, 2013). Urea washing successfully purified the oil bodies but indirectly changed the physical state of oil bodies intensely although, pre-soaked seed was carried out. Therefore, urea washing is not suitable to obtain good quality of oil bodies which probably formed hybrid oil. In addition, in food application, urea is not recognised as food grade and its use cannot be considered in food products.

However, it is reversed if washed with alkali i.e. sodium bicarbonate. It is clearly seen that small intact oil body droplets were obtained. Surprisingly, those unwanted non-soluble pigment was removed when washing with sodium bicarbonate solution. The oil body droplets repel each other and this indicates the stability of oil body suspensions against aggregation and coalescence. Additionally, the stability is due to strengthening of stearic hindrance and weakening of Van der Waals. Intact and discrete oil bodies provide large surface area which lowers surface pressure.

One of the objectives in this study was to recover good quality oil bodies. It is known that oil bodies are remarkably stable inside the cells or in an isolated aqueous preparation (Tzen and Huang, 1992; Tzen *et al*, 1992; White *et al*, 2008; Jolivet *et al.*, 2013). Obtaining small intact and stable oil bodies was evidenced in this work indicating high quality oil bodies being harnessed. It was also observed that the resultant alkali-washed oil body suspensions were highly surface charged indicating that very stable suspension phase behavior was achieved. Alkali-washing was capable of removing extraneous protein without destroying integral components to stabilise oil bodies.

5.4 Summary of the Results

Obtaining small intact oil bodies is crucial to avoid artefacts of oil droplets due to coalescence and hybrid droplets formation. Oil bodies were obtained by seed pre-soaking, wet milling, filtration, and centrifugation to produce a buoyant fat pad consisting of a crude preparation of the oil bodies. This work was undertaken to recover high quality oil bodies from sunflower seeds. Pre-soaking (2 hours, 6 hours 12 and 24 hours) before wet milling and multiple washing with different washing solutions (water, 9M urea and alkali (0.1M sodium bicarbonate)

was performed to isolate high quality oil bodies . The physical characteristics were analysed in their final suspension. Basic compositional analysis (total lipid content and protein content) along with physical measurement (particle size, zeta potential and microscopy) were measured. It was evident from different physical measurements such as particle size, zeta-potential and light microscopy that pre-soaking had a significant influence on the oil body suspensions with no significant sign of aggregation or coalescence.

Pre-soaking allows water uptake, the plasticising or softening effect may reduce oil body damage occurring during milling. It was also observed that the resultant washed oil body suspensions were highly surface charged indicating very stable suspension phase behaviour after alkali-washing. The physical state of the seed during wet milling has a significant impact on the quality of the oil body suspension. Despite this finding, the result obtained in Chapter 4 shows clear differences in oil body quality (damage) under cold storage. Perhaps, if pre-soaking was carried out on cold storage work therefore, may shed new light on the oil body integrity. It was hypothesised that intact oil bodies are more robust than hybrid droplets and expected to be destabilised due to weak association of the cellular surface active material that temporarily stabilised the damaged oil bodies. Retention of the size and surface chemistry of oil bodies after washing step was needed as a diagnostic of organelle integrity.

In order to obtain high quality oil bodies from partially-dehulled sunflower seeds, it is recommended to pre-soak the seeds for 6 hours at 4° C, isolate with cold deionised water (4°C) using a kitchen blender and washing with alkali (0.1M sodium bicarbonate).The extraneous protein located on the surface of oil bodies may protect the oil bodies from aggregation. However, during urea washing, this protein may loosen and is washed away during purification. Sodium bicarbonate is widely used in laboratory or in food applications as a raising agent. In fact, sodium

bicarbonate is edible and inexpensive. Perhaps sodium bicarbonate used in this work was selective in removal extraneous proteins without affecting integral elements such as PLs and oleosin. Therefore, small, intact and stable oil body droplets were gained in this work. This was sufficient evidence to highlight that high quality oil bodies.

CHAPTER 6

RECOMMENDED PROTOCOL TO RECOVER OIL BODIES FROM OIL SEED RAPE (OSR)

The properties of oil bodies from the seeds from a range of plant varieties may be similar, but it is already known that the size of oil bodies varies between plant species. The following work offers an insight into other plant material. The information gathered is useful for better understanding the general impact of processing on the oil body quality from oil seed rape.



Figure 6:1 Photo of oil seed rape. Oil seed rape (L. code PR46W21) were drilled on 3rd Sep 2012 and harvested on 14th Aug 2013.

Oil seed rape or *Brassica napus* is one of the important oilseed crops for production of vegetable oil in European countries. A photograph of oil seed rape is

presented in Figure 6.1. It is commonly known as oil seed rape (OSR) which contains a high amount of monounsaturated fatty acid (MUFA), α -linolenic acid (ALNA) and vitamin E (tocopherols) which are considered to be beneficial to health (McKevith, 2005). In 2015/2016, around 27.7 million metric tonnes of rape seed oil were produced globally, which became one of the four major types of the edible oils that dominate the world market (<http://www.statista.com/statistics/263933/production-of-vegetable-oils-worldwide-since-2000/>). The commercial way to produce rape seed oil is by crushing the seeds followed by a purification step (McKecith, 2005).

In this study, oil seed rape used were drilled on 3rd Sep 2012 and harvested on 14th Aug 2013. A germination test was conducted to test the viability of oil seed rape based on the same method applied for sunflower seeds. The germination rate of oil seed rape for this study was 95 %. This data was supported by germinated seeds observed in Figure 6.2. The results indicate that viable raw material was used for this work.

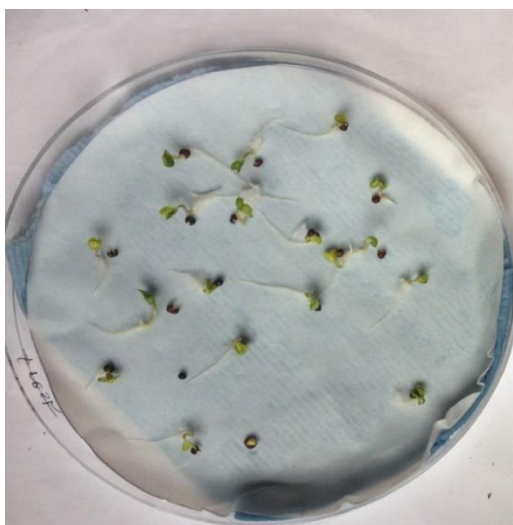


Figure 6:2: Germination test for oil seed rape based on “between paper methods” (ISTA, 1985).

6.1 Effect of Pre-Soaking on the Quality of Oil Bodies

It has been shown in this work that seed pre-soaking is crucial prior to extraction of oil bodies. The results in this work prove that during pre-soaking plasticisation is the reason for the decrease in shear force required during grinding. If pre-soaking was evident to have some effect on the quality of oil body in sunflower seeds, perhaps it also works in oil seed rape. In the pre-soaking of sunflower seeds, optimum pre-soaking time was 6 hours. However, it is unknown for oil seed rape. In order to determine the optimum time for pre-soaking oil seed rape, a set of experiments was carried out. The data obtained is useful to develop better processing parameters in future applications.

It is noted that contrary to sunflower seeds, de-hulling of rapeseed is an uncommon process since it expensive, due to the small size of the seeds (Matthaus, 2007). The set of experiments was conducted to optimise the pre-soaking time (2h, 6h, and 12h) for oil seed rape. The seed was subjected to the same soaking techniques as described in Chapter 5. The seed was pre-soaked at 4°C for the required time prior to oil body recovery. The best pre-soaking time was finalised followed by isolation of oil bodies. Oil seed rape was ground in 0.1M Tris-HCl buffer, at pH 8 and the buoyant crude fat pad was washed with 9M urea. Several measurements were analysed such as particle size and microscopy and any changes were recorded.

During the soaking experiment, the soaking water became brown and darker. The pH of the deionised water before and after soaking were measured, at the same time, an indirect measurement of the pH of seed tissue were achieved by measuring the pH after mortaring 2g of water-rinsed soaked-seeds in 3ml of distilled water. The results were displayed in Table 6.1 and showed that soaking would decrease the pH of soaking water and increase the pH of seed tissue.

Table 6:1 Changes in pH during soaking for the selected hours and pH of the mixture after mortaring.

Pre-soaking time (hours)	pH of the soaking water	pH after mortaring ¹
Non-soaking	-	5.50
2	6.20	6.00
6	6.10	5.90
12	6.24	6.27

Initial pH for deionised water, 6.60

¹Mortaring: 2g of water-rinse soaked seed with 3 ml of water (indirect measurement of pH seed tissues)

Studies showed that seed coats of rape seed contain condensed tannin (Rahman and McVetty 2011; Lipsa *et al.*, 2012) which is the major component contributing to the brown pigment. During soaking, some water soluble tannin on the coat might transfer into water to make the water slightly acidic and brown. Also, the pH of seed tissue was higher due to less tannin present on the coat. Because pH of the seed tissue did not decline, it could possibly be predicted that no significant enzymatic reaction occurred. However, it is possible that if fatty acids were generated through the action of lipases that they could become adsorbed to some of the seed matrix material and so not contributed to the reduction of pH. Further research should be done to detect the enzymatic reaction during soaking.

Volume mean diameter of oil body suspensions are presented in Table 6.1. Non-soaked seeds had slightly bigger oil body droplets compared to the rest of the

pre-soaking samples. Surprisingly, the size of oil bodies was larger (4.82 μm - 5.2 μm) in all washing treatment (non-soaked, 2h, and 6h-soaked) except for 12 hours where the size decreased from 4.82 μm in non-soaked seed to 3.42 μm after 12 hours of soaking. Previous studies indicated that the size of the seed oil bodies from a range of different plant ranged from 0.5-2.5 μm (Tzen and Huang, 1992). However, it was still larger than their corresponding crude oil bodies. In this study, the volume mean diameter (D 4, 3) other than surface mean diameter (D 3, 2) was measured. Previous study showed that while D (3, 2) was sensitive to the small particle, D (4, 3) tended to be more sensitive to larger particles (Courvereur and Hurtaud, 2007).

Table 6:2: Volume mean diameter of oil bodies at different pre-soaking times.

Pre-Soaking (hours)	COB ¹ (μm)	WOB ² (μm)
Non-soak	3.56 $\pm$ 0.2	4.82 $\pm$ 0.4
2	2.40 $\pm$ 0.0	5.50 $\pm$ 0.1
6	2.22 $\pm$ 0.0	5.27 $\pm$ 0.1
12	2.40 $\pm$ 0.0	3.42 $\pm$ 0.0

COB; crude oil bodies, extracted with 0.1M Tris/HCl, pH 8

WOB; washed oil bodies, (washed once with 9M urea, rinse with deionised water).

The results were supported by light microscopy images shown in Figure 6.3 and Figure 6.4. The images of each soaking time were compared. A large population of aggregation were observed in non-soaked, 2h and 6h-soaked seeds (a, b and c) after they were washed. Despite having smaller size after 12 hours of soaking, there were still groups of aggregation observed. Although the COB of non-soaked seed or after soaking for the minimum time (2h) showed larger oil body droplets however, these were not detected by the particle size analyser.

The reason can be explained that probably less volume of the larger oil bodies were present in these two suspensions therefore, it was not determined. It is proved that pre-soaking seed, and the duration of pre-soaking changes the nature of recovered oil bodies at different pre-soaking time. The number of aggregates could be associated with the centrifugation force. As a result, the other hypothesis was raised as ‘the centrifugation force used will have effect on oil body size’. An experiment was designed to test the hypothesis above.

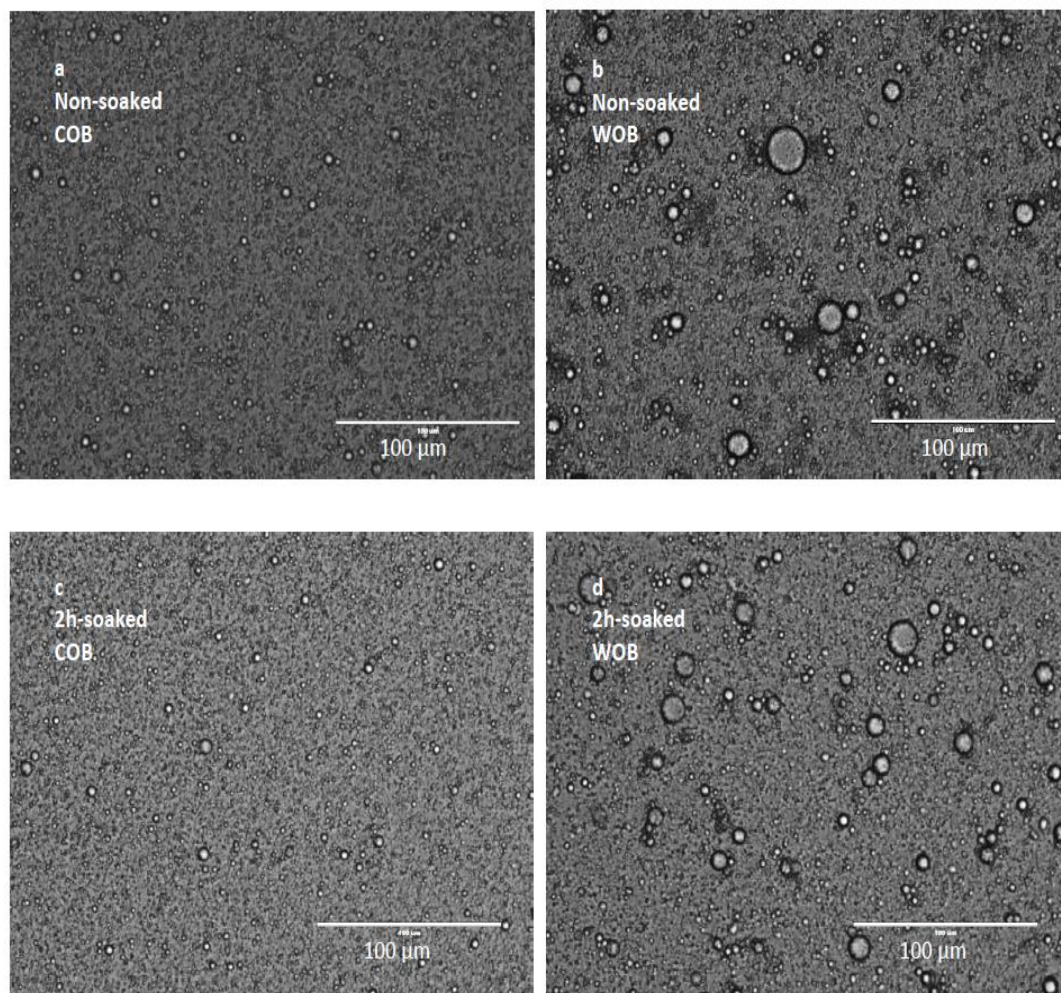


Figure 6:3: Microstructure of oil bodies extracted from oil seed rape after different soaking times. **(a)(c)** Represent crude oil bodies and **(b)(d)** represent washed oil bodies. **(a)(b)** Non- soak seed **(c)(d)** soaking for 2 hours . Scale bar represent 100 μm .

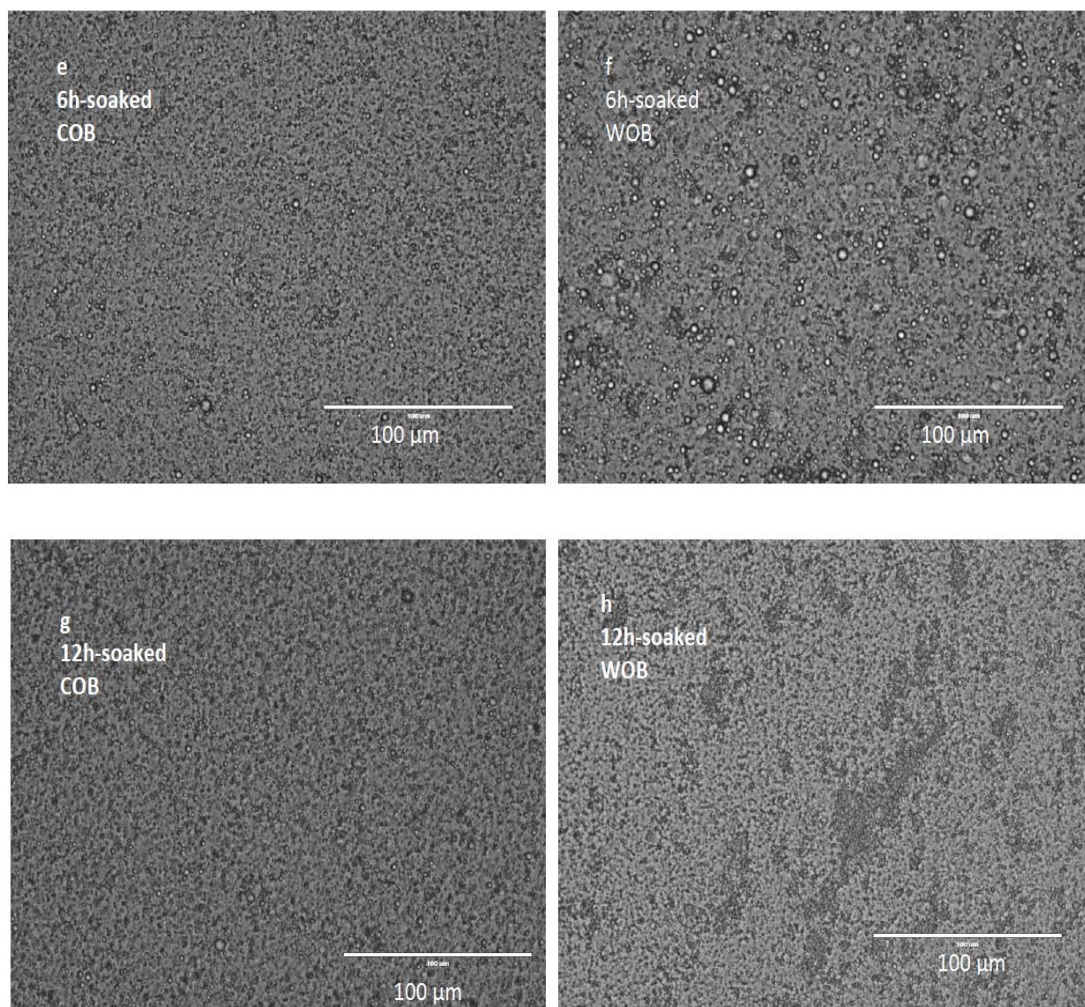


Figure 6:4: Microstructure of oil bodies extracted from oil seed rape after different soaking times. **(e)(g)** Represent crude oil bodies and **(f)(h)** represent washed oil bodies. **(e)(f)** Soaking for 6 hours **(g)(h)** soaking for 12 hours.

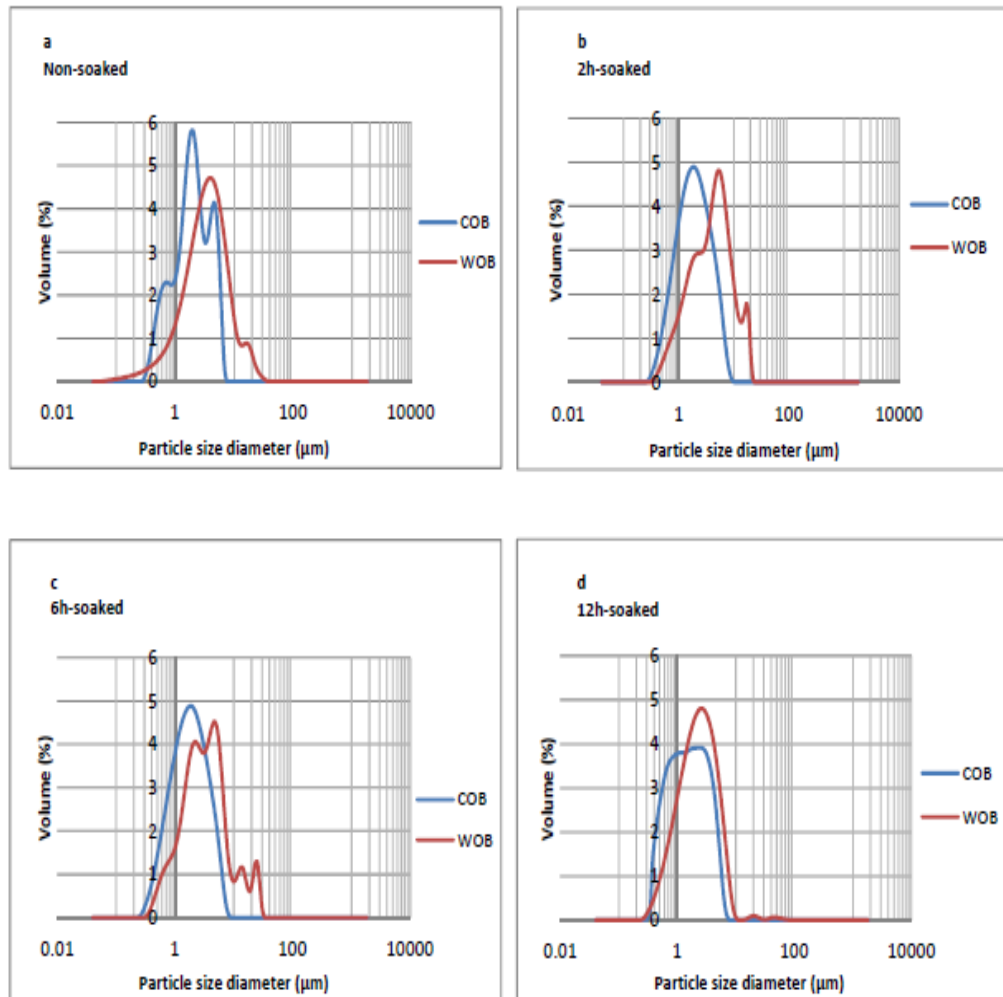


Figure 6:5: Particle size distribution of crude oil body (COB) suspensions and urea washed oil body (UWOB) suspensions a) non-soaked seed and seed soaked for b) 2 hours c) 6 hours d) 12 hours

The results of the particle size distribution were consistent with the optical microscopy images. For crude oil bodies from non-soaked, 2h and 6h-soaked seeds (Figure 6.5 (a), (b) and (c), a large population of particles were around 2 μm (> 57 vol % of non-soaked seeds, > 48 vol % of 2h and 6h soaked seeds). For non-soaked seeds, there was another population around 6 μm (> 40 vol %) which indicated the coalescence. The slightly larger COB particles found from 2h and 6h-soaked seeds in microscopy, on the other hand, were not detected by the particle size analyser, it was probably because there were lower volumes of the larger oil bodies in these two suspensions. After washing, the size of the oil bodies (non-soaked, 2h and 6h-soaked) increased, and the size of the most particles shifted from 2 μm to around 5 μm . In addition, there was another smaller population around 15 μm which might indicate the large coalescence as well the aggregation. For oil bodies from 12h-soaked seeds, there was no significant change of particle size between the crude and washed oil bodies, most particles were around 2 μm (Figure 6.5, (d). Additionally, a small group of particles around 17 μm (> 1 vol % of 12h-soaked seeds and > 0.8 vol % of 24h-soaked seeds) could be found, as no large oil bodies presented in the microscopy, these could be due to the aggregation. After washing, the number of 5 μm particles did not change (>40 vol %), however, a large population of WOB particles were around 2 μm (>43 vol %) instead of 1 μm , which might be due to the slight aggregation. Another group of particles around 20 μm (>5 vol %) also indicated the aggregation.

6.2 Effect of Centrifugation Force on the Quality of Oil Bodies

Compared to sunflower seeds, a centrifugation force of 2400 rcf during washing was insufficient to select small oil body droplets, therefore, larger oil bodies remained. To overcome this issue, washed oil body cream of rape seed from 12 hours of soaking was selected to be re-centrifuged either in 0.1M

Tris/HCl or 0.4M sucrose. Two centrifugation forces (2400 rcf and 16000 rcf) were applied for the study. Table 6.3 demonstrated the volume mean diameter (D_{4,3}) at selected centrifugation force and medium.

Table 6:3: Volume mean diameter, (D_{4,3}) of the emulsion at selected centrifugation force and medium.

Centrifugation force (rcf)	Medium	
	0.1M Tris-HCl(pH8)	0.4 M Sucrose
2400	3.8 ± 0.3µm	3.5 ± 0.3 µm
16000	2.4 ± 0.0 µm	3.1 ± 0.0 µm

Initial size of washed oil bodies (WOB; 9M urea) = 3.08 ± 0.4 µm.

The size of oil bodies was reduced in both media when the centrifugation force was increased to 16000 rcf. Compared to mean diameter of washed oil bodies before centrifugation (3.08 ± 0.4 µm), it was found that 2400 rcf was not high enough to select small oil bodies droplet while more small oil bodies would float to the top at 16000 rcf . The results were supported by images observed under light microscopy. The images are shown in Figure 6.6.

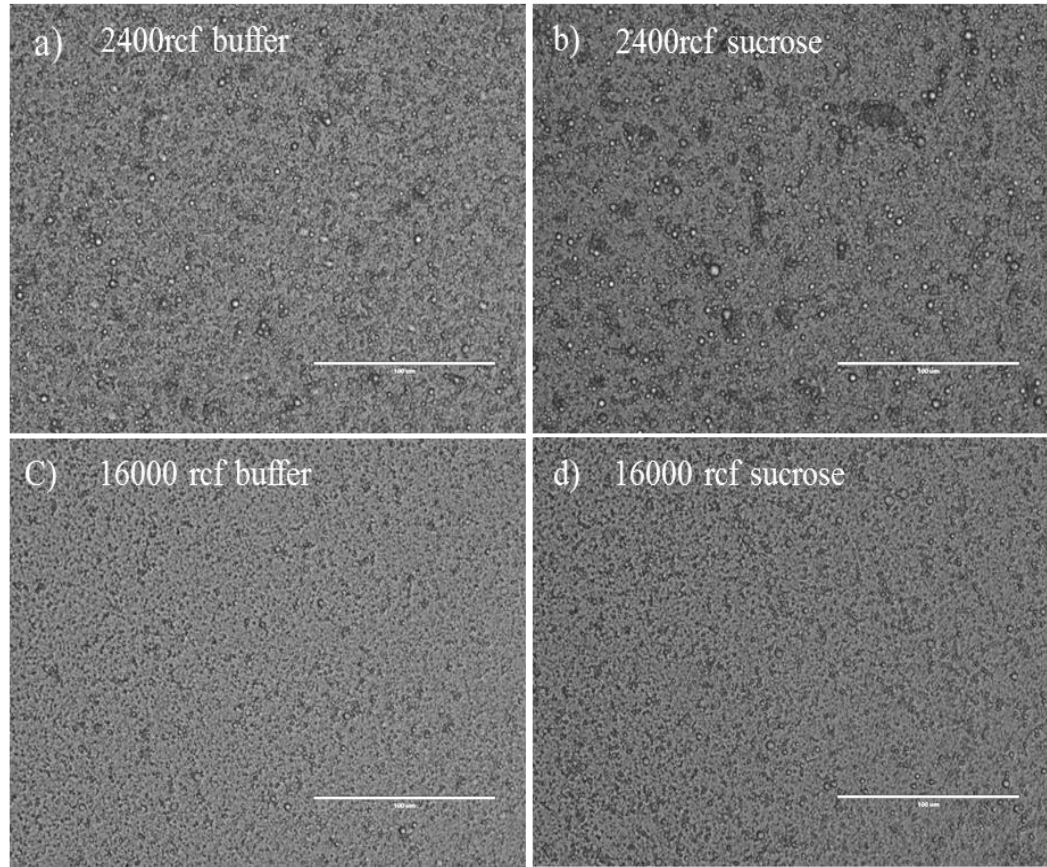


Figure 6:6 : Light microscopy represent oil bodies suspension under different centrifuge medium **a)** 2400 rcf in 0.1M Tris-HCl (pH8) **b)** 2400 rcf in 0.4M sucrose **c)** 16000 rcf in 0.1M Tris-HCl (pH8) **d)** 16000 rcf in 0.4M sucrose . Scale bar represents 100µm.

The volume-mean diameter of the oil bodies decreased with the increasing centrifugation forces in both medium. Using sucrose or buffer as the centrifugation medium resulted in no significant difference. The results indicate that higher centrifugation force could select more small oil bodies.

The phenomenon described above could be explained by Stoke's Law where $V_{\text{Stokes}} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta}$ where the (+) and (-) sign of creaming velocity V means the droplets moves upwards or downwards, g is the acceleration due to gravity, r is the radius of the droplets in the emulsion, ρ_2 and ρ_1 the density of the dispersed and the continuous phases, and η the shear velocity (McClements, 1999). The density of oil bodies is lower than water. As a result, the oil bodies would float to the top during centrifugation. When the centrifugation force was low (2400 rcf), a good number of the smaller sized particles could not float to the top because their radius is much smaller than large sized particles.

According to Dickinson & Stainsby (1982), the above equation is based on the ideal situation. When the particles are liquid instead of solid and the viscosity of the particles is much less than that of the continuous phase, the creamy rate could be 1.5 times faster than that predicted equation because when there is a force applied to the surface to the oil bodies, the liquid inside the oil bodies can move which can reduce the fiction force opposite to the movement of the particles. As a result, the creaming velocity can increase. In conclusion, the optimum centrifugation force for rape seed during washing was 16000 rcf.

Oil bodies were prepared by pre-soaking for 12 hours followed by extraction using 0.1M Tris-HCl and washed with 9M urea at 16000 rcf. After increased centrifugation force to 16,000 rcf, creaming velocity (g) would be higher so that a larger population of smaller particles could float to the top. As a

result, the average size of oil bodies from urea washed decreased from $3.8 \pm 0.3 \mu\text{m}$ to $2.4 \pm 0.0 \mu\text{m}$ after changing the centrifugation force to 16,000 rcf. Therefore, the size obtained in this work is in agreement with the nature size ($0.5 \mu\text{m} - 2.5 \mu\text{m}$) of oil bodies as published earlier in range of plant materials (Tzen *et al.*, 1992; Huang, 1992).

From standpoint of view, oil bodies can be extracted by aqueous extraction methods however, it is depends on the nature of plant material used. It was shown in this work that oil seed rape required a high centrifugation force in order to recover small oil body droplets. Unlike oil seed rape, sunflower seeds required lower force (2400 rcf) to obtain smaller oil body droplets. However, the higher centrifugation force of 16,000 rcf successfully attained smaller oil bodies from oil seed rape therefore; it was recorded in the protocols for further investigation. The recommended protocols to isolate good quality of oilseed rape are shown in Figure 6.6.

The characteristics of oil bodies from oil seed rape could be further investigated in the future. Several processing conditions could be adapted to enhance the oil body properties, such as nutritional values. The respective centrifugation force is vital to extract small and intact oil bodies. This information is useful in designing improved processing conditions prior to wet milling, or during washing treatment in order to produce good quality of oil bodies.

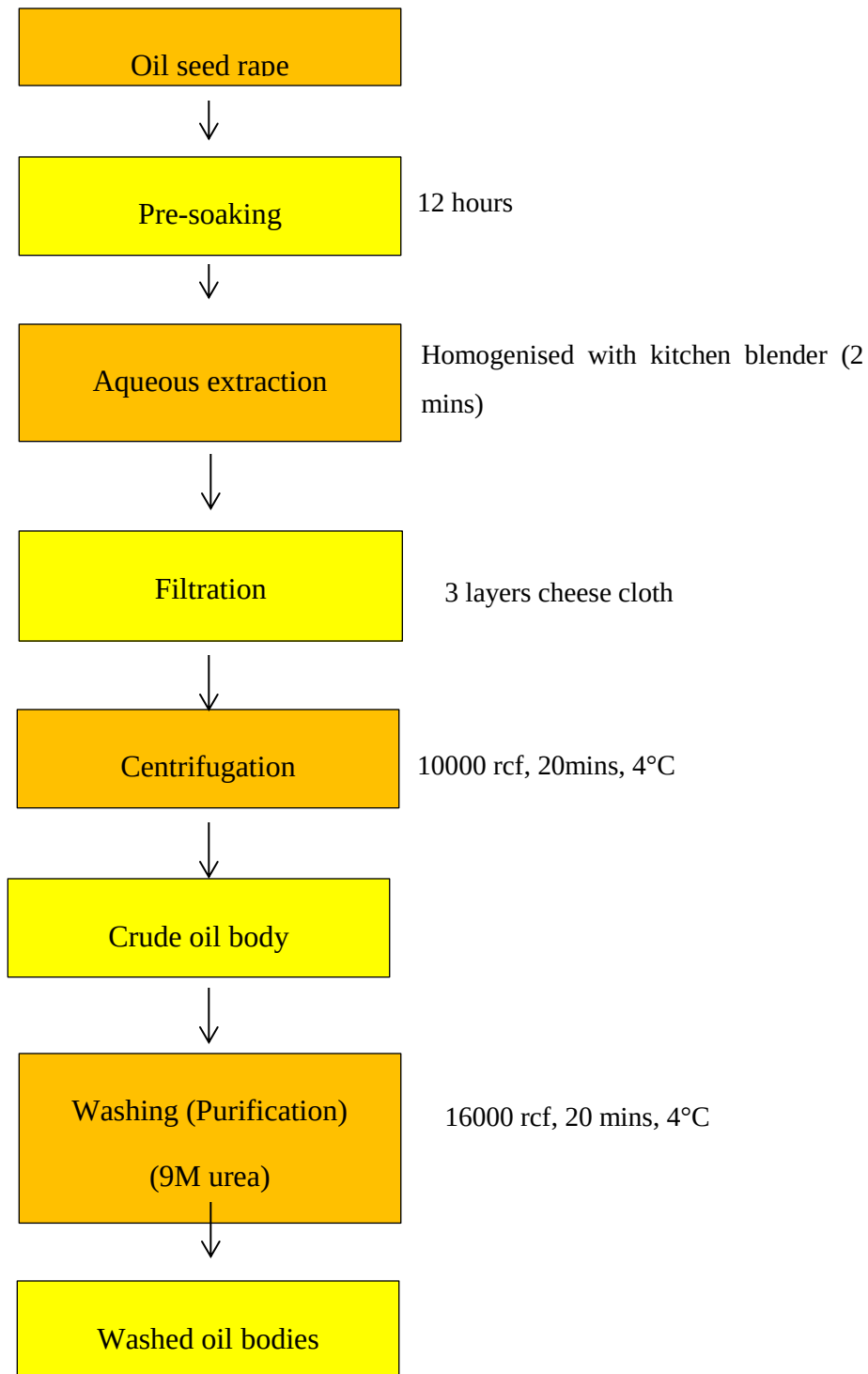


Figure 6:7: A proposed model of obtaining high quality of oil bodies in oil seed rape (OSR).

6.3 Summary of the Results

Based on the systematic experiments conducted, a conclusion could be drawn that pre-soaking seeds for 12 hours and using relative high centrifugation force (16000 rcf) during washing are the optimum methods to extract good quality of oil bodies from oil seed rape. High centrifugation force was able to select small sized oil bodies in oil seed rape.

CHAPTER 7

IMPACT OF SEED STORAGE ON THE QUALITY OF SEEDS AND ITS OIL BODIES

7.1 Effect of Seed Aging on the Seed Quality

Ambient temperature was selected as an ideal condition to store sunflower seeds. Storing the sunflower seeds at low temperature (4°C) resulted in damaging to the extracted oil bodies. Seed aging can be described as the loss of seed quality with time. However, whether storing sunflower seeds for long periods of time affects the quality of oil bodies is still unknown. A series of experiments was conducted to investigate, for the first time, if accelerated aging changes the quality of oil bodies. Accelerated aging is one of the most promising tests to evaluate the physiological potential of various species seeds. Seed aging occurs during storage and affects the physiological and physicochemical properties of seeds, leading to reduction in seed viability and a reduction in quality (Chojnowski *et al.*, 1997; Abreu *et al.*, 2013). The main external factors that influence seed damage during storage are temperature, relative humidity, oxygen, and water activity (Chojnowski *et al.*, 1997; McDonough *et al.*, 2004; Moncaleano-Escandon *et al.*, 2013).

Evaluation of seed quality has been conducted previously from wide range of plant species including maize, sorghum (McDonough *et al.*, 2004); *Jatropha curcas* L. (Moncaleano-Escandon *et al.*, 2013), sunflower seeds (Chojnowski *et al.*, 1997; Tasan *et al.*, 2011); pumpkin seeds (Souza *et al.*, 2013), rice (Kapoor *et al.*,

2011) and also almond (Zacheo *et al.*, 1998). Most of the studies concluded that the length of time of aging as well as storage conditions adversely affects seed quality.

The moisture content and temperature can be manipulated easily during the storage process. Because of rapid water absorption by seeds, salt solutions can be used to control the relative humidity around the stored seed. The salt saturated accelerated aging (SSAA) test promotes slow aging and ensures that the observed deterioration effects are basically due to temperature and exposure period, but not by the increase of water content in the seeds (Moncalleano-Escandon *et al.*, 2013). Therefore, the SSAA test is considered more precise than conventional test methods.

To ensure optimum humidity for aging reached 60% the change of weight over days was determined and demonstrated in Figure 7.1. The graph showed that the seeds are constant at days 5 to day 7. Therefore seeds aged in this way for 7 days will have reached optimum humidity and the seeds were stored up to 5 months at 40°C.

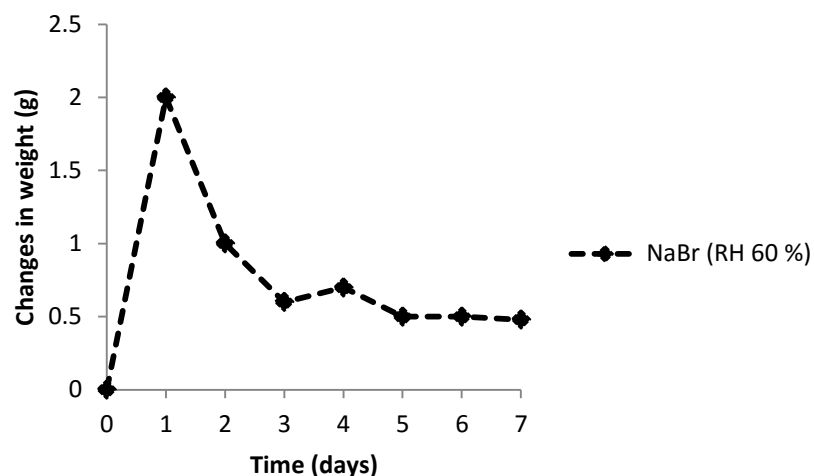


Figure 7:1: Changes in weight (g) of 5 g of sunflower seeds after Salt Saturated Accelerated Aging (SSAA) at 40°C. Relative humidity is 60% using sodium bromide. The seeds achieved the target humidity when the weight of seeds is constant.

7.1.1 Germination Rate

Germination rate is the most important index for testing seed quality. To test the viability of the seeds, the germination test was carried out after 5 months of storage under accelerated aging conditions. The relative humidity was 60%, and the temperature was kept at 40°C. The measurement was taken when the radicle started to grow and based by triplicate calculation in percentage. The germination of sunflower seeds during aging was presented in Figure 7.2; while the germination rate was displayed in Figure 7.3. The sunflower seeds were kept under accelerated aging for 5 months at 40°C and with Relative Humidity (RH) of 60%. The results of the germination test were reported as the mean of three replicates based on the “between papers” method (International Seed Testing Association 1985).

As shown in Figure 7.2 and Figure 7.3 extreme conditions of seed aging caused a significant reduction in germination of sunflower seeds after 5 months. Whereas the germination percentage of the fresh sunflower seeds remained at values higher than those had been artificially aging.

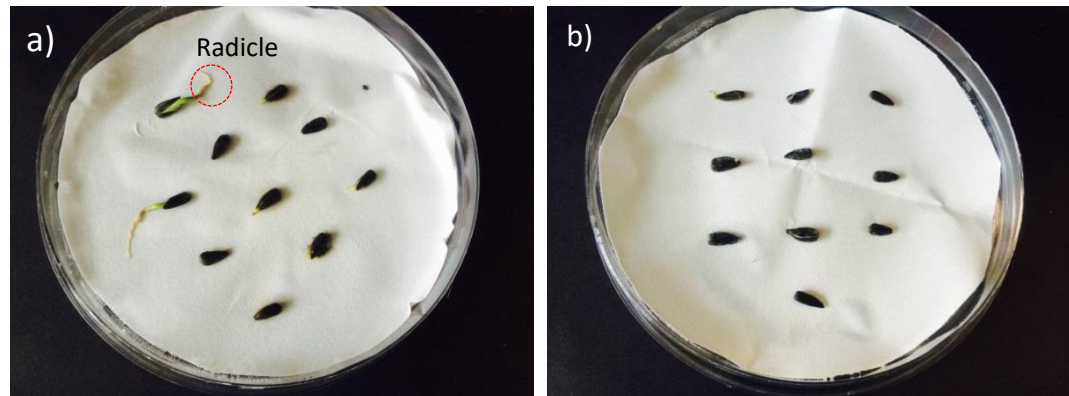


Figure 7.2: Germination test of sunflower seeds after 5 months storage under salt solution accelerated aging (SSAA) a) fresh sunflower seeds (control) b) aging sunflower seeds.

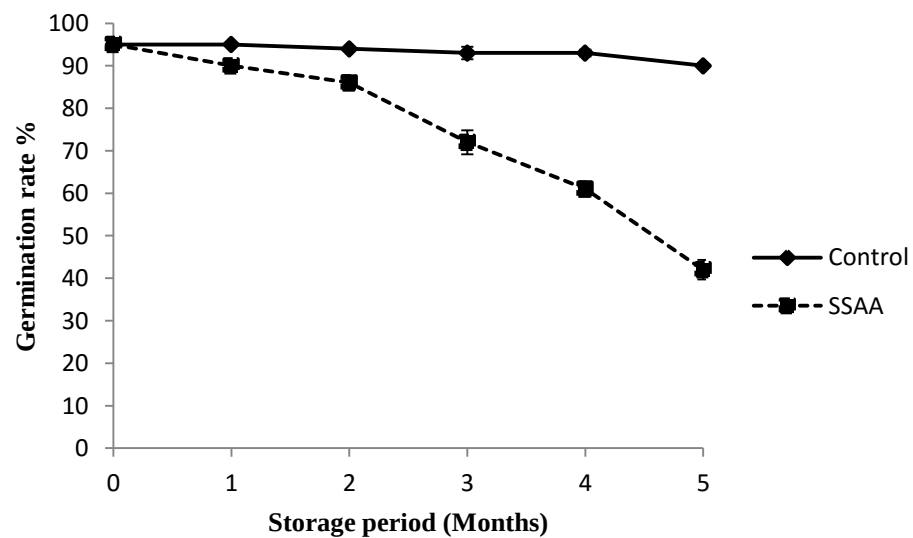


Figure 7.3: Effect of accelerated aging on the germination rate during the 5 month storage trial.

There are three phases of seed reduction that can be identified in this study. At the beginning, the seed from the accelerated aging batch was slightly reduced in first 2 months. After 2 months of storage under the extreme conditions, the seeds germination had significantly decreased from 86% to 72% as indicated by the results from the standard test. Finally, at the end of the storage (after 4 months) the germination dropped to approximately 42%. The sunflower seeds after accelerated aging is still viable but at low germination rate.

It has been reported that a decreased germination rate of soybean seeds can be explained by the high temperature and relative humidity conditions which cause metabolic changes such as rate of respiration or inefficiency of free radical-scavenging enzymes (Bailly *et al.*, 1998; Silva *et al.*, 2008). Seeds are still metabolically active soon after harvesting and the respiration rate is very high. The complete loss in ability to germinate is the critical result of seed deterioration.

Previous studies have shown that storage duration, combined with temperature, had strong effects on the germination rate of *Jatropha* seeds under salt accelerated aging (Moncaleano-Escandon *et al.*, 2013). Only 2 % of the seeds germinated after 12 months in storage at 25° C. The reduction in seed germination rate after 5 months in this study can be attributed to the more stressful conditions at high temperature, such as 40°C. In the standard laboratory, germination of normal seed might form a normal seedling which is expected due to optimum humidity and temperature during testing. However, the reduction in germination rate shown in this work was a result of stress caused by extreme temperature, therefore the seed damage caused low seed vigour. The results indicate that aged sunflower seed have a slower germination rate than the fresh ones. It is important to note that the aim of this work is to study the storage duration of viable seeds, both fresh and aged seed; hence not the dead seeds. Therefore, accelerated aging performed in this work was successful.

7.1.2 Effect of Accelerated Aging on the Basic Composition of Seeds

In general, a higher nutritional value of oil is an indicative of high quality of oil (Sisman and Delibas, 2005). The differences of lipid and protein composition between fresh seeds and aging seeds was measured and displayed in Table 7.1.

Table 7:1: Basic composition of fresh and aging seed in percentage of dry weight basis.

Sample	Lipid content (%)	Protein content (%)
Fresh seeds	55.00 ± 4.2 ^a	30.55 ± 1.2 ^a
Aging seeds	39.75 ± 0.9 ^b	20.70 ± 1.8 ^b

Note: ^{a-b} Means that do not share a letter are significantly different.

It can be seen that fresh seed contained relatively high percentages of lipid and protein, approximately 55 % and 30.5 %, respectively. There is a significant ($P < 0.05$) reduction in the lipid and protein contents after storage for 5 months. The lipid and protein content was 39.75% and 20.70% respectively. The decrease was about 27% in lipid of seed aged and 32 % for protein in aged seeds.

A similar trend was also observed in almond seeds during accelerated aging where the lipid content decreased 31 % from 41mg/g dry weight for aged seeds after storage for 40 days and protein content decreased 7.9% from 151mg/g

respectively, at 80% RH (Zacheo *et al.*, 1998). The results suggest that the reduction of lipid and protein content in aged almond seeds is due to high humidity. The results in this work show similar trends of decreased lipid and protein contents of partially de-hulled sunflower seeds. Potentially rapid water adsorption might affect the lipid and protein measurement especially after seeds desiccated for 5 months under extreme temperature (40°C). Therefore, the loss of some lipids and protein content in this work could potentially due to handling and uncontrollable rapid water adsorption from the surrounding area. The changes of lipid constituents in 14 seeds species including onion, peanut, rape, pepper, watermelon, soybean, sunflower, lettuce, flax, tomato, tobacco radish, sesame and corn has been well documented previously for long- term storage (11-31 years) (Vertucci, 1991).

7.1.3 Effect of Accelerated Aging on Free Fatty Acid (FFA) in Seeds

The major lipid in seeds is triacylglycerol (TAGs). TAGs are hydrolysed to free fatty acids (FFA) and glycerol which normally would alter the quality of oil. Accumulation of free fatty acids (FFA) is particularly related to seed deterioration. In plant physiology, free fatty acids can be analysed using a range of methods. Acid-base titration is one of the analytical procedures used to determine the free fatty acid content. This method is fast and easy, and uses chemicals such as phenolphthalein to determine the endpoint of the titration. However, it is not suitable for the titration of coloured samples. In this study, the free fatty acid content was measured based on the colour change of phenol red by using spectrophotometer and oleic acid as a reference. The advantages of this method are that it is both simple and economic, due to using low volumes of solvent (Walde and Nastruzzi,1990).

The percentage of free fatty acid during accelerated aging is presented in Table 7.2. In this study, the free fatty acid content significantly increased ($P<0.05$) during storage for 5 months. Non-aged seed contained 11.6 % of free fatty acid from total lipid as expressed as a percentage of oleic acid and increased to 19.1 % during accelerated storage.

Table 7:2 : Free fatty acid of seed during storage.

Sample	Free fatty acid (%) ¹
Fresh seeds	11.6 ± 0.9 ^a
Aging seeds	19.1 ± 0.6 ^b

Note: ^{a-b} Means that do not share a letter are significantly different.

¹ Expressed as percentage of oleic acid.

These results are not surprising as a similar pattern of accumulation of free fatty acid has been reported in lipid-rich *Coffee arabica* seed (Laffargue *et al.*, 2007). The dramatic increase of free fatty acid from 3mg/g lipid in coffee to 15mg/g lipid using solid-phase extraction gas chromatography (SPE-GC) was reported after the beans had aged for 18 months, 60% RH at 15° C. The FFA increase observed in this study suggests that lipid hydrolysis occurred during seed storage.

7.1.4 Effect of Accelerated Aging on Fatty Acid (FA) in Seeds

To test the quality of TAG in partially de-hulled sunflower seeds during accelerated aging, fatty acid profiling was undertaken. Fatty acids can be divided

into generic groups by the number of double bonds in their carbon chains. For determination of fatty acids, TAG were transesterified to methyl esters prior to analysis because the esterified form is less polar than the corresponding fatty acid. Fatty acid methyl ester (FAME) analysis by Gas Chromatography with flame ionization detection (FID) is a common analytical method that provides valuable information regarding the net fatty acid chain composition of a sample. Because of the highly complex nature of lipid mixtures, mass spectrometry is used for identification of molecular species within various lipid classes. In the present work, the methyl ester profiles of fatty acids are illustrated in Figure 7.4.

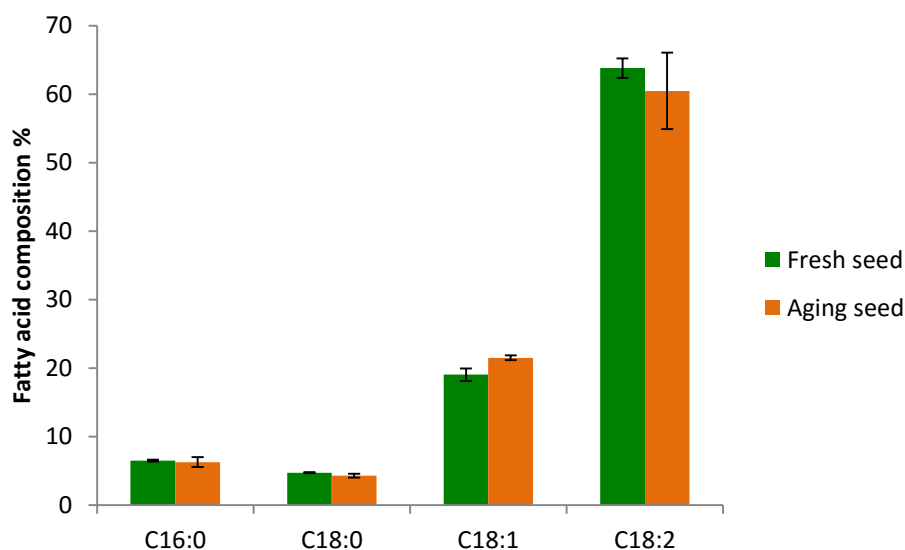


Figure 7:4: Fatty acid profile of seed under storage condition. Error bars represent standard deviation.

Four types of fatty acid were detected with predominance of unsaturated fatty acid; oleic (C18:1) and linoleic as (C18:2) as well palmitic (C16:0) and stearic (C18:0). The fatty acids oleic and linoleic have totaled more than 80% of the total fatty acids in fresh seeds. It can be seen that there were no changes in

saturated fatty acids (palmitic and stearic) obtained in this work but there was a slight increase in the monounsaturated fatty acid (oleic acid) during storage. However, there was no significant difference in linoleic acid between fresh and aging seeds.

Fatty acid changes were reported earlier between two hybrid sunflower seeds (Helio 250; high oil content and Helio 251; low oil content) accelerated for twelve months at 10°C under 40% relative humidity (Abreu *et al.*, 2013). The fatty acid profiles in these two hybrid sunflower seeds were also found to remain stable. It is noted that salt saturated accelerated aging (SSAA) is a slow process therefore; the quality of TAG fraction extracted from partially de-hulled sunflower seeds in this work was preserved.

7.1.5 Effect of Accelerated Aging on Peroxide Value (PV) in Seeds

Despite having no difference between TAG fatty acid as discussed in the previous section (section 7.1.4) the quality of oil extracted from partially de-hulled sunflower seeds is still unknown. Lipid peroxidation can be major problem of oil quality, safety and nutritional value (Frankel, 2005). Oxidative rancidity in oils and fats can be measured using the peroxide value (PV) test. Peroxide value (PV) is a measure of the concentration of peroxides and hydroperoxides, that are formed in the initial stages of lipid oxidation (Li *et al.*,2013). The peroxide value of the seeds during aging are presented in Table 7.3.

Table 7:3 : Peroxide value of seeds during storage

Sample	Lipid hydroperoxide (mmol kg ⁻¹ lipid)
Fresh seeds	0.07 ± 0.02 ^b
Aging seeds	0.16 ± 0.02 ^a

Note: ^{a-b} Means that do not share a letter are significantly different.

Fresh seeds contained 0.07 mmol kg⁻¹ of lipid hydroperoxide and after 5 months this value was 0.16 mmol kg⁻¹ lipid . There is significant difference (P<0.05) between the fresh and aging seeds under salt saturated accelerated aging. The results obtained in this work suggests that induced high temperature and humidity causing oxidative rancidity during storage. Hydroperoxides are the primary products formed during oxidation, but they are labile intermediate compounds that decompose into several secondary oxidation products. However, secondary oxidation products were not determined in this work.

Thus, even though peroxide value is a common indicator of lipid oxidation, its use as a measure of oxidation is limited to the earlier stage of oxidation (Tassan *et al.*, 2011, Crapiste *et al.*,1999). Peroxide formation is one of the major concerns in industrial applications and nutritional properties. High values of peroxide indicate that low quality oil has been extracted. Peroxide value in crude oil should not exceed 2.5 mmol kg⁻¹ lipid (Frankel, 2005). During accelerated seed aging, lipid hydroperoxide values in obtained in this work was less than the standard set to detect oil rancidity. Although the aged seed are not rancid, it is still poor compared to the quality of fresh seed. The results suggest, however, that the quality of oil extracted from partially de-hulled sunflower seeds was still good quality after accelerated aging. If bulk oil is still in good quality

against oxidation there is potentially the presence of compounds that protect the oil against oxidation. To test this, Vitamin E analysis was carried out and discussed next.

7.1.6 Effect of Accelerated Aging on Tocopherol in Seeds

Vegetable oils are one of the main sources of tocopherol. α - tocopherol and γ - tocopherol are two most common homologs found in vegetable oils. These natural antioxidants play a role as lipid oxidation inhibitors in food and biological systems. Tocopherols were determined by high-performance liquid chromatography (HPLC). Tocopherol was identified by comparing the retention times of samples with pure standards of α -and γ -isomers of tocopherol. Additionally, they were quantified on the basis of peak areas of the unknown with those pure standards. The amounts of tocopherol were calculated using external calibration curves obtained for each standard. The tocopherol content of seeds stored under accelerated storage conditions are presented in Table 7.4.

Table 7:4: α -tocopherol and γ -tocopherol content during aging.

Sample	α -tocopherol (mg/kg)	γ -tocopherol ((mg/kg)
Fresh seeds	102.32 $\pm$ 3.2 ^a	0.90 $\pm$ 0.1 ^c
Aging	75.65 $\pm$ 3.7 ^b	0.39 $\pm$ 0.1 ^c

Note: ^{a-c} Means that do not share a letter are significantly different.

It was clear that fresh seeds contained more α -tocopherol than the seeds stored at high temperature, with values of approximately 102.3 mg/kg and 75.6 mg/kg, respectively. Sunflower oil, which has approximately 732.6 ± 10.3 ppm of its tocopherol in the α -configuration, could possibly have improved the oxidative stability of sunflower oil in fresh material (Tasan and Demirci, 2005). However, the individual tocopherol contents in this work seem to be affected by accelerated aging at the extreme temperature of 40°C. Although there is a significantly lower concentration of α -tocopherol in aged seed during 5 months storage, there is no significant difference in γ -tocopherol for both type of seeds. Tasan *et al.*, (2011) mentioned that tocopherols were sensitive to light, temperature, heating time, pressure, alkali, air, and metal such as iron and copper. The results obtained in this work indicate that the reduction of α -tocopherol during storage was probably due to the high temperature (40°C) causing stressed environment conditions in the seeds.

7.2 Effect of Accelerated Seed Aging on the Quality of Oil Bodies

7.2.1 Optimisation of Seed Pre-Soaking During Accelerated Aging

During accelerated aging, sunflower seeds were stored for 5 months at extreme temperature (40°C) along with salt solution (sodium bromide) to control the relative humidity. It is important to note that Saturated Salt Accelerated Aging (SSAA) test is hypothesised to more accurately evaluate the effect of storage on small seeds (Moncalleano-Escandon *et al.*, 2013) like sunflower. One of the advantages of this technique was to reduce water adsorption and technically leave the seed desiccated.

A set of experiments was performed to optimise the pre-soaking time for aging seeds. Since 6 hours of soaking is sufficient to hydrate fresh seeds, therefore it was expected that a longer soaking time due to seed desiccation, would be

required promote water uptake after storage for 5 months at 60% relative humidity (RH). Figure 7.5 shows the size distribution of the oil body suspension during accelerated aging. The size distribution of the oil body suspension was shifting to larger particle sizes after 6 hours of soaking. The longer soaking time reduced the particle size in both crude and washed oil body preparations.

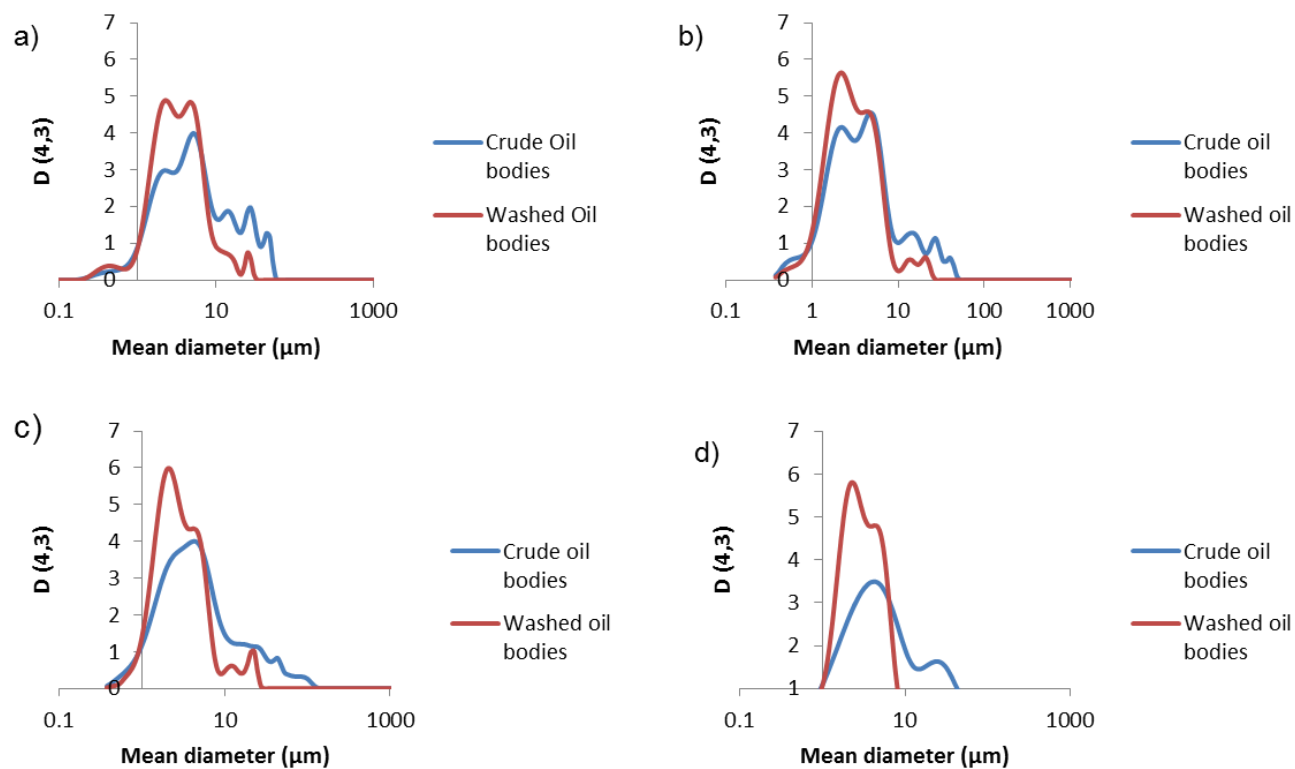


Figure 7:5: Size distribution of oil bodies extracted from accelerated aging seeds that had also been subjected to a pre-soaking step prior to oil body extraction. The size distribution of a) non-soak seed b) 6 hours c) 12 hours d) 24 hour of soaking.

The size distribution results were supported by the light microscopy images shown in Figure 7.6. As can be seen in Figure 7.6, crude oil bodies contain larger particle size of oil bodies in all crude oil body preparations. There was some insoluble material (carry over from seed coat) observed. However, those materials were washed away during purification. With regards to this result, volume mean diameter (D 4, 3) of oil bodies was measured and was summarised in Table 7.5.

The size of oil bodies was slightly bigger in crude oil body preparation however; the size of oil body was reduced once the oil body fat pad was repeatedly washed with 0.1M sodium bicarbonate. As increasing the soaking time from 12 hours to 24 hours progressively reduced the particles size to 3.4 μm . The oil bodies obtained were intact and small discrete organelles. The results suggest that under accelerated aging (5 months), water molecules required 24 hours to hydrate the cell matrix by softening the seeds and by promoting plasticisation.

Table 7:5: Volume mean diameter of oil bodies during seed pre-soaking.

Pre-soaking time (hours)	Volume mean diameter, D (4,3)	
	Crude oil bodies ¹	Washed oil bodies ²
Non soak	9.26 $\pm$ 2.1	8.14 $\pm$ 1.1
6	8.97 $\pm$ 1.6	5.54 $\pm$ 1.8
12	9.13 $\pm$ 0.9	3.97 $\pm$ 1.7
24	9.07 $\pm$ 1.4	3.41 $\pm$ 1.3

¹Crude oil bodies was homogenised in deionised water

²Oil bodies was repeatedly washed with 0.1M sodium bicarbonate

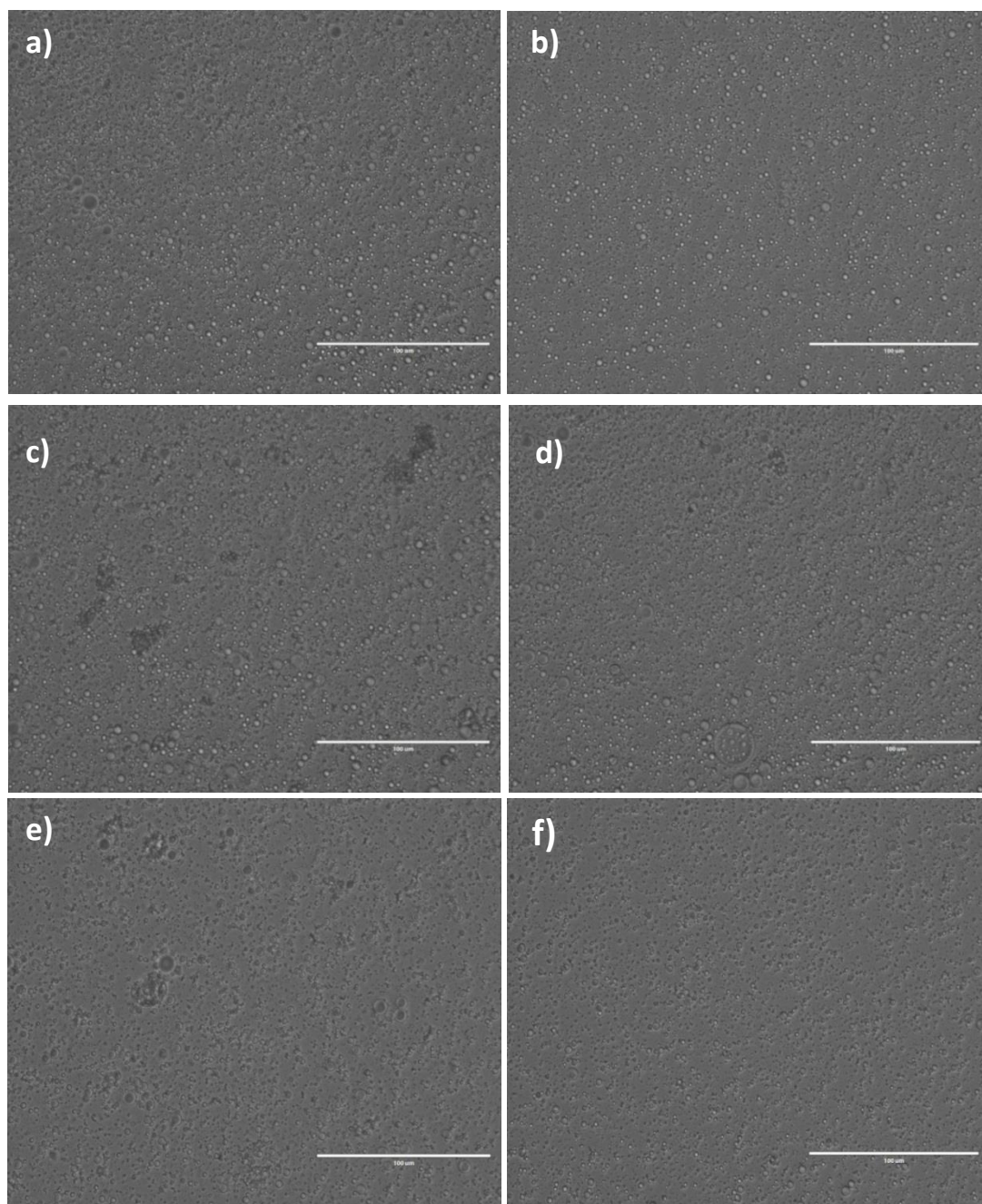


Figure 7:6: Images of oil body suspension from sunflower seeds kept under accelerated storage conditions for 5 months. Scale bar represents 100 μm . **(a) (c) (e)** crude oil bodies **(b) (d) (f)** washed oil bodies. **(a)(b)** pre-soaked for 6 hours **(c) (d)** pre-soaked for 12 hours **(e)(f)** pre-soaked for 24 hours.

7.2.2 Effect of Accelerated Aging on the Zeta Potential in Oil Bodies

An aliquot of each oil body sample from the pre-soaking trials was subjected to zeta potential measurement as shown in Figure 7.7. The oil bodies were dispersed in deionised water and zeta potential values were recorded. All crude samples of the oil body suspensions are highly negatively charged ($\sim$ pH7) in all pre-soaking seeds. The results indicate that the suspensions are stable. However, the zeta potential values decreased after the washing treatment which was not surprising and were also observed, as described previously (Fisk, 2007). Despite having slightly decreased surface charge values in all final suspensions obtained from different soaking times, however all of the suspensions were stable without any sign of aggregation or coalescence. Kapchie *et al.* (2013) reported that the surface charge of oil bodies extracted from soybean was approximately -14.4 mV at pH 7 in the presence of 100 and 500 μ M ferric ions. The decrease in zeta potential was due to the additional transition metal ion added during the recovery process. The results showed that oil bodies recovered at different conditions showed different properties and were related to the extrinsic protein in recovered oil bodies (Zhou *et al.*, 2016).

Extraneous protein associated with the surface of oil bodies may affect the surface charge of the oil body droplets, since a slight reduction in the charge was observed after washing the oil bodies. There was no evidence that increasing the soaking time affects the zeta potential values in crude and washed oil bodies in sunflower seeds.

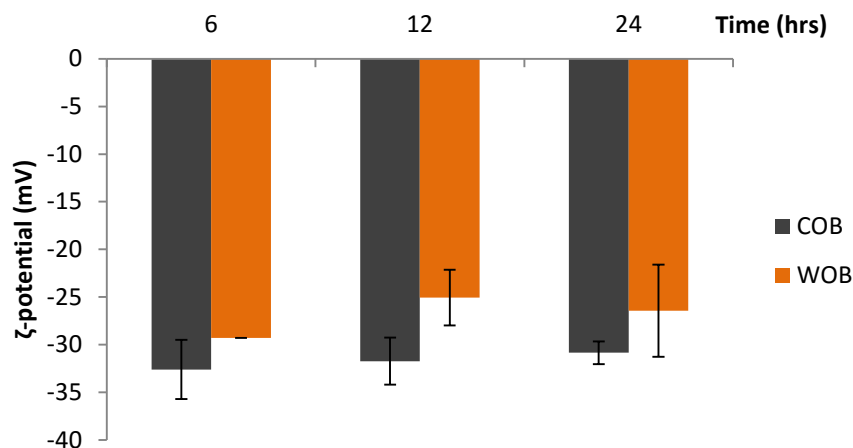


Figure 7:7: Zeta potential values during seed pre-soaking. Error bars represent standard deviation. Oil bodies were obtained by pre-soaking, grinded in deionised water and called crude oil body (COB). The crude fat pad was repeatedly washed with 0.1M sodium bicarbonate (WOB). pH of COB suspension, pH 7.13 and pH of WOB suspension, pH 7.26.

7.2.3 Effect of Accelerated Aging on the Basic Composition of Oil Bodies

The basic composition of crude and washed oil bodies after 5 months of accelerated aging was measured and presented in Table 7.6. The concentration of lipid and protein in oil bodies initially was 87.05 % and 8.10 %, respectively. The lipid content of washed oil body was increased significantly ($P < 0.05$) to 93.5%. However, the amount of lipid in washed oil bodies materials was reduced significantly ($P < 0.05$) after aging. However, there was no significant difference ($P > 0.05$) was observed in both crude and washed oil body preparation accelerated aging.

Initial total protein content of crude oil bodies isolated from fresh seeds was 8.1%. However, the amount of protein content was reduced significantly

($P < 0.05$) to 1.9 % after washing treatment. The protein content in aging seeds also showed similar trends, indicating that the samples were successfully purified. The protein content was reduced significantly ($P < 0.05$) from 5.8 % to 1.6 % in crude and washed oil bodies, respectively. Despite of removing extraneous protein during washing, there was also some unaccountable materials were washed away includes cell wall or any carbohydrate material.

Table 7:6: Basic composition of oil bodies during accelerated seed aging.

Sample	Lipid content (%)	Protein content (%)
Fresh seed	55.3 $\pm$ 4.5 ^c	30.5 $\pm$ 2.7 ^a
Crude oil bodies	87.3 $\pm$ 1.8 ^b	8.1 $\pm$ 0.5 ^b
Washed oil bodies	93.5 $\pm$ 2.1 ^a	1.9 $\pm$ 0.3 ^c
Aging seeds	40.1 $\pm$ 1.6 ^d	21.1 $\pm$ 2.6 ^c
Crude oil bodies	84.9 $\pm$ 1.7 ^b	5.8 $\pm$ 0.7 ^d
Washed oil bodies	88.1 $\pm$ 1.8 ^b	1.6 $\pm$ 0.3 ^d

Note: Means that do not share a letter are significantly different ($P < 0.05$).

Furthermore, the amount of protein was significantly difference ($P < 0.05$) in oil bodies extracted from fresh sunflower seed. To summaries, the progressive reduction in the total protein content highlighted the important of washing regime as part of the purification process.

7.2.4 Effect of Accelerated Aging on the Free Fatty Acid (FFA) in Oil Bodies

The major lipids in oleaginous plant material are triacylglycerols. The concentration of free fatty acid hydrolysed from triacylglycerol was measured. Free fatty acid analysis was conducted with oleic acid as the reference. No free fatty acid was detected in the oil body samples extracted either from fresh seeds or from the aging seeds. There are number of reasons attributed to this. Potentially, some of the acid has been leached out to the surrounding medium during pre-soaking or the acid has been removed during alkali-washed later, leaving behind samples which do not have carry-over of free fatty acid. As consequence, the amount of free fatty acid was not detected during the measurement. Thus, it is evidenced that washing treatment successfully purified the oil bodies into clean preparation.

7.2.5 Effect of Accelerated Aging on the Fatty Acid in Oil Bodies

Analysis of the fatty acid profiles was undertaken on the oil body samples. The fatty acid profile of oil bodies either extracted from fresh or aging seeds were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). The peak of fatty acids at different retention times is shown in Figure 7.8. Retention time of fatty acids is shown in Table 7.7

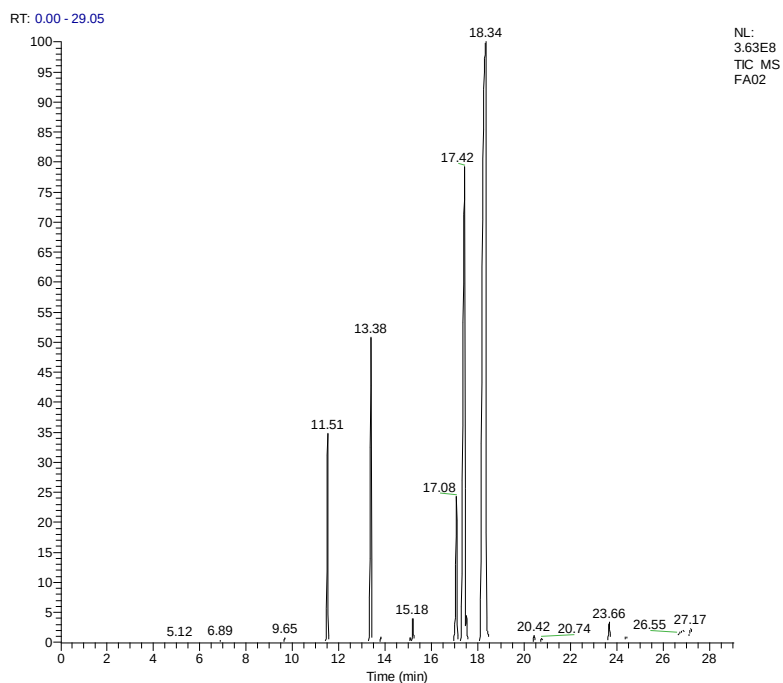


Figure 7:8: Peaks showing fatty acids present in partially de-hulled sunflower seeds by Gas Chromatography Mass Spectrometry (GC-MS).

Table 7:7: Retention time of fatty acids.

Peaks	Retention time (mins)
Methyl pentadecanoate (Internal standard)	11.51
Palmitic acid (C16:0)	13.38
Stearic acid (C18:0)	17.08
Oleic acid (C18:1)	17.42
Linoleic acid (C18:2)	18.34

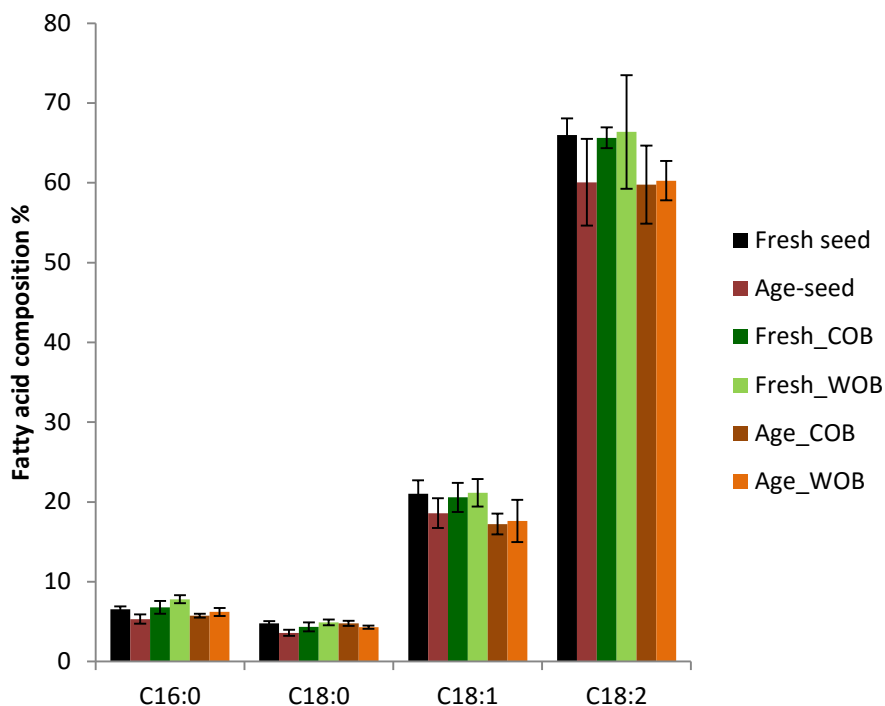


Figure 7:9: Fatty acid profile of oil bodies and seeds during storage. Error bars represent standard deviation.

The fatty acid composition of seeds and oil bodies were compared in Figure 7.9. Both samples have a similar fatty acid composition, in fact there were no differences ($P > 0.05$) observed between the fatty acid compositions in seed oil or in oil bodies. It was noted that the bulk oil present in seeds will be neutral lipid stored in oil bodies. Although there are some differences in fatty acid composition especially from different seed type or variety, it is suggested that it is probably due to different cultivar used or differences in the environment that they are exposed to.

Fatty acid composition of fresh oil bodies is slightly reduced after accelerated aging for 5 months. Compared to seed, linoleic acid in fresh oil bodies

was reduced from 66.85 % to 62.3 % however seed oil decreased to 54.06 % from initial amounts of 64 %. The amount of stearic acid (C18:0) in aging seeds (1.6%) was reduced about half from fresh seeds (4.75%) however there was no significant difference ($P>0.05$) in the oil body samples.

7.2.6 Effect of Accelerated Aging on the Tocopherol in Oil Bodies

Tocopherol is one of the vital components for antioxidant activity present in oil bodies. It is expected that if tocopherol was an intrinsic component in oil bodies it would not be lost during seed aging. The amount of tocopherol retained after accelerated seed aging is presented in Table 7.8. Tocopherol was enriched on an oil per unit dry mass compared with seed, and is further enriched after washing of oil bodies; this is consistent with tocopherol being an intrinsic component of oil bodies. Fisk *et al.*, (2008) reported 368 mg/kg α -tocopherol and 1.93 mg/kg γ -tocopherol of sunflower seeds without a pre-soaking treatment.

Table 7:8: Tocopherol content of oil body preparation after accelerated seed aging.

Sample	α -tocopherol(mg/kg)	γ -tocopherol(mg/kg)
Fresh seeds	102.32 $\pm$ 2.6 ^d	0.90 $\pm$ 0.1 ^d
Crude oil bodies	252.60 $\pm$ 40.3 ^b	14.5 $\pm$ 1.4 ^c
Washed oil bodies	491.6 $\pm$ 21.2 ^a	36.3 $\pm$ 3.0 ^b
Aging seeds	75.65 $\pm$ 4.2 ^d	0.39 $\pm$ 0.0 ^d
Crude oil bodies	264.6 $\pm$ 18.0 ^b	10.7 $\pm$ 0.3 ^d
Washed oil bodies	211.3 $\pm$ 9.60 ^c	11.4 $\pm$ 2.0 ^a

Note: Means that do not share a letter are significantly different ($P<0.05$).

In this work, tocopherol (25%) appears to be lost in the seed overall during aging, and the washed oil body preparation has less than 50% of the tocopherol compared with washed oil bodies from fresh seed. During seeds aging, the amount of α -tocopherol obtained was 264.6 mg/kg and γ -tocopherol obtained was 10.7 mg/kg, respectively in crude preparation however, reduced after washing treatment.

In comparison with the control, most of the samples have significant correlation on α -tocopherol. A similar trend was clearly seen in γ -tocopherol except for aging seeds where the amount of γ -tocopherol did not significantly correlate. A slightly decreased amount of γ -tocopherol in aging seeds was recorded compared to the fresh ones, therefore the results were not significant ($P < 0.05$). The results indicate that oil bodies were not affected during seed aging and tocopherol are thought to play a structural role in preventing oil body from oxidation. It is suggested the present of tocopherol on the surface of oil bodies delayed the aging process in partially de-hulled sunflower seeds.

7.2.7 Effect of Accelerated Aging on Peroxide Value (PV) in Oil Bodies

Oxidation of oil bodies during storage was measured by lipid hydroperoxide concentration. The lipid hydroperoxide value of ferric thiocyanate method, based on the oxidation of ferrous to ferric ions, is more sensitive and requires a smaller amount of sample than standard iodometric method measured by titration (Frankel, 2005). Figure 7.10 the hydroperoxide concentration in sunflower seeds and its oil bodies. It is important to note that the quality of oil bodies was assessed based on hydroperoxide concentration level as suggested by Frankel (2005), which is less than 2.5 mmol.kg^{-1} lipids, which indicates high quality. During storage, both crude and washed oil bodies in aging seeds showed

raised hydroperoxide levels as compared to both crude and washed oil bodies extracted from fresh sunflower seeds.

Lipid hydroperoxide values obtained in this work, ranged from 0.1 to 0.13 mmol.kg⁻¹ lipids in crude preparation and approximately 0.08 to 0.113 mmol.kg⁻¹ lipids in washed preparation, respectively. It was expected that the partially de-hulled sunflower seeds had a higher peroxide concentration as the hull (act as physical barrier) had been removed during de-hulling process. The highest concentration of hydroperoxides was observed in crude oil body extracted from aging sunflower seeds, which on average of 0.13 mmol.kg⁻¹ lipids. Purified oil body preparation extracted from fresh seeds, on the hand showed good oxidative stability, where hydroperoxide level reached only 0.08 mmol.kg⁻¹ lipids.

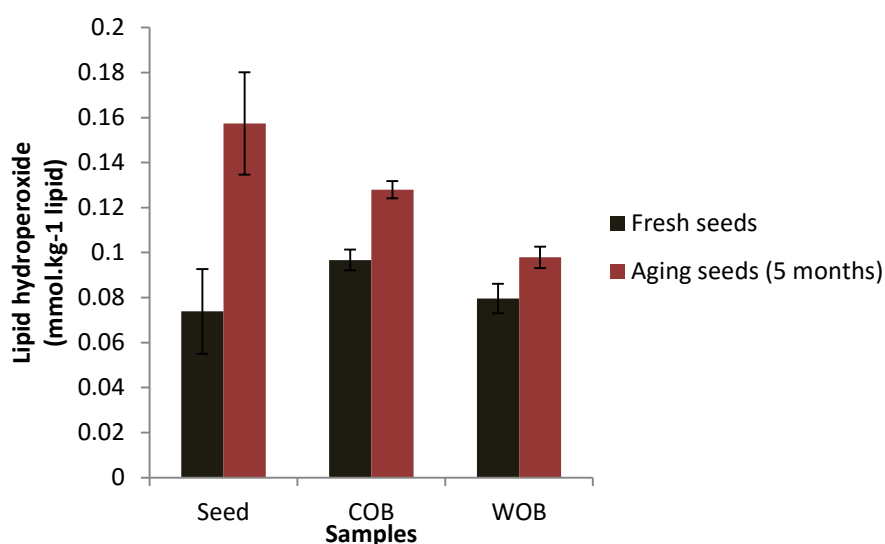


Figure 7:10: Lipid hydroperoxide value of crude and washed oil bodies during seed aging. Error bars represent standard deviation.

However, the formation of hydroperoxide concentration was more pronounced in partially de-hulled sunflower seeds stored at high temperature (40°C). In addition, maximum level of hydroperoxide produced in seeds was almost two times as much as the level of hydroperoxides in fresh seeds. It is unsurprising that bulk sunflower oil showed higher rates of oxidation during storage as compared with crude oil body preparation, where the oil is covered by oleosins and phospholipids. The lipid hydroperoxides formation in crude and washed oil body preparations might be retarded with the presence of oleosin at the lipid-water interface. The presence of oleosin may retard the oxidation by acting as a physical barrier against pro-oxidant or chelating metals by preventing them from gaining access to unsaturated fatty acid inside the oil bodies. Besides, the presence of antioxidant also contributed to the low lipid oxidation during storage.

In order to visualise the correlation between seeds and oil bodies during accelerated aging, Principle Component Analysis (PCA) test was undertaken. PCA was discussed in next section.

7.3 Correlation of Seed Storage and Chemical Properties by Principal Component Analysis (PCA)

Post hoc analysis by Tukey and Dunnett were applied to determine differences ($P < 0.05$) between samples and variables (XLstat Version 2015.1.01). The results are summarised and represented in Table 7.9. One Way ANOVA of the data showed significant differences at $P < 0.05$. In order to determine the significant pairs, a Tukey post hoc was performed.

Table 7:9: Analysis of the differences between control (fresh seeds) and the other categories using Dunnett (two sided) on main chemical composition in sunflower seeds and its oil bodies.

Samples	Lip %	Pro %	α -T	γ -T	C16:0	C18:0	C18:1	C18:2
Aging seeds	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Fresh-COB	Yes	Yes	Yes	Yes	No	No	No	No
Fresh-WOB	Yes	Yes	Yes	Yes	Yes	No	No	No
Age-COB	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Age-WOB	Yes	Yes	Yes	Yes	No	Yes	Yes	No

Lip:Lipid, Pro: Protein, α -T: α -tocopherol, γ -T: gamma-tocopherol, C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid)

Principle component analysis (PCA), in general, allows visual as well as statistical analysis of data which combines multivariate modeling and analysis (Shin *et al.*,2012). In order to verify which variable (sunflower seeds and oil body) were more related with the main chemical compositions (*i.e.* lipid, protein and others), a principal component analysis (PCA) was applied. Principal component analysis (PCA) was applied to the analytical and descriptive ratings in order to determine relationships between sunflower seeds and its oil bodies during storage.

In general, the first Principal Component (PC) contains the most representative information in the data set, while the second PC is the next largest amount of variation accounts. In order to confirm the PCA results, ANOVA analyses were used as a discriminatory tool.

In this work, sunflower seeds and its oil bodies were separated into different group categories and were presented in Table 7.10. Figure 7.11 represents bidimensional of PC1 and PC2 of the tested variable.

Table 7:10: Group classification of sunflower seeds and its oil bodies

Group 1	Fresh seed
Group 2	Aging seed (5 months)
Group 3	Fresh _Crude oil bodies
Group 4	Fresh _Washed oil bodies
Group 5	Aging _Crude oil bodies
Group 6	Aging _Washed oil bodies

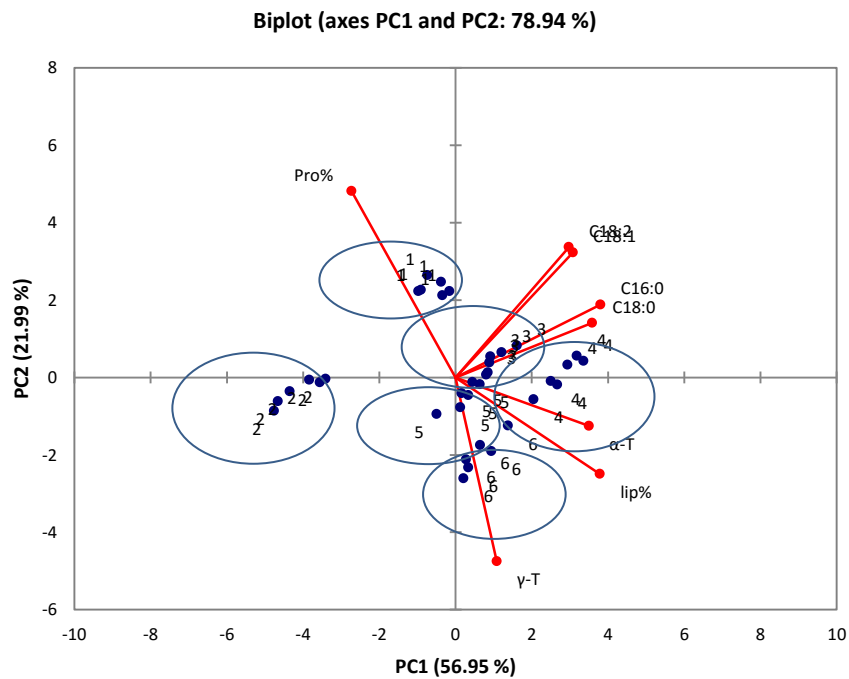


Figure 7:11: Principle Component Analysis (PCA) of sample variable.

In the bi-plot graph (Figure 7.11), resulting from the PCA, showed the correlation of the cluster analysis on the chemical components of the seed and oil bodies into two distinct groups. Based on Figure 7.11, the two principle components (PC1 and PC2) were able to account for 78.94 % (56.95 % by PC1 and 21.99 % by PC2) of the original variability. The results of PC1 and PC2 plots show discrimination between the 6 categories tested. PC1 separated seeds and its oil bodies based on their chemical compositions such as tocopherol content (α - and γ tocopherols), lipid, protein and fatty acid content under different condition (fresh seeds or accelerated). It explained 56.95 % of the total variance. However, PC 2 based on the storage condition of the seeds. It explained 21.99%.

Washed oil bodies (Group 4) extracted from fresh partially de-hulled sunflower seeds has high percentage of chemical composition. Oil bodies at this stage were higher in purity compared to the crude oil body preparation (Group 3). Specifically, the main characteristics of oil bodies at different source of seeds (fresh or accelerated) were shown at extreme right position of the plot explaining the high correlation between these variables.

The protein content was more pronounced in seed (Group 1 and Group 2) rather than its oil bodies. Some of the extraneous protein was removed during the washing treatment therefore; oil body appeared to have less amount of protein. With this regard, they are negatively correlated against each other.

In the middle axis of PC1, Group 4 (washed oil bodies extracted from fresh seeds) presented highest amount of α -tocopherol, and was correlated positively. Group 3 and Group 5 representing crude oil body preparation. Concerning the composition of fatty acid, Group 3 was positively correlated to C16:0 (palmitic acid), C18:0 (Stearic acid), C18:1(oleic acid) and C18:2 (linoleic acid). However,

Group 5, correlated negatively on PC1 axes. Nevertheless, despite detected some differences in individual compounds, the results of nutritional parameter such as fatty acid and tocopherols, were less affected in oil body sample preparations caused by storage.

7.4 Summary of the Results

The storage of sunflower seeds for an extended period of time (5 months) under salt saturated accelerated aging (SSAA) was successfully undertaken. The quality of oil in terms of lipid hydroperoxide was less than the standard set (2.5 mmol kg⁻¹ lipid). The presence of antioxidant components (α -tocopherol and γ -tocopherol) in sunflower seeds is apparent because the seeds had been protected from rancidity during seed aging. Therefore, the results suggest that the seed should be used as fresh as possible in order to obtain high quality of oil bodies.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The primary objective of this research was to obtain high quality oil bodies from sunflower seeds. Sunflower seeds are widely known for its oilseed while sunflower meal, by-product of the oil extraction was used for livestock feed especially for ruminant. In this study, sunflower seed which was partially de-hulled was used throughout the study. Therefore, the potential of this partially de-hulled sunflower seed was observed and is recommended as a potential raw material for oilseed recovery, as it behaves similarly to the de-hulled material. Thus, the data generated in this study, possibly can be used for scaling-up for industrial production.

During oilseed processing, sunflower seed is mechanically pressed to obtain the oil, prior to a de-hulling step to increase the protein level to 30% (based on Sunflower National Association). Hulling is the process of removing hulls from the oilseed to obtain high quality edible oil by the processing of kernels (Gamea, 2013). For the commercial approach, this technique increases the value of the sunflower oil and sunflower meal in the market, depending on variety.

The effect of de-hulling of sunflower seeds have been reported earlier on its quality of oil and yield (Gunasekaran *et al.*, 1988; Parde *et al.*, 2002). It was shown in this study, that during the de-hulling process of sunflower seeds, the impact created by a hammer mill (Glen Creston), followed by separation of the

kernel and seed coat, did not overly damaged the kernels and preserved the oil bodies. If de-hulling had damaged the seeds, therefore, directly damaging the oil bodies. De-hulling is advisable to be carried out however; it is method dependent for each plant materials. It is noted from previous research that *Brassica napus* (hull content 7%) which undergo de-hulling process is high in nutritional value and has health benefits (Batista *et al.*, 2011). Although the de-hulling process was not successful in this study due to the plasticity of the hull which makes it adhere strongly to the kernel, therefore partially de-hulled seeds were shown to be an alternative and suitable substitute. Thus, highlight the new values of partially de-hulled as subject of plant material attributed in oilseed productions.

On the other hand, the potential of partially de-hulled sunflower seeds not only limit as vegetable oil, but also can be used as thermal energy storage (TES) medium for cooking application (Mawire, 2016). Currently, oilseeds phospholipids are one of the major components of lecithin that are by-products isolated during extraction of vegetable oil (Cabezas *et al.*, 2011). In food industry, sunflower lecithin acts as a surfactant (Chen and Zhong, 2015) and also as an emulsifier (Pan *et al.*, 2013) in emulsion systems.

Storage lipids from oilseed plants accumulate in the embryo and endosperm during seed development. They provide source of carbon and energy for germination and called oleosomes or oil bodies. In food applications, their tendency to form stable emulsions in water without the need to add emulsifiers highlight oil bodies as an important ingredients for formulation (Watchatko *et al.*, 2012). Therefore, oil bodies are extracted from various plant materials including seeds, nuts or leaves for potential utilisation such as in food, personal care or pharmaceuticals (Zhou *et al.*, 2016; Shukotu *et al.*, 2014; Batista *et al.*, 2011). A pilot-scale study extraction of soybean oil bodies reported yields up to 93.4 %

(Towa *et al.*, 2011). However, the focus of this investigation was on the integrity of oil bodies recovered from sunflower seeds.

The hypothesis tested was that the quality of the seeds plays a major role in producing high quality oil bodies. Oil bodies exhibit physical stability when the seeds themselves are of high quality. The quality that been discussed in this work relates to the physical and chemical state of the oil bodies, which optimised the suitable temperature and age of seeds. Although the recovery process of oil bodies reported previously seemed easy using flotation centrifugation (Huang, 1997), however observation throughout this project was that it was not straightforward and needed a careful extraction procedure so that, intact and small oil bodies could be obtained. The fundamental of this process of recovery was towards environmentally friendly methods without any interference of toxic solvents during the recovery process.

Initially, seed pre-treatments *i.e.* pre-soaking, are crucial steps before commencing the isolation process of the oil bodies. It was shown that oil body integrity could be altered if high forces during grinding were imposed on the tissue during their recovery. It is speculated that tissue disruption of hard materials *i.e.* seeds requires more force than the disruption of softer materials. As consequence, oil bodies embedded in the cytoplasm could be damaged which may lead to destabilisation when dispersing oil bodies in aqueous medium. It is suggested that pre-soaking is recommended as a standard step prior to wet-milling. In fact, plant materials each have their ideal soaking time but need to be optimised to make sure the tissues is at the best condition (softened) before wet milling. The data generated in this study, demonstrated that the physical state of seeds during wet milling had a significant impact on the quality of the oil body suspension.

The key question of this study was whether or not oil bodies are disrupted during processing and so form hybrid oil bodies with an altered surface chemistry. When oilseeds are crushed in the presence of water, a milky emulsion is generated, but it is uncertain whether this emulsion is composed of intact oil bodies or could be any lipid droplet which is formed through the release of oil from the damaged oil bodies. Then, the oil was stabilised by a mix of natural surface-active materials present in the seeds. The hypothesis tested in this study was that wet-milling hard oilseeds resulted in the breakage of oil bodies and the *in-situ* formation of artefact oil droplets with a physical stability and surface properties distinct from intact oil bodies.

One should assume that if the diameter of the recovered oil bodies is in-line with those seen *in-vivo* in the parent seed, then the recovered dispersion is made up of intact oil bodies. It is possible, however, that artefactual or hybrid droplets, formed during recovery, adopt a similar diameter to the parent organelles, thus, undermining this simple association as proof of the recovery of intact oil bodies. The effect of the recovery regime and post-recovery (downstream) processing on the quality of oil bodies has not been extensively reported. The assumption being that intact oil bodies remains the same size during rigorous washing, but that artefactual or hybrid droplets will undergo a change in size distribution due to a looser association between the surface active materials takeover to stabilise the oil droplets and the interfacial region of the oil.

With regards to the data generated in this thesis, it is evident that removal cell contaminant or extraneous protein by the harshest agent such as 9M urea triggered widespread droplet coalescence. The ability of 9M urea as cleaning agent for crude oil body preparation have been reported earlier with some reporting no loss of sunflower seed oil body integrity (White *et al.*,1998; Lacey *et al.*, 1998; Milichip *et al.*, 1996). However, in 2013, Nikiforidis and his co-worker

observed and reported fragility of oil bodies of sunflower seeds after removal extraneous protein using 9M urea and visualised under advanced microscopy. Intact oil bodies can be recovered if the seeds are suitably plasticised through pre-soaking the seed in an aqueous medium. However, pre-soaking was not performed in earlier report or non-optimised soaking time was conducted (Nikiforidis *et al.*, 2013) prior to wet milling but still, the same observation was obtained as in this study, indicating that 9M urea diminished the hydrophobic effect to form aggregation or coalescence in the suspension or emulsion. Suspension or emulsion instability could limit the use of sunflower seed oil bodies as functional food ingredients.

The work presented in this study showed evidence that pre-soaking is one of the key steps prior to extraction of intact oil bodies. Recent research published on extraction of oil bodies from different type of seeds (sunflower, rapeseed, castor bean, jicama and sesame) at different ranges pH values and for varying washing power (Zhao *et al.*, 2016). All of the seeds were simply soaked in deionised water for 18 hour at 4°C respectively, prior to extraction. In the present study, it was sufficient to soak sunflower seeds (any variety) for 6 hours to soften the seed tissues. Further increasing the soaking time to 18 hours at lower temperature would increase the energy and also time consuming for the next extraction processes.

To best of our knowledge this is the first work that reports in detail how to obtain high quality (in terms of their size distribution, physical and chemical properties) of oil bodies, especially for sunflower seeds. A recommended protocol to produce high quality of oil bodies from sunflower seeds is presented in Figure 8.1. If we now turn to this new protocol and its modification, perhaps intact oil bodies can be recovered from any oil seed species.

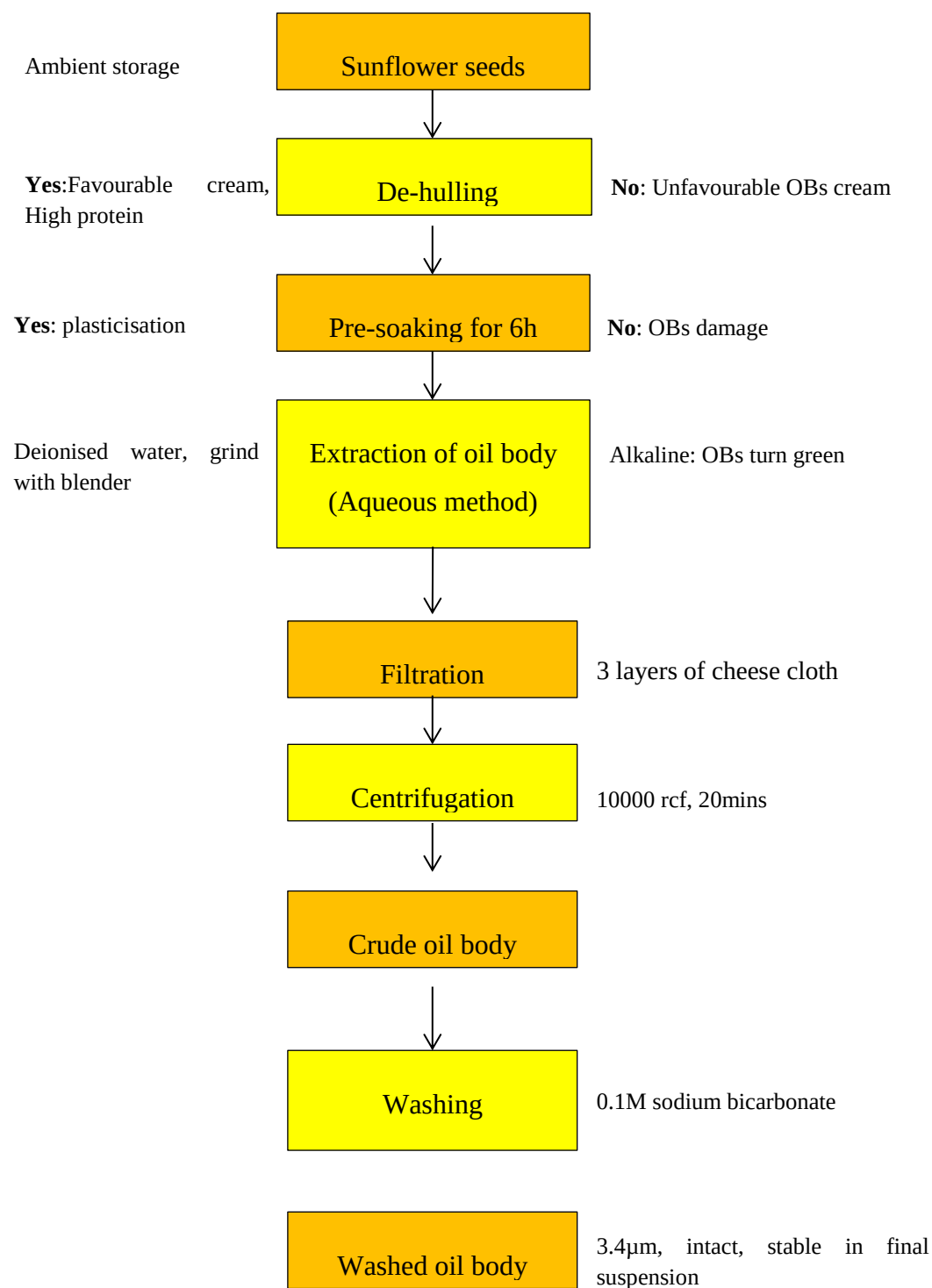


Figure 8:1 A proposed model to produce high quality oil bodies from sunflower seeds.

Oil bodies protect the stored lipids from oxidation and hydrolysis to occur (Fisk *et al.*, 2008). It is likely that unsaturated fatty acid can rapidly oxidise however, the oxidation may occur faster in oil rather than oil bodies. The comparison of fatty acid of total lipids and oil bodies from sunflower seeds and literature was presented in Table 8.1

Table 8:1: Comparison of fatty acid of total lipids and oil bodies from sunflower seeds

Fatty acid	Composition (%)			
	Sunflower oil ¹	COB ²	AlkaliWOB ³	Literature ⁴
Palmitic	6.55	7.40	7.80	8.00
Stearic	4.85	4.70	4.90	5.00
Oleic	21.80	21.40	21.15	20.00
Linoleic	66.90	66.55	66.46	62.00
Saturated fatty acid	11.40	12.10	12.70	13.00
Unsaturated fatty	88.70	87.95	87.61	82.00

¹Extracted from partially de-hulled sunflower seeds by Bligh and Dyer (1959)

²Recovered with deionised water, 7500 rcf, 20mins

³Washed repeatedly with 0.1M sodium bicarbonate

⁴Adapted from Fisk (2007)

The results presented in this work showed that high quality of seeds were used and was reflected in the quality of oil bodies inside the seeds. In addition, oleosin (protein) located on the surface of oil bodies acts as a physical barrier to

protect oil body TAG core and prevent any contact with lipase. In total lipids, unsaturated fatty acid constitutes more than 80% of the fatty acid glycerides of oil bodies. The amount of unsaturated fatty acid obtained in the literature is lower than the present study and could be due to the nature of sunflower seeds (non-oil seed type) used to recover the oil bodies.

In this study, the integrity of oil bodies was tested under extreme storage condition under controlled condition (Salt saturated accelerated aging). This condition (SSAA) was more systematic than conventional methods and was suitable to be adapted for small seeds. Although considerable amounts of literature have been published on the storage of sunflower seeds little has been reported on oil bodies. This work was carried out to investigate the quality of oil bodies during seed aging. The data generated in this work provides an insight into the structure and functionality of oil bodies.

In order to maintain the quality of oil bodies, it is important to know the amount of free fatty acid in the sample therefore; formation of free fatty acid was analysed. It was noted that crude and washed oil body preparations contained less free fatty acid (FFA) than bulk oil from seeds during seed aging. Free fatty acid formation is responsible for lipid deterioration in oil bodies however, relatively low FFA levels, compared with the oil extracted from the same seeds, was recorded in this study. The possible explanations for obtaining low level of FFA in oil bodies rather than bulk oil are as follows:

- a) FFA are derived from lipids other than those in the oil body
- b) The nature of the sample somehow binds the FFA and prevents it from being observed in the assay performed.

Tocopherol can increase oxidative stability of lipids through increasing induction period by scavenging lipid peroxy radicals (Frankel, 2005). One of the intrinsic components of oil bodies assessed in this study was vitamin E (tocopherol). The presence of tocopherol in oil bodies support the protection of polyunsaturated fatty acid (PUFA) against oxidation in sunflower seeds (Fisk *et al.*, 2006). The study of tocopherol was previously reported on de-hulled sunflower seeds (Fisk, 2006), however so far no data is available for oil bodies recovered from partially de-hulled sunflower seeds. The concentrations of tocopherol in the present study and in the literature were compared in Table 8.2.

Table 8:2: Comparison of tocopherol content on recovered oil bodies from sunflower seeds.

Sunflower seeds	Pre-soaking	Washing regime	Tocopherol (mg/kg) lipid	
			α -tocopherol	γ -tocopherol
Dehulled ¹	No	9M urea ²	368.00 $\pm$ 50.00	1.93 $\pm$ 0.23
Partially de-hulled	6 hours	0.1M NaHCO ₃	491.60 $\pm$ 21.20	36.30 $\pm$ 3.00

Note: ¹ Adapted from Fisk *et al.*, 2006

²Oil bodies was washed once with urea, and repeatedly washed with water

³Oil bodies was repeatedly washed with 0.1M sodium bicarbonate (NaHCO₃)

The concentration of tocopherol in the present study (pre-soaking, alkali-washed) was higher than in the literature (without soaking, urea-washed). In the present study, the washed oil body preparation contains approximately 491.6 mg/kg of α -tocopherol and 36.3 mg/kg of γ -tocopherol. Fisk (2007) introduced urea washing step to purified crude oil bodies and obtained less tocopherol than the present study. Since tocopherol appeared to be physically associated on oil bodies *ex vivo* therefore, some of the extraneous protein enriched in tocopherol might wash away during the purification process. It also provides an evidence that 9M urea wash is too harsh for sunflower seeds. Therefore, the results indicate that sunflower seeds that undergo pre-soaking step and washing with 0.1M sodium bicarbonate were more enriched in tocopherol than the oil bodies extracted from 9M urea. Thus, partially de-hulled is comparable with de-hulled sunflower seeds and alkali-washed obtained more tocopherol than chaotropic washing regime.

The presence of tocopherol on the surface of oil bodies contribute to the stability of oil bodies against oxidation, although the sunflower seeds were exposed to extreme temperatures during storage. The robustness of oil bodies under environmental stress, affect the seeds without damaging the oil bodies housed within the cytoplasm. The phytochemical present on the surface of oil bodies suggest a protective role against oxidation *in vivo*. Overall, the work presented in this thesis identifies major factors that affect the quality of oil bodies recovered from oilseeds. Through this study partially de-hulled sunflower oil bodies were found to be: extremely stable in the final suspension, contain high level of tocopherol as antioxidant and robust subcellular organelles during environment stress.

8.1 Conclusions

From all the chapters comprising this research project a number of conclusions can be drawn:

- De-hulling process did not causing impairment on oil bodies.
- Cold storage (4°C) damages the oil body particle size, especially after washing (9M urea).
- Ambient temperature was suitable to store seeds rather than cold storage.
- Seed pre-soaking allows water uptake; the plasticising or softening effect reduces the damage to oil bodies during milling.
- The optimum soaking time for sunflower seeds is 6 hours, and 12 hours for oil seed rape: pre-soaking times may need to be optimised on a batch by batch basis.
- Deionised water is suitable grinding medium to extract oil bodies of partially de-hulled sunflower seeds.
- The washing regime produces clean preparation and remove cell contaminant and extraneous protein.
- Washing with deionised water was not efficient to remove extraneous protein and cell contaminant. Washing with 9M urea was effective in removing extraneous protein, but damage the oil bodies *ex vivo*. Washing with 0.1M sodium bicarbonate successfully removes the extraneous protein and cell debris but preserved the biological nature of oil bodies *ex vivo*.
- The smallest oil body size was observed after pre-soaking for 6 hours then washing with sodium bicarbonate.
- The storage of sunflower seeds at extreme temperature (40°C) increased the free fatty acid and lipid peroxide values of bulk oil.
- Hydrolysis of TAG to FFA was detected in fresh and aging seeds.
- Storage sunflower seeds for 5 months increased the lipid peroxide value.

In detail therefore: This study was carried out to establish protocols for obtaining intact oil bodies from sunflower seeds. Pre-soaking prior to wet-milling was recommended as a standard step but was dependent on seed type and quality. This suggests that pre-soaking allow penetration of water through cell matrix and leading to plasticisation of seeds.

It was shown in this work that, if un-soaked or dry seeds are ground in the presence of water (or aqueous medium), an effective dry-grind takes place and this leads to extensive damage to oil bodies or oleosomes. On the other hand, pre-soaking seeds allowed water hydration of the seed tissues, causing plasticisation or softening of the seed, resulting in reduced damage to oil bodies. The seed pre-soaking required varies depending on the plant species. At least 6 hours of soaking is recommended for sunflower seeds. At this point, the sizes of oil bodies are intact and small. This study highlights the importance of a carefully determined extraction procedure to produce high quality oil bodies from sunflower seeds.

Despite obtaining high lipid containing? and clean oil bodies preparations when washing with 9M urea, it could be described as unsuitable due to its effect on oil body size. In this thesis the disadvantages have been demonstrated; the existing protocols using 9M urea for purification increased the size oil bodies in sunflower seeds and affect the integrity of oil bodies causing destabilisation in the final suspension or emulsion.

Storing sunflower seeds in low temperature (4°C) and employing extraction processes using alkali solution may causing physical changes (greenish colour) and increase the size of oil bodies. Therefore, sunflower seeds should not be stored for too long before extraction procedures are conducted. The protocols for sunflower seeds (harvest treatment, age, storage temperature and time,

soaking, extraction medium and washing medium) may affect the properties of oil bodies. The fact that based on this study, different seeds would require different treatments and optimisation is recommended before commencing extraction process.

Therefore, overall it can be concluded that pre-soaking shows promise in improving the extraction of oil bodies of sunflower seeds with low temperature of distilled water. To obtain purer crude extract with high physical and chemical quality such as 0.1M sodium bicarbonate as a washing solution is recommended.

8.2 Future work

As normally the case in research of this nature, there are many areas that can be identified that warrant further investigation as well as new areas that have yet to be explored. Based on the experience and findings gathered from the current study, several recommendations can be made. Future work may encompass the following studies:

- Is a change in oil body size during washing a robust diagnostic of oil body integrity?
- Can one use advanced imaging techniques to visualise the difference in the surface composition of intact and hybrid oil bodies?
- Are enzymes activated during pre-soaking, and if so, do they affect the quality of recovered oil bodies?
- Are FFAs tied up in the oil body preparations, preventing them from being released in the assay and so leading to erroneous results?

- If aged seed retain high quality oil bodies, does aging inactivate enzymes that may be active during pre-soaking of seeds?
- If oil bodies are not affected during aging, what lipids in the seeds are being hydrolysed and oxidised?
- Why are sunflower seed oil bodies susceptible to urea-induced damage, and oilseed rape oil bodies appear not to be?
- Can the pre-soaking time be optimised to minimise nutrient loss and enzyme inactivation?

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