# Physicochemical Characterisation of Sunflower Seed Oil Bodies Ex-Vivo

By Ian Fisk, BSc (hons)

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# Table of Contents

# List of Figures

# List of Tables

# List of Equations

# Abstract

Oilseeds store energy as triacylglycerides during periods of dormancy in preparation for germination and the early stages of development. The triacylglyceride is stored in discrete organelles termed oil bodies. Oil bodies are formed during the synthesis of neutral lipids within the bilayer of cellular endoplasmic reticulum (ER); as lipid is synthesised it forms droplets of oil that swell distending the ER membrane and at a critical diameter separate from the ER by vesiculation forming independent organelles. These organelles are structurally stabilised by a phospholipid monolayer originating from the ER and the addition of highly amphiphilic oleosin proteins.

Oil bodies have been shown previously to be extremely stable organelles that can be easily extracted and purified from oilseeds; our aim was to develop an understanding of the physical and chemical properties of sunflower oil bodies ex-vivo prior to their subsequent use in commercial products. Several novel findings were elucidated through this work: oil body phytochemical composition, their physical and oxidative stability and their ability to store and deliver flavour compounds.

It was hypothesised that tocopherol is tightly associated with sunflower oil bodies. This was tested by recovering oil bodies from sunflower seed and washing them to remove extraneous proteins and associated phenolic compounds. Tocopherol remained with washed oil bodies (392 mg tocopherol.kg$^{-1}$ oil body oil) and this population of tocopherol represented 38% of the total seed tocopherol. It was hypothesised that this high tocopherol concentration and its intrinsic association to oil body structures would contribute to an increased level of oxidative stability.

Sunflower seed lipids were significantly more resistant to thermally induced oxidation when stabilised in oil body suspensions compared to sunflower oil emulsions stabilised by a range of commercial emulsifiers (sodium dodecyl sulfate, polyoxyethylenesorbitan monolaurate (tween 20) and dodecyltrimethylammonium bromide). Oxidative stability was assessed

through lipid hydroperoxide concentration and the concentration of headspace hexanal. Maximum lipid hydroperoxide concentration in surfactant stabilised emulsions after 8 days at 45°C ranged between 26 and 333 mmol lipid hydroperoxide.kg$^{-1}$ oil whereas lipid hydroperoxide concentrations in oil body suspensions did not exceed 12 mmol lipid hydroperoxide.kg$^{-1}$ oil. In addition there was no development in oxidative rancidity over the 8 day storage trial of oil bodies stored at 5°C.

The composition of phospholipids in a range of oil body preparations was assessed; purified oil bodies contained principally phosphatidylcholine (91%) and a smaller fraction of phosphatidylethanolamine (9%). Less purified preparations contained other phospholipid species; the presence of which was explained by contamination with either non-intrinsic cellular phospholipids or phospholipase D that catalysed the breakdown of phospholipids to phosphatidic acid.

Mechanisms and the extent of oil body physical stability were assessed using charge analysis and resistance of oil body preparations to changes in temperature and pH. Oil bodies are stabilised by a combination of steric hindrance and electrostatic repulsion provided by the surface proteins and phospholipids. Oil bodies had a zeta potential of -30mV at neutral pH and the surface charge was pH dependant with an apparent isoelectric point of between pH3 and pH6 was calculated from electrophoretic mobility, streaming potential and creaming stability measurements. Purified oil bodies were physically stable to thermal stresses up to 45°C for 2 days, although less purified preparations lost structural integrity at temperatures above 25°C.

When assessed for their ability to delivery flavour molecules, oil bodies had comparable bulk phase properties to artificial emulsions stabilised by tween 20. Oil bodies did show a greater rate of flavour delivery during headspace dilution, when compared with the model artificial emulsions, suggesting commercial benefits may be gained through the incorporation of oil bodies into commercial emulsions.

The key findings of this work are that oil bodies are extremely stable organelles that are resistant to thermal stress and physical processing. When lipid is stored within oil bodies it has greater resistance to the onset of lipid oxidation which may be explained by the intrinsic association of phospholipids, proteins and phytochemicals (vitamin E).

# Acknowledgements

# Abbreviations

| | |
|---|---|
| DAG | Diacylglyceride |
| dwb | Dry Weight Basis |
| ER | Endoplasmic Reticulum |
| FFA | Free Fatty Acid |
| IEF | Isoelectric Focussing |
| PA | Phosphatidic acid |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| pI | Isoelectric Point |
| PI | Phosphatidylinositol |
| pK | Acid-Base Ionization (or Dissociation) Constant |
| PS | Phosphatidylserine |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SW-OB | Salt-Washed Oil Bodies |
| TAG | Triacylglyceride |
| TEM | Transmission Electron Microscopy |
| UW-OB | Urea-Washed Oil Bodies |
| wwb | Wet Weight Basis |
| WW-OB | Water-Washed Oil Bodies |

# 1. Introduction

All biological organisms are classically defined as carbon-based, although some researchers (Brack 1993; Cosgrove and Rijsberman 2000) suggest that they could be defined as water-based as water is a ubiquitous component of all biological systems and essential during formation and development. This study focuses on the understanding and exploitation of biological lipid reserves. Due to the low solubility limit of lipid in water and the predominance of water as the bulk component in biological systems any specific internalised regions high in lipid concentration will form an emulsion (oil droplets suspended in water). This emulsion may be in the form of one droplet or a large number of droplets suspended throughout the structure. There are many examples of biological emulsions: lipoproteins deliver lipids and nutrients within animal blood to various biological tissues, they are structurally stabilised by phospholipids and proteins and range in size from 8 to 80nm; milk fat globules (MFG) deliver nutrients and energy to developing infants, they also are stabilised by proteins and phospholipids and are 0.2-20µm in diameter (Mulder and Walstra 1974); oil bodies found in desiccation tolerant oilseeds are stabilised by oleosin proteins and phospholipids and are 0.2-2µm diameter (Tzen, Cao et al. 1993).

Given time, native emulsion droplets will coalesce (fuse) forming larger droplets that will float to the surface to form a layer of oil above the water phase. The free energy of an emulsion is a function of the interfacial surface area between the oil and water and there is an entropic drive for the emulsion droplets to coalesce. Emulsions although thermodynamically unstable, may exist in a stable metastate. This is achieved though the addition of surface active agents that may produce a kinetically stable system.

The presence of surface active agents (phospholipids, proteins, carbohydrates) on the surface of an emulsion will increase the activation

energy required for full phase separation. These emulsions are therefore transiently stable.

Additional compounds may also be present that may alter the density of an emulsion droplet or the viscosity of the continuous phase therefore controlling the rate of gravitational separation (creaming). In the example of lipoproteins, MFG and oil bodies, all contain proteins and phospholipids which kinetically stabilise the emulsions through a range of mechanisms.

There are many industrial applications of emulsions, some of which may be encompassed under the broad topics of food applications, pharmaceutical drug delivery and paint formulations. Others examples include bitumen, insecticides, hair and skin creams and the use of transportation emulsions in mineral processing. Food emulsions vary in size from 1mm to 100nm and include oil in water (o/w) water in oil (w/o) and more complex multiphase (e.g. o/w/o) forms.

## *1.1. Oilseed Oil Bodies*

All plants have metabolic and structural requirements for lipids of varying classes. Triglycerides are a major plant lipid class and are principally used for long term energy storage. Phospholipids are required in cellular membranes to maintain membrane structure. Cuticular lipids, such as waxes, may be present to control water migration between cells and the external environment; other functional lipids may include cellular signalling molecules, metabolic intermediates and protective lipids, such as tocopherol and flavonoids. Lipid classes occur over a range of concentrations depending on the species of the plant and the environmental influences during its development. In sunflower seed, the plant tissue of interest in this work, the principle lipid component is triacylglyceride (97% of lipid component), with diacylglyceride (1.2%), phospholipid (1.3%) and free fatty acids (0.4%) also present but at lower concentrations (Corbineau, Gay-Mathieu et al. 2002).

Neutral lipid accumulates as droplets within the cytosol of plant cells. These neutral lipid droplets are probably ubiquitous components of all plant cells but are found in highest concentration in the seeds of oilseeds. Oilseeds accumulate neutral lipid in preparation for seed dormancy as cytosolic droplets. The presence of these discrete cytosolic droplets was first identified in 1880 by Hanstein who identified microsomes in non seed tissue; these cellular plastids are now defined as oil bodies. Cytosolic oil bodies normally contain a central core of neutral lipid and an outer coat of both phospholipid, in the form of a monolayer, and surface-active proteins (oleosin and caleosin). Oil bodies have been found in a range of plant tissue, including tubers, leaves, roots, root nodules, anthers, pollen grains and bulbs (Huang 1996; Murphy 2001), but the highest concentration is found in oilseed seed cotyledons.

Oilseeds can accumulate large concentrations of neutral lipid (40-45% in sunflower seeds) which is stored in oil bodies in a range of structures. In sunflower plants the major zone of oil body deposition is the seed embryo cotyledon and axis (Murphy 1992; Huang, Tzen et al. 1993). Other plants also accumulate oil bodies, for example in cereals the major area of accumulation is the embryo scutellum and the aleurone layer (Pollard, Mckeon et al. 1979; Qu, Wang et al. 1986).

### 1.1.1. Oil Body Composition

Oil bodies are primarily composed of neutral lipid (94-98% w/w) with smaller fractions of surface active phospholipids (0.5-2% w/w) and proteins (0.5-3.5% w/w) (Tzen, Cao et al. 1993). The surface active proteins are present to reduce the droplet size of the oil bodies, facilitating an increased rate of energy release during germination. The proteins also stabilise oil bodies from osmotic and physical stresses principally during the desiccation-rehydration cycle prior to and after seed dormancy. The density of proteins and phospholipids is therefore dependent upon the seed type and the

environmental influences that the seed is exposed to during dormancy. Seeds that stabilise themselves by desiccation during periods of dormancy require a greater level of stabilising proteins to maintain the structural stability of oil bodies during the desiccation-rehydration cycle.

#### 1.1.1.1. Protein

Oleosin is the major protein associated with oilseed oil bodies (oleosin has a molecular weight of 15kDa-24kDa and can contribute up to 10% of the seed cell protein (Murphy 2001)). Oleosin is a highly amphiphilic protein that is strongly bound to the surface phospholipid membrane of the oil body. Oleosin's major characteristic feature is the presence of a central hydrophobic domain of 68-74 residues containing a proline knot motif. Oleosin can be schematically visualised as a T-shaped protein (Figure 1-1) with its hydrophobic central core extending into the lipid core of the oil body and the two hydrophilic terminal domains exposed on the surface. The conformation of the central core has been described as either anti-parallel alpha-helices that extend into oil body core (Vance and Huang 1987); a beta-strand running under the surface of the oil body (Murphy, Keen et al. 1991) or an anti-parallel beta-strand forming a loop that enters the oil body neutral lipid core (Tzen, Lie et al. 1992).

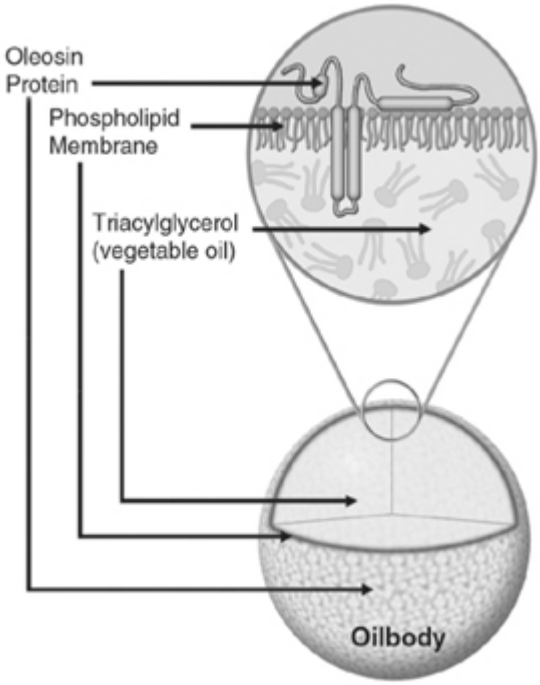



**Figure 1-1 Schematic representation of oleosin topology at the surface of an oil body (Markley, Nykiforuk et al. 2006)**

The N-terminal tail lies outside of the oil body and is required for oleosin targeting to the endoplasmic reticulum (ER) during oleosin synthesis (this is further discussed in section 1.1.2) (van Rooijen and Moloney 1995; Abell, Holbrook et al. 1997; Beaudoin, Wilkinson et al. 2000). The C-terminal tail is not required for oleosin targeting to the ER, but is necessary for the correct orientation of oleosin in the ER membrane and consequently oleosin orientation on the oil body surface (Hills, Murphy et al. 1989; Loer and Herman 1993; van Rooijen and Moloney 1995; Beaudoin, Wilkinson et al. 2000). The three structural domains of oleosin maintain the association of the protein to the oil body surface stabilising the oil body through a reduction in the surface tension. Millichip (1996) suggests that oleosin occupies 4% w/w of the oil body structure although other authors suggest lower values of 0.5-3.5 % (w/w) (Tzen and Huang 1992; Tzen, Lie et al. 1992; Tzen, Cao et al. 1993). In oilseeds oleosin can contribute up to 8% of the total seed protein (Tzen, Cao et al. 1993).

Caleosin protein is also found on the surface of oil bodies, although structurally similar to oleosin it has the ability to bind to calcium. Caleosin shall not be addressed further in this work as it is only present in low concentration. Excellent reviews by Fransen (2001) are available that discuss caleosin biosynthesis, functionality and topology.

In addition to oleosin and caleosin, there are some suggestions of other proteins being associated to oil bodies, these may be remnants from the endoplasmic reticulum membrane or may be specific to oil body functionality. Tzen (1997) suggests that there may be associated ER membrane proteins, glyoxisome receptors, inactive lipases, or lipase receptors present in the membrane. It is hypothesised that these residual proteins are lost by current oil body purification techniques; therefore, it cannot be conclusively explained whether these are integral components of oil bodies or artefacts of the extraction process.

#### 1.1.1.2. Phospholipid

Phospholipid is an integral component of oil bodies; in species with low concentrations of oil body associated proteins (recalcitrant oilseeds), phospholipids stabilise the oil body from coalescence. In species with elevated levels of oil body associated protein, protein is the major stabilising component and the presence of phospholipids act primarily to provide an environment for the protein to locate. The protein and phospholipid molecules interact due to the charge and polarity distribution within the molecules (Vance and Huang 1987; Qu and Huang 1990). In reconstituted oil bodies, oil bodies formed from neutral lipid stabilised with phospholipid and oleosin are stable, whereas oil bodies formed without phospholipid and those without oleosin are unstable and rapidly coalesce (Tzen and Huang 1992) illustrating the importance all three constituents confer to oil body stability.

Oil body phospholipids originate during oil body biogenesis (discussed further in section 1.1.2) and current literature suggests that the phospholipid content

of oil bodies from diverse species ranges between 0.5% and 2% on a dry weight basis (Tzen, Cao et al. 1993).

Tzen (1993) details phospholipid composition of a range of seed oil bodies including rapeseed, mustard, cotton, flax, maize, peanut and sesame. Tzen concludes that the major phospholipid species is phosphatidylcholine (PC) (41%-64.1%) with phosphatidylserine (PS) (18.3-33.1%), phosphatidylinositol (PI) (6.9-20.9%) and phosphatidylethanolamine (PE) (2.8-15.8%) also being present. The presence of PC as the major phospholipid and PE and PI as minor components was later confirmed by Millichip (1996) who suggests for sunflower oil bodies that the major phospholipid fractions are PC (79%), PE (13%) and PI (8%) although Millichip did not find the presence of PS in any oil body preparations, confirming previous work on safflower and maize oil bodies (Millichip 1995).

### 1.1.2. Biogenesis and Functionality in Vivo

Oil bodies are formed by vesiculation from the endoplasmic reticulum (ER) of the cell. Triacylglyceride (TAG) is synthesised by TAG biosynthesis enzymes, such as acyltransferases, within the membrane of specific domains of the ER (Herman 1987; Whitfield, Murphy et al. 1993; Lacey and Hills 1996). TAG then accumulates within the ER phospholipid bilayer and consequently forms a separate globular domain of neutral lipid, this area of hydrophobicity attracts oleosin proteins synthesized in association with the rough ER. The presence of oleosin reduces the surface tension and allows the swollen ER to bud and form independent organelles, classed as oil bodies. The targeting of oleosin proteins to oil bodies is principally due to the highly hydrophobic 70-residue central domain. There are some suggestions that the association of oil bodies to the ER is dynamic and that oil bodies can re-fuse with the ER membrane to undergo further TAG modifications prior to seed maturation (Kohn, Hartmann et al. 1994; Stobart, Mancha et al. 1997).

Oil bodies are extremely stable organelles in-vivo. In oilseeds that are desiccation tolerant (orthodox species: Helianthus Annus L., Sterculia setigera Del., Brassica napus L.) elevated levels of oleosin protein contribute oil body stability during the desiccation-rehydration cycle. In recalcitrant species (oilseeds that are desiccation sensitive and generally do not experience desiccation during their life cycle: Theobroma cacao L., Quercus rubra L.) low levels of oleosin protein are present and consequently oil bodies on rehydration coalesce to form large oil droplets that result in a loss of cellular integrity (Leprince, van Aelst et al. 1998). The loss of cellular integrity only occurs during the rehydration phase and not during the process of seed desiccation (Berjak and Pammenter 2003).

#### 1.1.2.1. Functionality in Vivo

The main biological role for oleosin proteins on the surface of oil bodies is to stabalise the neutral lipid in small diameter droplets for storage in preparation for germination. These droplets are found to be extremely physically stable and resist elevated temperature, cold shock, desiccation and rehydration. There were previous suggestions that oleosin was involved in the biogenesis and mobilisation of oil bodies, but there is no evidence for this and recently researchers have dismissed this theory (Murphy 2001). The surface covering of oleosin also protects the oil body from enzymic degradation by phospholipase A2 and phospholipase C.

#### 1.1.2.2. Current Oil Body Surface Model

The current oil body model as proposed by Tzen (Tzen and Huang 1992; Tzen, Lie et al. 1992) for maize oil bodies suggests that the surface of an oil body is stabilised by the interaction of proteins (principally oleosin) and phospholipid species. The two surface active species (phospholipid and oleosin) interact between species and interact with themselves. The surface concentration of each species is the thermodynamic minimum required for stabilisation. Tzen's model proposes a mathematical model of the surface of

oil bodies based on the surface density of phospholipids and the experimentally derived ratio of protein and lipid concentration.

Tzen's model proposes that the oil body surface contains thirteen phospholipid molecules for each oleosin molecule, with 3/5 of the oleosin molecule protruding from the surface to stabilise the oil body by electrostatic repulsion and steric hindrance; and 2/5 of molecule embedded in the neutral lipid core, achoring the oleosin molecule to the surface and stabilising the oil body.

## *1.2. Physical Characterisation of Emulsions*

Emulsions are thermodynamically unstable, given time any emulsion will separate into two phases unless there is a physical mechanism preventing coalescence. Emulsion instability can be generally separated into two separate mechanisms: the coalescence, or joining, of two or more emulsion droplets to form one larger droplet; and creaming when an emulsion separates into regions of different densities. Both of these processes may occur independently or concurrently with each other and can be controlled and manipulated, for example creaming can be controlled by the addition of density modifiers or the addition of viscosity modifiers to reduce the rate of movement and speed at which creaming occurs. Coalescence can be controlled by preventing oil droplets coming into contact with each other, this can be achieved through the presence of small molecule surfactants that firstly stabilise the oil-water interface reducing the thermodynamic drive to coalesce, and secondly may repel the oil droplets from each other, preventing the droplets from touching and reducing the potential for coalescence.

The stability conferred by surfactants to oil droplets can be achieved though several different mechanisms. The presence of a surface charge will repel droplets; the presence of long chain molecules on the surface may contribute

steric hindrance (a physical or structural protection) to coalescence through physical or osmotic barriers.

### 1.2.1. Surface Characterisation

#### 1.2.1.1. Isoelectric Point

When charged species are exposed to media of varying pH, their charge varies due to binding or release of hydrogen ions. In media of a specified pH the binding of hydrogen ions will result in the species having a net charge of zero; although within the structure there may be charged regions, the number of positive and negative regions cancels each other out. This pH is defined as the isoelectric point of the species.

In an emulsion, the isoelectric point is critical to its colloidal stability. For an oil body suspension which is stabilised by surface charge, loss of charge will result in oil body aggregation. Knowledge of the isoelectric point of a charged electrostatically stabilised emulsion is critical for design of future applications as it will impinge on the breadth of possible applications or may offer novel processing-induced functionality (exploitation of aggregation as a novel method of oil body purification).

Two methods of assessment of surface charge and isoelectric point are discussed here: streaming potential difference and zeta potential.

#### 1.2.1.2. Streaming Potential Difference

The technique of streaming potential difference can be used to measure the surface charge of a colloidal system. In brief, a colloidal suspension is bound to a polystyrene piston that oscillates in cloud of counter ions. As the piston oscillates the counter ions move to counter the movement of the charged colloidal particles, the movement of counter ions is measured by the changing potential difference across the measurement cell.

If this technique is applied to an oil body suspension, the oil bodies will attach to the polystyrene piston and, upon oscillation, a potential difference will be formed across the aqueous media. With the use of known amounts of polyelectrolyte a measure of surface charge density can be calculated, or by titrating with dilute hydrochloric acid and measurement of the pH, a point of zero streaming potential can be recorded. This pH is defined as the isoelectric point by streaming potential difference and is related to the true surface charge of an oil body suspension.

The method of streaming potential difference although measuring fundamentally the same phenomena (surface charge) as zeta potential (discussed further in section 1.2.1.3) does so by a different mechanism. The Helmhotz-Smoluchouski (H-S) equation defines the relationship between streaming potential difference and that of zeta potential. The relationship is a function of many factors including the pressure drop applied to the solution, the dielectric constant of the suspension, the viscosity of the aqueous solution and the geometry of the measurement cell (Levine, Marriott et al. 1975).

#### 1.2.1.3. Zeta Potential

Zeta potential is a measure of fundamentally the same physical phenomena as streaming potential, it also measures hydrodynamic mobility but uses a measurement cell of known geometry with accurate blanks. This allows a more accurate measurement of surface charge.

Zeta potential is calculated by placing a sample of emulsion in a known geometry measuring chamber then applying an oscillating voltage across the chamber, any suspended particles or emulsion droplets that are charged will move opposing the charge flow of the applied voltage. The rate of movement of the particles can be used, with prior knowledge of the solution's viscosity and conductivity, to calculate a measure of the surface charge.

When a charged object is present in suspension, counter ions from the continuous phase will migrate to the surface forming an immobile layer of charged species and interfacial material. This layer is not affected when the charged species is moved relative to the continuous phase (as during measurement of zeta potential and streaming potential difference). This immobile layer is defined as the stern layer, the extremity of which is termed the shear plane. The radius of the hydrodynamic shear plane is the true hydrodynamic radii of the species and accounts for the true electrophoretic charge of the species as measured by zeta potential. The surface charge at the stern layer is lower than at the true surface of the colloid and is termed the zeta potential (shown in Figure 1-2).

The thickness and viscosity of the stern layer is dependant upon many factors. If large amounts of fibrous proteins or large adsorbed molecules are present on the surface they may entrap further charged ions leading to an increase in the thickness of the stern layer. Large numbers of divalent counter ions in solution may compress the stern layer, leading to aggregation (Wang 2004).

Surface charge is highest at the surface, but decreases, as shown in Figure 1-2, from the true surface charge ($\psi_o$) to the surface charge outside the shear layer ($\psi_\delta$) as measured by zeta potential ($\zeta$).

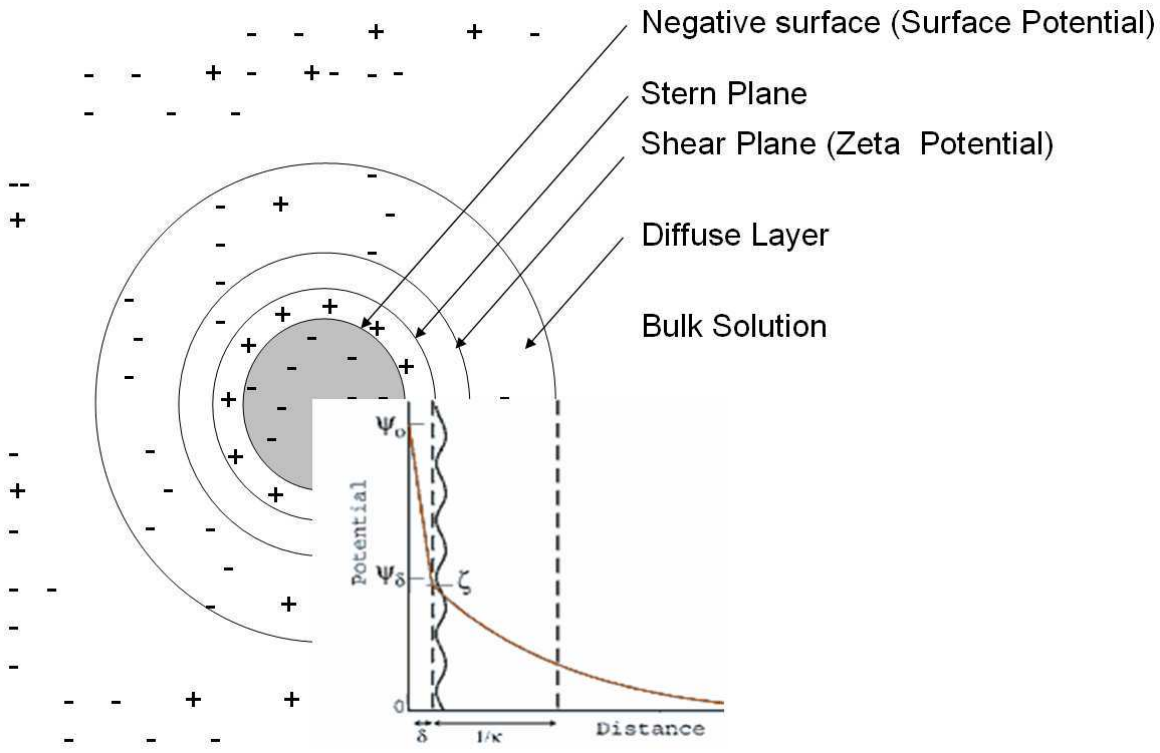



**Figure 1-2 Illustration of surface charge reduction with distance from a charged species (as proposed by the electrical double layer theory (Lyklema 1993))**

### 1.2.2. Stability Assessment

Oil bodies are extremely stable organelles; they are stable in a range of solvents, elevated temperatures and media of varying pHs.

Murphy (1989) showed that rapeseed oil bodies are stable when suspended in a range of detergents (SDS (sodium dodecyl sulphate), DOC (sodium deoxycholate), CHAPS (3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid)) used at greater than their critical micellar concentration (detergent:protein 5:1 w/w). SDS is able to solubilise oil body associated protein but only when elevated temperatures are used. Tzen (1992) confirmed this and showed the presence of small amounts of SDS increases oil body stability.

Chen (2004) detailed the resistance of oil bodies to sonication, and confirmed that oil bodies can be reformed after defatting to form stable emulsions that

are stable to elevated temperatures up to 50°C for 30 minutes. This was confirmed by Peng (2003) who showed that sesame seed oil bodies were stable up to 50°C and their thermostability could be further extended to 90°C by the addition of cross-linking agents such as genipin or gluteraldehyde.

Peng also showed that if the pH of a suspension of oil bodies is varied, the oil bodies may aggregate but do not coalesce further illustrating the intrinsic stability of oil bodies. This is again detailed by Valencia-Turcotte (Valencia-Turcotte and Rodriguez-Sotres 2001) who showed that freeze dried oil bodies can be rehydrated although no stability assessments were carried out.

### *1.3. Oxidative Stability*

Although oil bodies have been studied extensively for their fundamental biochemical properties and more recently for their potential as a commercial pharmaceutical product (Deckers 2001), it is not known if oil bodies recovered from oilseeds are stable against lipid oxidation and the development of oxidative rancidity.

Oil bodies contain a number of potentially antioxidant compounds that combined could offer a significant level of protection to oxidation. These may include tocopherol and tocotrienols; proteins; phospholipids and residual compounds present as bi-products of cellular metabolic activities; an example of which is the lipoxygenase pathway.

The presence of proteins associated with oil bodies will play a crucial role in oil body oxidative stability. Proteins present may include pro-oxidant lipoxygenase enzymes, anti-oxidant metal ion chelator and charged species such as oleosin that could repel or attract metal ions. Phospholipids are also closely associated with oil bodies and are an integral part of their structure. Oxidation chemistry typically suggests that interfacial lipids are the principle species to undergo oxidation and that the formation of phospholipid hydroperoxides is often a point of initiation of oxidation.

Spatial location and availability of oxidatively active molecules will also affect the overall oxidative stability of oil bodies. Factors may include the interaction of oxidatively active compounds and their physical location; for example, the interaction of surface proteins and surface phospholipids results in a net negative charge at neutral pH. The voltage of this surface charge is dependant upon a range of factors which include media pH and the presence of contaminating proteins (Fisk, White et al. 2005). A net negative charge will attract prooxidant metal ions which may affect oil body oxidative stability.

In formulated emulsions the type of surfactant used has the potential to significantly affect the development of oxidation through the promotion of metal catalyzed oxidation (Mancuso, McClements et al. 1999) or the presence of residual surfactant hydroperoxides (Nuchi, McClements et al. 2001). A study by Mancuso et al. (1999) used a range of surfactants at equimolar concentrations of positively charged, negatively charged and non-ionic surface charge to illustrate the effect of surface charge on oxidative stability. The negatively charged surfactants attracted free metal ions which promoted oxidation. In the suspensions stabilised by neutral and positively charged species, oxidation did not develop as rapidly.

Oil bodies contain a range of lipids including free fatty acids, diacylglycerides, triacylglycerides and glycerophospholipids, such as phosphatidylcholine and phosphatidylethanolamine. All lipid classes have the potential to promote oxidative stability which could significantly impact on the end product quality.

Oxidation of food lipids is a common problem within the food industry and a range of mechanisms have been used to control the development of oxidative rancidity. It is not the intention of this work to reproduce a detailed description of all factors associated with lipid oxidation as there are many excellent texts in this area (StAngelo 1996; Frankel 1998); rather the important factors that are likely to impinge on the oxidative stability of oil body emulsions will be illustrated, highlighting the effect of antioxidants and the

effects of a structured matrix (emulsion) on the development of oxidative rancidity.

### 1.3.1. Lipid Oxidation

Autooxidation of lipids is a chain reaction occurring autocatalytically through the interaction of unsaturated lipids with oxygen. Lipid hydroperoxides are the initial products; these either breakdown to form volatile products or polymerise to form large molecular weight products. Lipid oxidation occurs spontaneously and is often initiated by trace metals, light, heat, peroxides or hydroperoxides (Frankel 1998).

Lipid oxidation can generally be separated into three phases, initiation, propagation and termination. The initiation phase of lipid oxidation occurs when unsaturated lipids are in the presence of oxygen and an initiator. Lipids lose a hydrogen or hydroxyl radical $R^{\bullet}$ to form a lipid radicals $L^{\bullet}$ as defined in Equation 1-1. The most common mechanism in food emulsions by which free radicals may form, and initiate oxidation, is through the metal-catalysed decomposition of preformed lipid hydroperoxides species as detailed in Equation 1-2.

$$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$$

**Equation 1-1 Formation of lipid radicals from unsaturated fatty acids (Initiation) (L=lipid)**

A) $LOOH + M^{2+} \rightarrow LO^{\bullet} + OH^{-} + M^{3+}$

B) $LOOH + M^{3+} \rightarrow LOO^{\bullet} + H^{+} + M^{2+}$

**Equation 1-2 Decomposition of hydroperoxides catalysed by redox metals (Initiation) (M=metal ion)**

Alkyl radicals of unsaturated lipids $L^{\bullet}$ react rapidly with molecular oxygen to form peroxyl radicals $LOO^{\bullet}$ which can then react with further unsaturated

lipids $LH$ to form hydroperoxides as the principle primary product of lipid oxidation (Equation 1-3).

A) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

B) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

**Equation 1-3 Formation of lipid hydroperoxides from alkyl lipids (Propagation)**

The formation of hydroperoxides from peroxyl radicals (Equation 1-3(b)) is the rate limiting step in the propagation of lipid radicals. The rate of oxidation is critically dependant on, and limited by, the availability of allylic hydrogens and their relative ease by which they react with peroxyl radicals to form hydroperoxides.

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

**Equation 1-4 Reaction of hydroperoxide with chain breaking antioxidant (Termination)**

Once formed, peroxyl radicals may undergo a range of fates. They may return and proliferate the propagation phase of oxidation (Equation 1-3 (A)) or form one of a range of termination products, including non-radical products through the Russell mechanism, stable alcohols ( $LOH$ ) through reaction with unsaturated lipids ( $LH$ ), fragmentation to form unsaturated aldehydes, or though binding to an antioxidant thereby removing the radical species from further decomposition (Equation 1-4). As oxidative rancidity develops, a range of volatile products may be formed that may impinge on the end product quality in food systems. These include carbonyl compounds and alcohols; one classic example of a volatile produced from the oxidation of a C18:2 hydrocarbon is hexanal and is shown in Figure 1-3 .

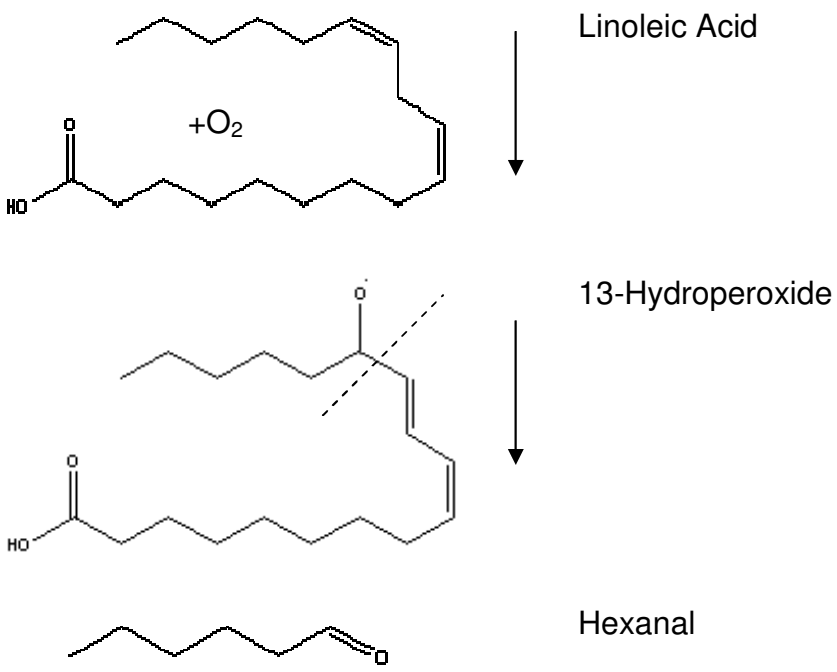



**Figure 1-3 Hexanal formation during linoleic acid autoxidation**

### 1.3.2. Measurement of Oxidation

There are a wide range of methods available to measure oxidation at various stages, but no method can be used exclusively to define the state of oxidation. If the concentration of a compound associated with oxidation is followed then the concentration must change with the progression of oxidation. The use of one marker compound is possible but there are major limiting steps, for example hydroperoxides can be followed to indicate the first stages of oxidation, but in the later phases hydroperoxides are broken down into one of many secondary products. If the secondary products (e.g. hexanal) are followed the concentration increases only during the termination phase of oxidation, resulting in a time lag between the two curves (as illustrated by Figure 1-4).

To fully estimate the progression of oxidation at least two methods must be employed simultaneously looking at different products of oxidation. Two methods employed for analysis are discussed below.

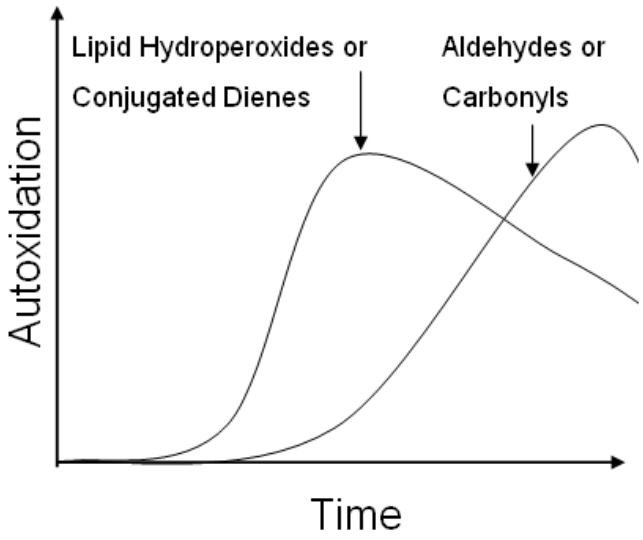



**Figure 1-4 Progression of oxidation as measured by hydroperoxides and headspace hexanal concentration (Frankel 1998)**

#### 1.3.2.1. Lipid Hydroperoxides

Measurement of lipid hydroperoxides is the classical method for following oxidation. Traditionally, the amount of iodine liberated from potassium iodide by the oxidised sample is measured, from which peroxide values can be calculated. Although a robust technique, it is not especially sensitive to low levels of hydroperoxides and may also be affected by non lipid peroxides.

More sophisticated methods are currently available; they still rely on colorimetric assays but have the ability to resolve lower concentrations of lipid hydroperoxides and are more sensitive to small variations in hydroperoxide concentration. The ferric thiocyanate method (Nuchi, McClements et al. 2001) is based on the oxidation of ferrous to ferric ions, this method is used hereinafter and is sensitive to low lipid hydroperoxide concentrations and small changes in lipid hydroperoxide concentration.

#### 1.3.2.2. Headspace analysis

This is a rapid method that assesses the production of volatile bi-products of lipid oxidation. It is a powerful technique limited by the partitioning of volatile components into the headspace and the possibility of further degradation

reactions occurring during extraction. Volatiles are typically quantified by solid phase microextraction (SPME) fibres or through the use of Tenex traps and then gas chromatography mass spectroscopy.

### 1.3.3. Oxidation of Oil-in-Water Emulsions

Critical factors controlling lipid oxidation vary depending on the structural matrix in which the lipid is situated. In oil-in-water emulsions lipid is suspended in an aqueous continuous phase, which may affect a range of factors principally based on the mobility and spatial location of oxidatively active molecules. Factors that may impact on the rate or development of oxidation include oxygen permeability into the lipid phase, location of free radicals, location of polyunsaturated fatty acids, the presence and location of radical generators and location of antioxidants.

Oil-in-water emulsions are often stabilised by the addition of surfactants, the presence of which may vary the viscosity at the membrane or impart a surface charge on the oil droplets, surfactants may contain oxidation promoting metal ions as contaminants, the surfactants may also oxidise. The overall effect of addition of surfactant may promote or limit the rate of development of oxidation depending on the state of the system.

In biological and food emulsions the initiation of oxidation is often catalyzed by the presence of metal ions such as $Fe^{2+}$ (McClements and Decker 2000). Metal catalyzed oxidation within an emulsion is dependant on the surface charge of the stabilizing surfactants; anionic suspensions attract cationic metal ions thereby promoting oxidation (Frankel 1998). Although this is true in complex matrices containing trace metal ions, in a ‘pure system’ other factors including light and elevated temperatures are the primary drivers in initiating lipid oxidation.

Generally, in an emulsion, the point of initiation of lipid oxidation is at the oil-water interface. The presence of surface active proteins can therefore act in

many ways to affect the progression of oxidation. If the surface active agents are fibrous, they will effect the viscosity of the media surrounding the droplet, this will affect oxygen diffusibility, the mobility of oxidation products and the ability of metal ion initiators to come into contact with lipids (Silvestre, Chaiyasit et al. 2000). In addition, proteins may chelate free metal ions or oxidise preferentially over, or co-currently with, the lipid species, resulting in inhibition of lipid oxidation (Faraji, McClements et al. 2004). The process of oxidation may also be promoted through the formation of peroxyl radicals or reactive carbonyls from proteins (Villiere, Viau et al. 2005).

Physical parameters of the emulsion droplets may also impinge on the development of oxidation. Droplet size is suggested to affect the development of oxidation as the surface area to volume ratio changes with droplet size. If the ratio increases in a system of high hydroperoxide concentration then more hydroperoxides will be available at the surface to promote oxidation. A reduced ratio will limit the number of surface hydroperoxide molecules at the surface, reducing the rate at which oxidation develops (McClements and Decker 2000).

### 1.3.4. Antioxidants

The presence of antioxidants ($A$) at a range of concentration in any lipid system will reduce the propagation of oxidation through the system. Most antioxidants are defined as chain breaking antioxidants that operate by interfering with chain propagation or initiation by donating hydrogen to lipid peroxyl radicals.

Phenolics, such as tocopherol are effective antioxidants as they can compete effectively with lipid species ($LH$) to produce stable and unreactive antioxidant radicals ($A^{\bullet}$) (Equation 1-5) that do not propagate further oxidation. Other antioxidant classes include preventative antioxidants that operate through mechanisms such as a metal ion chelation; hydroperoxide

destroyers and ultraviolet light destroyers that absorb irradiation without the formation of radical products.

A) $LOO^{\bullet} + AH \leftrightarrow LOOH + A^{\bullet}$

B) $L^{\bullet} + AH \rightarrow LH + A^{\bullet}$

C) $A^{\bullet} + LOO^{\bullet} \rightarrow non - radical$

D) $A^{\bullet} + A^{\bullet} \rightarrow non - radical$

**Equation 1-5 Activity of antioxidants (A) on free radicals and the formation of non-radical products**

Tocopherol is an amphiphilic molecule that in a surfactant stabilised emulsion will locate in the membrane interface (Wang and Quinn 1999). This spatial location contributes to the high efficiency of tocopherol in controlling lipid oxidation, as oxidation typically initiates at the interface, its location will also limit the propagation of free radicals between emulsion droplets. Vitamin E isoforms have varying levels of antioxidant potency, efficacy of action and physical properties. It is proposed by Serbinova and co-workers (1991) that tocopherol may cluster at the interface. This, depending on the surface chemistry, may increase or decrease the antioxidant strength of the molecule.

In oilseeds, tocopherol molecules generally associate to membrane structures and are principally associated with the storage lipid fraction (Yamauchi and Matsushita 1976) and with plastid membranes (Munne-Bosch and Alegre 2002). Tocopherol acts not only to prevent oxidative rancidity but also to maintain membrane integrity and may affect intracellular signalling (Munne-Bosch and Alegre 2002).

## *1.4. Flavour Retention and Release*

### 1.4.1. Flavour Retention and Release

The volatilisation of flavour compounds from single phase matrices is dependant on many interacting factors. The main controlling parameters are the rate of mass transfer within the matrix, the physico-chemical parameters of the flavour molecule itself and the thermodynamic parameters at the macroscopic level (vapour pressure, solubility, partition coefficient and activity coefficients).

$$K_i = \frac{Y_i}{X_i}$$

**Equation 1-6 Partition coefficient (K) of compound *i.* Where the concentration of *i* in the vapour phase (Y) and in the liquid phase (X).**

The partitioning of a volatile between a matrix and its headspace will dictate the level of sensory perception of the volatile. This is defined by the partition coefficient (Equation 1-6). The equilibrium concentration in both phases will be heavily dependant upon the chemical composition of the liquid phase. The addition of lipids and proteins will effect the relative concentrations and consequentially the partitioning coefficient, leading to significant changes in volatile availability and flavour perception.

When flavour volatiles are added to a cloud emulsion (oil-in-water suspension) a fraction of the flavour volatile will partition into the lipid phase, the level of which is dependant on the hydrophobicity of the molecules. Very hydrophilic compounds will not partition into the lipid and will effectively increase in concentration in the aqueous phase. A volatile of high hydrophobicity will partition significantly into the lipid phase, and although the bulk concentration will be the same, the air-emulsion partition coefficient will reduce and the volatile concentration in the air phase will reduce. The strength of this association was detailed by a range of authors (Buttery,

Guadagni et al. 1973; Doyen, Carey et al. 2001; Carey, Asquith et al. 2002) who suggest that the physical parameters of the flavour volatile and the oil fraction are the most significant factors in predicting the effect of lipid on a suspension of volatile in a lipid system.

### 1.4.2. Spray Dried Flavoured Oil Body Suspensions

When long term storage of a flavour compound is required, the compound of interest is often encapsulated in a glassy matrix or spray dried to form a dry powder from which only small amounts of flavour compound are lost during storage. Ideally flavour release is initiated by a controllable mechanism such as hydration, heat or shear; and in an ideal encapsulant, all of the flavour compound should then be released.

We propose that oil bodies could be a novel and effective method for entrapping flavour compounds for long term storage. In order to assess a flavour encapsulant several parameters should be considered, these include the ability to release flavour on consumption, secondly the ability not to release during the storage state, and thirdly the dynamic association of flavour compounds to the matrix in question.

Spray drying of emulsions is typically achieved using a carrier matrix to stabilise the emulsion and protect it from elevated temperatures, Carriers are often carbohydrate based as they are stable at low water contents and offer a reduced rate of diffusion of flavour compounds when dehydrated, typical examples include maltodextrin, gum arabic and hydroxypropyl cellulose.

Efficiency of encapsulation will depend on a large number of factors including: processing conditions of the spray dryer, composition of raw material, physical properties of the volatile to be encapsulated and the composition and spatial location of compounds in the spray dried product.

Despite all the work that has been carried out previously on oil body structure, oil body synthesis and oleosin biochemistry; no studies have been reported on oil body oxidative stability and their ability to deliver flavour within foods; both are key characteristics of emulsions in food. There is also no literature covering the commercial exploitation of oil bodies beyond pharmaceutical applications. An understanding of the basic physical and chemical nature of oil bodies is fundamental to developing future food applications and appreciating the commercial limitations of any oil body containing product. Some of the key issues highlighted in this work include composition of suspension media, long term chemical stability, resistance to processing conditions such as elevated temperatures, emulsion stability, and oil body functionality in the end product. The work described in this thesis was aimed at characterising sunflower oil bodies for their ultimate use in food applications.

# 2. Materials and Methods

## 2.1. Materials

Capsul (oxylsuccinated starch) was sourced from National Starch, Yorkshire, UK. Morex (Maltodextrin 10-19DE) and limonene was sourced from Firmenich SA, Geneva, Switzerland. Dehulled sunflower seeds (China) were less than 20 months old and viable (as supported by germination tests detailed below). Seeds were sourced from Lembas Ltd, UK. All other chemicals were purchased from Sigma-Aldrich, Gillingham, UK.

Germination tests (Zarbakhsh, Prakash et al. 1999) were performed on 25 seeds (n=5) on hydrated filter paper by the 'between paper' method (International_Seed_Testing_Association 1985). Dehulled sunflower seeds had 60% germinability as shown in Figure 2-1.

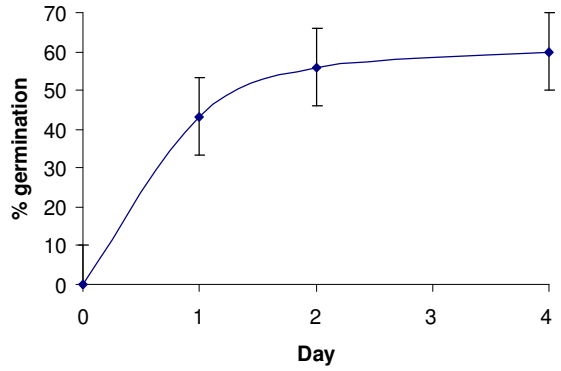



**Figure 2-1 Sunflower seed viability as assessed by germination over 4 days.**

## 2.2. Oil Body Isolation

Oil bodies from dehulled sunflower seed were extracted and purified by the method of Tzen (1997) with slight modifications as described. Seeds (100g) in grinding medium (0.5L, 10mM sodium phosphate buffer pH 7.5, 0.6M sucrose) were ground in a laboratory blender (Kenwood BL315 full power for

60s). The resultant slurry was filtered through 3 layers of cheesecloth and the filtrate centrifuged in 400mL batches (8000 RCF, 30 min, 5°C). The upper layer was isolated using a chilled metal spatula and dispersed (5mL) in washing buffer (25mL, 10mM sodium phosphate buffer, pH 7.5, 5°C) then centrifuged at 5°C (A swinging bucket rotor at 2000 RCF for 20min is used for all other centrifugations unless stated otherwise). The upper layer was isolated and designated as a water-washed oil body preparation. This isolate was then further purified by suspending the water-washed preparation (5mL) in washing buffer (25mL, 9M urea, 10mM sodium phosphate buffer, pH 7.5) and centrifuging at room temperature. The fat pad was isolated, resuspended (10mM sodium phosphate buffer, pH 7.5) and centrifuged at room temperature to remove residual urea and extraneous proteins. After centrifugation the centrifuge tube was then chilled on ice for 5 min and the upper solidified phase (urea-washed oil body preparation) was removed, mixed with sodium azide (0.02mM) and stored at 5°C under nitrogen.

To increase purity oil body preparations can be further washed in urea or buffer a number of times, unless otherwise stated, oil body preparations designated as ‘urea-washed’ or ‘salt-washed’ are washed in urea or salt once and then washed in buffer once to remove residual urea or salt.

## *2.3. Size Distribution*

A Malvern Mastersizer S (Malvern Instrument, Malvern., England) equipped with a small sample dispersion unit and a 300RF lens was used to study droplet diameter of oil body samples. The volume particle size distribution was estimated using the Malvern Mastersizer S polydisperse analysis model and 3NAD presentation code (real refractive index and imaginary refractive index were 1.095 and 0 respectively, dispersant refractive index was set at 1.33), obscuration was maintained at 15% for all samples by dilution with 10mM sodium phosphate buffer, pH 7.5.

Samples were introduced into the small sample dispersion unit after sonication (1min), samples were allowed to equilibrate (1 minute) prior to measurement to ensure samples had fully dispersed. A hydro S dispersion unit (Malvern Instrument, Malvern., England) was used to test the effect of sonication on oil body stability. Particle size was measured over 50 min with two sonication pulses (full power, 2 min) at 10min and 20min.

#### 2.3.1. Surface Area Calculation

Mean surface area was calculated from the sauter mean diameter D(3,2) using the equation proposed by McClements (2000).

$$Mean\ \ surface\ \ area = \frac{6 \times \phi}{D(3,2)}$$

$$Where : \phi = lipid\ \ content$$

**Equation 2-1 Emulsion Mean Droplet Surface Area (McClements 2000).**

## *2.4. Imaging*

#### 2.4.1. Light Microscopy

Oil bodies (1g) were suspended in 10mM sodium phosphate buffer (25mL) at pH 6.5 and pH 7.5 with and without trypsin (1mg trypsin added then the samples were imaged after 1hr and 24hr) then observed under a Nikon LABphoto 2 (Kanagawa, Japan) light microscope at 40 x magnification. Images were recorded using JVC KY-F55B imager (Tokyo, Japan) and analysed on Optimas software v 6.0 (Optimas Ltd., Washington, USA).

#### 2.4.2. Electron Microscopy

Sunflower seeds were fixed in glutaraldehyde (2.5% in cacodylate buffer, pH7.4), 1mm sections were then fixed in glutaraldehyde (5%) under vacuum (1hr). Following washing, sections were post-fixed in osmium tetroxide (2%

for 3hr) before dehydration in a graded ethanol series and embedding in Spurr's resin. Ultrathin sections (80nm) were then taken from representative areas of the seed and observed by electron microscopy (Joel 1010 TEM)

## *2.5. Compositional Analysis*

### 2.5.1. Freeze Drying

Moisture content estimation and preparation of freeze dried samples was achieved by freeze drying until constant weight. Samples were flash frozen in liquid nitrogen and stored at -80$^{o}$C for 4 hours prior to freeze drying, samples were freeze dried (Edwards Freeze Dryer Super Modulyo Pirani 1001) at -50$^{o}$C under vacuum (48hr or until constant weight).

### 2.5.2. Vacuum Oven Drying

Samples were prepared in pre-dried containers, and dried in a Gallenhamp vacuum oven at -900mBar and 80$^{o}$C (48hr or until constant weight).

### 2.5.3. Soxhlet Lipid Extraction

Total fat was extracted by weighing oil body (2g) or seed (8g) samples into cellulose thimbles (22mm x 80mm, Whatman, Fisher Scientific Ltd, Loughborough) samples were capped with cotton wool and placed into soxhlet glassware. 200mL of (40$^{o}$C-60$^{o}$C) petroleum ether was used to extract total fat by refluxing on a heating plate for 18h.

### 2.5.4. Cold Solvent Lipid Extraction

Samples (500mg or 0.5mL) were dried by freeze drying at -50$^{o}$C (24hr) and lipid extracted (~50mg) with diethyl ether (1ml). Samples were vortexed (2min) centrifuged (13,000 RCF) and diethyl ether removed, extraction procedure was repeated 4 times and resulting pooled diethyl ether was evaporated with nitrogen (30$^{o}$C) in a rotary evaporator under vacuum. Lipid

content was determined gravimetrically and samples stored under nitrogen (-80$^{o}$C) for further analysis.

### 2.5.5. Pulsed Field Low Resolution Nuclear Magnetic Resonance

A Bruker minispec PC 110 bench top pulsed NMR spectrometer (25mm diameter probe head) was used to assess lipid content of intact sunflower seeds using the spin–echo method as described by Jones (2001). Spin-echo curves were formed from milled sunflower seeds and lipid extracted by cold solvent extraction (diethyl ether) from seeds from the same batch (as previously described). Pulsed field low resolution NMR assessment was carried out by Leticia Andrad, University of Nottingham.

### 2.5.6. Lipid Profile Thin Layer Chromatography (TLC)

Urea-washed sunflower oil bodies were isolated and total lipid extracted by soxhlet extraction. The extracted oil was re-suspended at 1% v/v in hexane and spotted (100µL) onto a thin layer chromatography plate (ALUGRAM SIL G, Machery-Nagel GmbH + Co., Duren Germany). The TLC plate was developed in a solvent system of hexane : diethylether : acetic acid (80:20:2) (Chen, Tai et al. 1998). After development the plates were dried at 40$^{0}$C for 20 min, stained with 4.2M sulphuric acid and allowed to char at 40$^{0}$C for 15min.

### 2.5.7. Fatty Acid Analysis

Fatty acid analysis was performed on the total lipid extracts of seed and oil body samples after freeze drying. Freeze dried samples (1g) were ground to homogeneity, and total lipids extracted by cold solvent extraction (diethyl ether, 8mL) using a laboratory roller for 24hr, solvent phase was aspirated and extraction was repeated 4 times. Pooled lipid extracts (500 mg) were re-suspended in chloroform (1mL), vortexed and centrifuged (2000 RCF, 5min), the solvent layer was transferred to individual bijou bottles by aspiration and the solvent evaporated. Lipid (20µL) was diluted in chloroform and trimethyl

sulfonium hydroxide (0.25M suspended in methanol) was added to derivatise the triglycerides to fatty acid methyl esters, to a final concentration of lipid : chloroform : TMSH (in methanol) (1:500:200). Samples were allowed to derivatise for 10min, then syringe filtered (0.45µm nylon filter, Whatman Ltd.), and injected (1µL) into a CG-MS for fatty acid analysis (CP-WAX GC column 25m 0.25mm ID; [oven temperature 120$^{o}$C for 1 min ramped at 10 $^{o}$C.min$^{-1}$ ,ramped to 260$^{o}$C at 10 $^{o}$C.min$^{-1}$] source temperature 200 $^{o}$C, interface temperature 250 $^{o}$C in a Carlo Erba GC 8000, Milan, Italy in positive electron impact mode). Samples were analysed in triplicate and compared via residence time and mass spectra to a standard mix of fatty acid methyl esters (Sigma, California, USA)

### 2.5.8. Phospholipid Composition

Oil body and seed samples (0.5g) were extracted by cold solvent extraction (diethyl ether (1mL)) as previously described. Excess solvent was evaporated using nitrogen (2hr) and samples re-extracted as by Bligh and Dyer (1959), 750uL of chloroform methanol (2:1 v/v) was added samples were agitated, vortexed and centrifuged (13,000 RCF), lower chloroform layer was removed, and samples re-extracted in methanol : water (1:1 v/v). Samples were then spotted (50uL) onto a BioRad Silica gel 60A TLC plate.

TLC plates were developed in hexane/diethyl ether/acetic acid (80/20/2 v/v) then allowed to dry under nitrogen and further developed in chloroform/acetic acid/methanol/water (70/25/5/2 v/v) to separate phospholipids. Plates were sprayed with molydbenum blue reagent (1.3% molydbenum oxide, 4.2 M sulfuric acid Bio-Rad, Hercules, USA) and imaged. Authentic standards were obtained from Avanti Polar Lipids Inc., Alabaster.

Resulting plates were analysed using a BIO-RAD GS-800 calibrated densitometer, processed using a salt and pepper filter, and subsequent gaussian filter. Spot density was calculated by removing localised

background and calculating optical density. Images were processed using PDQuest Quantity-one (Bio-Rad, Hercules, USA).

### 2.5.9. Protein Content

The protein content of the defatted dried oil bodies was determined using the BCA assay following solubilisation of proteins (500mg dry defatted oil body) in 2% sodium dodecyl sulfate (1mL) solution (90 ℃ for 15min). Samples were then vortexed (1min), centrifuged (13,000rpm for 1min), supernatant was then aspirated, diluted (to within the range of the standard curve) and assayed for protein content per the BCA assay (Smith 1985) at 562nm.

### 2.5.10. Protein Separation by SDS-PAGE

Isolated oil bodies (0.1g) were suspended in distilled water (1mL) and vortexed (2min); to which 100µL of 100% trichloroacetic acid (TCA) (w/v) was added. The tube was chilled on ice for 30 min to promote the precipitation of proteins and centrifuged (13,000rpm for 5min). The supernatant was aspirated and discarded and the pellet vortexed with 200µL SDS solution (10% w/v SDS, 10mM β-mercapto-ethanol, 20% v/v glycerol, 0.2M tris-HCl, pH 6.8, 0.05% bromophenol blue.) Proteins were resolved by SDS-PAGE using 15% and 4.0% polyacrylamide gels in the separating and stacking gel respectively. After electrophoresis the gel was stained with BioRad coomassie blue (R-250) and destained with excess methanol:water:acetic acid (9:9:2 v/v).

### 2.5.11. Tocopherol and Tocotrienol

Sunflower seed was ground in a pestle and mortar after freezing in liquid nitrogen then homogenised in a sample mill (2 x 2s burst), the homogenised seed (5g) or fresh oil body cream (5g) was then freeze dried. Methanolic extraction was carried out with freeze dried sample (1g) in 100% methanol (8mL) using roller mixer for 12 hr, the supernatant was then aspirated after

centrifugation (2000 RCF for 10min). Methanolic extraction was repeated twice and pooled samples stored under nitrogen at –20$^{o}$C.

Tocopherol content and composition were analysed by HPLC as described by Bryngelsson (2002). Methanolic extract (1mL) was evaporated to dryness in a rotary evaporator at 40$^{o}$C and resuspended in hexane (1mL) then syringe filtered (0.45µm nylon filter).

HPLC was performed using a Waters 2695 separation module equipped with a Waters 996 photodiode array detector and a Jasco intelligent fluorescent detector P-920 (excitation set at 294nm and emission at 326nm, gain set at 10). Separations were performed using an Inertsil 5 silica ChromSep HPLC column SS 250x 4.6 mm (Varian BV) with a ChromSep guard column. Mobile phase was hexane/1,4 dioxane (95:5, v/v) and a flow rate of 1.5 mL.min$^{-1}$. Samples (50µL) were injected with a run time of 20min at 25$^{o}$C. Identification and quantification were made using authentic standards of α-, β-, γ-, δ-tocopherol (Sigma Ltd, Gillingham). Coefficient of variation of standards was acceptable at < 4.1%.

### 2.5.12. Total Phenolic Content

Folin Ciocalteau reagent (0.25ml) was allowed to react with methanol extract (50µL) for 1min. Sodium carbonate solution (0.75ml, 20% w/v) was added, the sample vortexed and left for 1 min then resuspended in distilled water (3.95ml), vortexed and left for 2hr. The sample was passed though a syringe filter (0.45µm nylon filter) and absorbance measured using a quartz cuvette at 760nm using gallic acid as a standard.

### 2.5.13. Phenolic Acids

Samples (2g) were extracted in 100% methanol (15mL) on a mechanical stirrer (20 min); the supernatant was then aspirated after centrifugation and pooled. Reverse-Phase High Performance Liquid Chromatography was used to identify phenolic acids in methanol extracts (Bryngelsson, Mannerstedt-

Fogelfors et al. 2002) (n=3) following alkaline hydrolysis to cleave soluble esters (Krygier, Sosulski et al. 1982). In brief, compounds were separated (Waters Alliance Separation Module) on a HP ODS Hypersil column (5µm, 125mm, ID 4mm) using a gradient mobile phase (1ml $min^{-1}$) of 0-40% acetonitrile in 0.01M phosphate buffer (pH 2.8, 5% acetonitrile) over 60min. Compounds were detected at 320nm (DAD module) and quantified using authentic standards and employing Waters Millenium Software.

## *2.6. Physical Stability*

### 2.6.1. pH Stability

Oil Body suspensions were diluted to 2% lipid w/w in phosphate-citrate buffer at a range of pHs, allowed to equilibrate (24hr), vortexed, then allowed to equilibrate (5 min).

Post storage, emulsions were assessed for stability against creaming by measuring the total height of the emulsion ($H_E$) and the height of the clear serum layer ($H_S$) that may have formed due to emulsion creaming. Creaming index was determined by; $100 \times (H_S/H_E)$.

### 2.6.2. Thermal Stability

Samples were prepared as described in section 2.6.3 and stored (48 hr) at 5°C 15 °C 25 °C 35 °C 45 °C. Samples were analysed for size distribution, creaming stability, and physical appearance.

### 2.6.3. Streaming Potential

A Mütek Particle charge analyser (PCA-01) was used to measure the colloidal streaming potential (10 $g.L^{-1}$ 10mM sodium phosphate buffer, pH 7.5, dry weight basis, 0.2µm filtered) and titrated with HCl (0.5M, 50mM and 0.5mM) to the point of zero streaming potential which is defined the

isoelectric point of the emulsion by streaming potential. (Mohammed, Hill et al. 2000).

### 2.6.4. ζ-Potentials

Zeta potential measurements were carried out using a Malvern Zetasizer IV (Malvern instruments, UK) (Illum, Jacobsen et al. 1987; Stolnik, Davies et al. 1994). Oil bodies (20mg dry weight basis) were suspended in 10mM sodium phosphate buffer, pH 7.5 (10ml, 0.2µm filtered) and sonicated (10 min). The suspension was then further diluted to achieve a final concentration of 30µg.mL$^{-1}$. Suspension buffer was adjusted previous to dilution to pH values ranging between pH 2.0 and pH 8.0. Settings used in the micro-electrophoresis instrument were: temperature = 25$^{o}$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. Three true replicates were taken for each sample.

### 2.6.5. Artificial Emulsion

An emulsion was formulated by mixing water (780mL) and phospholipid (30mL) in a high shear mixer until dispersed, sunflower oil (190mL) was then added dropwise, under shear, until an emulsion was formed. The crude emulsion was passed through a homogeniser (Emulsiflex C5, Glen Creston, Stanmore, UK) twice to form an emulsion that was uniform, monodisperse and had a droplet diameter of 1µm. The emulsion was stored at 5$^{o}$C under nitrogen.

### 2.6.6. Rheology

Viscosity (steady shear-rate) measurements were obtained using a controlled stress Bholin Rheometer (CS10 and CS BRCS 09:07 Electronic Unit) with a double gap geometry (DG 40/50)) at 25ºC. Samples were prepared in sodium phosphate buffer (pH 7.5) and allowed to equilibrate (20min)

The viscoelastic properties of urea-washed oil body creams were characterised with a 40 mm parallel plate measuring system with a gap height of 1000 µm (CVO-Rheometer, Bohlin Instruments, Gloucestershire, UK). The dynamic stress sweep for determination of the linear viscoelastic domain (lve) was conducted at a frequency of oscillation of 1 Hz. Subsequently, a dynamic frequency sweep in the range of 0.1-5 Hz applying a stress amplitude from inside the lve domain (1 Pa) was carried out.

The viscosity of diluted emulsions (≤20 % lipid) at various pH (2-7) and $CaCl_2$ (0-150 mM) concentrations were measured using a concentric cylinder geometry (bob diameter 14 mm; cup diameter 15.4 mm; measurement gap 150 µm (CVO-Rheometer, Bohlin Instruments, Gloucestershire, UK). The shear stress was then recorded as the shear rate was increased from 1 to 200 $s^{-1}$ (30 seconds equilibration time for each measurement). The measurement temperature was constant 25 $^{o}$C, and all measurements were conducted in triplicate.

## *2.7. Flavour Incorporation*

### 2.7.1. Static Headspace Analysis by APcI-MS

Apparatus (APcI-MS) for analysis of static headspace intensity are described by Linforth (1998; 1999) in brief, samples (10ml) were placed in a capped Schott Bottle (volume = 123mL) with a plugged hole in the lid (as shown in Figure 2-2 without the dilution gas port), after equilibrium (2hr) the plug was removed and the interface probe for the APcI-MS was passed though the hole. The interface sampled the headspace and measured the concentration of volatiles present in the headspace. Calibration was achieved by direct injection of the volatile in hexane into the mass spectrometer.

The variation in relative headspace intensity was measured for a range of volatiles which were chosen to cover a range of hydrophobicities, physiochemical properties and volatilities in an oil body suspension and an

artificial emulsion (as described previously). Intensity was directly compared and relative headspace intensity (RHI) calculated.

Solutions of volatile (10ppm) were prepared by shaking (15 min) (SF1 flask shaker, Stuart Scientific, Redhill, UK). Suspensions and emulsions were then diluted to achieve a final lipid concentration of 1% w/w and a volatile concentration of 1ppm (100ppm for dimethyl pyrazine). Linearity of the MS response was tested by preparation of a standard curve of a range of volatile concentrations in buffer (10mM sodium phosphate buffer pH 7.5) for each volatile.

Relative headspace intensity (RHI) for a volatile compound is calculated as (Doyen, Carey et al. 2001)

$$RHI\% = \frac{C_E}{C_w}$$

Where RHI% = relative headspace intensity; $C_E$= concentration of the volatile in the headspace above an emulsion sample and $C_w$= concentration of the volatile in the headspace above a buffer only sample.

Limonene headspace concentrations above the spray dried powder were above the saturation limit of the mass spectrometer; to measure the volatile headspace concentration the sampling gas flow was diluted with nitrogen at a flow rate of 3L.min-1 in a 2L container. Absolute headspace concentration was calculated by direct injection of limonene standards in hexane (10ppbv - 10ppmv) into the MS detector.

### 2.7.2. Dynamic Headspace Analysis by APcI-MS / PTR-TOF-MS

Dynamic headspace analysis was performed according to the method by Doyen (2001). Samples (100mL) were dispersed in solution and stored in a Schott bottle (123mL), allowed to equilibrate (4hr) and then the headspace measured for volatile concentration during dynamic headspace dilution.

Dynamic headspace dilution was achieved by passing nitrogen gas (70mL.min$^{-1}$) through the dilution gas port (see Figure 2-2) at the same time the gas exiting from the sampling gas port was measured by APcI-MS (cone voltage 20V) or PTR-TOF-MS for limonene concentration (sampling flow rate 20mL.min$^{-1}$). Headspace dilution was continued for 10min. Samples were stirred throughout the measurement period.

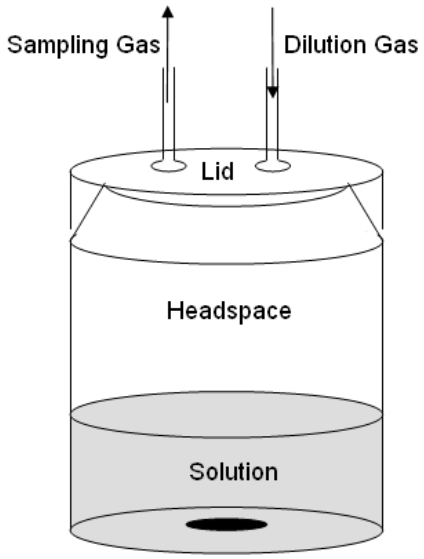



**Figure 2-2 Dynamic headspace dilution apparatus**

### 2.7.3. Limonene Content by Standard Addition

Limonene content in spray dried powders was calculated by standard addition and measurement of headspace concentration (20mL) by gas-chromatography-mass-spectroscopy (GC-MS) (Agilent model G129013, G2613A, G1530A, G2578A). Samples (10mg) were diluted in water (1mL) sealed and shaken. Dilution water contains a serial dilution of limonene, concentration of limonene in the headspace is calculated by peak area on the GC-MS, and the standard curve is extrapolated to the point of no added limonene to calculate the concentration of limonene in the sample. (all data points are taken in triplicate, $R^2$<0.999). (Oven temperature ramped from

60°C to 240°C at 40°C.min$^{-1}$, helium gas flow rate 15mL.min$^{-1}$, Sampling flow rate 75ml.min$^{-1}$, Agilent column 19091S-433 HP-5MS, 0.25mm, 30m).

#### 2.7.4. Spray Drying and Emulsion Formulation

Oil (oil body or sunflower oil), water and carrier (capsul or lecithin) were homogenised (Ultraturax T25; Janke-Kunkel, IKA labortechnik) for two minutes at either 8,000 rpm or 24,000rpm (low or high homogenisation power) allowed to dissolve (2hr) with limonene in a thermally controlled (35°C) air-tight water bath and stirred with a dissolver disk (Vorsicht RW20) at 240rpm. Morex was added and stirred for a further 30min, samples were further homogenised (Ultraturax T25; Janke-Kunkel, IKA labortechnik) for one minute at either 8,000 rpm or 24,000rpm (low or high homogenisation power) prior to spray drying.

Spray-drying was performed using a Buchi B-191 Laboratoriums-Technik (Flawil, Switzerland) test spray dryer. The spray dryer was operated at 180°C and 85°C inlet and outlet temperatures respectively; aspirator at 90% and sample pump at 10%. Drying air was filtered nitrogen at 650ml.min-1 and filter pressure drop maintained below 30mBar.

Moisture content of spray dried products was calculated using the Karl-Fisher method by Firmenich SA, Geneva.

Niro minor spray drying was performed at 210°C and 90°C inlet and outlet temperatures respectively; aspirator at 70% and sample pump at 80%. Drying air was filtered nitrogen at 80kg.hr-1.

## *2.8. Sensory Evaluation*

#### 2.8.1. Dressing Formulation

Samples were formulated as described below:

| | Oil Body Dressing | Sunflower Oil Dressing | |
|---|---|---|---|
| | 25% lipid | 25% lipid | 75% lipid |
| Oil | 251 mL | 200 mL | 600 mL |
| Oil Body (50% oil) | | | |
| Lecithin | | 48 mL | 48 mL |
| Vinegar | 125 mL | 200 mL | 200 mL |
| Water | 124 mL | 200 mL | |
| Mustard | 5 g | 6 g | 8 g |
| Pepper | 2 g | 2.5 g | 3.5 g |

**Table 2-1 Salad dressing formulation**

45% lipid commercial dressing: Schwartz French Vinaigrette (with cracked black pepper)

Vegetable oil, water, white wine vinegar, extra virgin olive oil, Dijon and course grain mustard 7% (mustard flour, spirit vinegar, mustard seed, malt vinegar, white wine vinegar, salt, sugar, mustard bran, colour, (turmeric) spices, herb) sugar, salt, black pepper, stabiliser (Xanthan Gum). [44.9g fat per 100mL]

Dressings were homogenised in a commercial Kenwood blender for 3 min (oil in water dressing) and 30s (oil body dressing).

Samples were produced one day prior to consumption and stored at 5$^{o}$C under reduced lighting.

### 2.8.2. Sensory Evaluation

Assessors were an untrained panel of 60 nieve assessors approximately balanced for age, ethnic background, gender and technical knowledge of sensory techniques. Panellists were filtered only by the prerequisite that they purchased, made or had consumed salad dressing over the last year.

Samples were presented in 10 mL samples in 30mL semi-translucent plastic container and panellists requested to consumer a fraction of the sample

using a bread finger matrix, method of application was controlled across all panellists to minimise variation in sample load (approximately 3mL).

Samples were presented to the panellist individually in a randomised balanced order and they were requested to rate the dressing on a 9 point verbal hedonic scale (sensory evaluation techniques 3rd Ed) as illustrated below. Results were recorded using computerised data acquisition software (FIZZ Biosystemes, France), and all assessments were carried out at in an air conditioned room, under northern hemisphere daylight and in individual booths.


**Figure 2-3 Verbal hedonic likeability scale**

Panellists were requested to cleanse their palette between samples during a 1 minute break with cracker, dilute lime cordial, and water to minimise carry over effect. In addition to the likeability test, panellists were requested to explain why they liked or disliked a given sample using the form presented below during the session.

**Please could you describe what type of salad dressing you normally purchase or make.**

**Please could you comment on what you particularly liked or disliked about the samples presented.**

Sample 162 (not in order sampled)

Sample 513 (not in order sampled)

Sample 864 (not in order sampled)

Sample 045 (not in order sampled)

Thank you for participating in this sensory test, please come through to the lounge for some chocolate or sweets.

**Figure 2-4 Sensory feedback sheet**

Results were analysed as using one way analysis of variance (ANOVA) and after ranking using Friedman's two factor ranked analysis of variance (Friedman's test), to identify statistical differences in the ratings. All data was analysed at a 5% confidence level.

## *2.9. Oxidative Stability*

Oil body suspensions at 10% (w/w) lipid were formulated with sodium azide as a antimicrobial agent (0.02mM) and stored at 5$^{o}$C under nitrogen prior to use. Equivalent artificial emulsions were formulated from sunflower oil extracted from the same batch of dehulled sunflower seeds by diethyl ether extraction, excess solvent was removed by drying under nitrogen. Extracted lipid was added slowly to surfactant solutions (dodecyltrimethylammonium bromide, tween 20 and sodium dodecyl sulphate predissolved for 24 hours at 17mM in deionised water) using an Ultraturax homogenizer (IKA-WERKE, Staufer, Germany) at 10,000 rpm, the emulsion was further homogenized by three passes through an Emulsiflex-C5 (Glan Creston, Stanmore, UK) at an emulsification pressure of 10,000kPa.

Samples (10% w/w lipid) were stored at 5$^{o}$C, 25 $^{o}$C and 45 $^{o}$C with restricted lighting in closed 20mL glass vials.

### 2.9.1. Hexanal Headspace Concentration

Hexanal concentration was calculated from the headspace concentration using solid phase micro-extraction gas chromatography mass spectroscopy (SPME-GCMS) (Beltran, Aguilera et al. 2005). This was achieved by adding 1,2 dichlorobenzene (100ppmv) as an internal standard (10µl) to the emulsion or oil body suspension (1mL) and capping in a 20mL vial. Headspace concentration was determined using a CTS Analytics PAL system autosampler and a DSQ and Trace GC Ultra (Thermo Electron Corporation) Samples were agitated (50$^{o}$C for 3 min) prior to extraction (20

min at 50°C), desorption was achieved in 5 min (250°C). Compounds were separated with 30mL.min$^{-1}$ nitrogen, a ZB-5 Phenomenex gas chromatography column (Macclesfield, UK) and oven temperatures controlled at 40°C (1 min) then ramped (3°C.min$^{-1}$) to 140°C, ramped (15°C.min$^{-1}$) to 210°C and held at 210°C for 1min. Volatiles were quantified with authentic standards.

### 2.9.2. Lipid Hydroperoxide Concentration

Peroxide value (PV) was measured using the method of Shantha and Decker (1994) as modified by Nuchi et al (2001).

# 3. Chemical Characterisation of Sunflower Oil Bodies

Although oilseed oil bodies have been extensively studied in the literature (Baker 1957; Huang 1992; Huang 1996; Galili, Sengupta-Gopalan et al. 1998; Frandsen, Mundy et al. 2001; Murphy, Hernandez-Pinzon et al. 2001) there has often been contradictory results in regard to their formation, proximate composition, functionality of structural proteins, method of physical stability and physical arrangement (Huang 1996; Zweytick, Athenstaedt et al. 2000; Frandsen, Mundy et al. 2001).

In this overview the chemical composition of sunflower oil bodies' major components (protein, neutral lipid and phospholipid) and the presence of minor components not previously detailed (tocopherol and phenolic acids) are discussed. This fundamental compositional data is essential for the discussion of results presented later in this study: oxidative stability (chapter 5), stability to desiccation (section 6.3) and characterisation of physical stability (chapter 4)).

## *3.1. Chemical Composition*

Sunflower oil bodies were extracted by wet milling, filtration, centrifugation and isolation of the buoyant oil body fraction. This crude oil body preparation was washed with buffered aqueous solution followed by sodium chloride (1M) or urea (9M) to produce three fractions (water-washed, salt-washed and urea-washed). Washing treatments remove residual cell debris and passively associated proteins to a range of extents depending on the aggressiveness of the treatment. Aggressiveness of washing protocol, as measured by the amount of protein removed, increased in the order water-washed<salt-washed<urea-washed (see Table 3-1).

Lacey (1998) concluded that the extent of purification by washing was species dependant. Lacey compared the effect of salt and urea-washing on sunflower and safflower oil bodies. Safflower oil bodies could be purified with salt-washing so that their protein fraction was principally oleosin isoforms; sunflower oil bodies required treatment with >8M urea to achieve preparations of similar purity. Salt-washing disrupts charged based interactions occurring on the oil body surface releasing some passively associated compounds. Urea breaks hydrogen bonds releasing compounds more strongly associated with oil bodies.

The presence of salt exhibits non-specific effects on electrostatic interactions through screening of charged groups (Lapanje 1978) leading to a weak chaotrophic effect on proteins in solution. Urea acts as a strong chaotrophic agent at high concentrations (>8M) but does not effect all proteins at low temperatures and neutral pH (Lapanje 1978). It is proposed that oleosin molecules embedded in oil body membranes are resistant to urea denaturation and do not unfold and enter solution. It has also been shown that for some proteins urea denaturation is reversible (Anson and Mirsky 1929) and this could be the case for any isoforms of oleosin that are partially denatured, reforming to their original conformation and functionality during subsequent water-washing of urea-washed oil bodies.

It has also been suggested that uncharged urea is less effective at denaturing charged protein species than charged chaotrophs of similar strength (Monera, Kay et al. 1984). Bowie (2004) states that urea does not disrupt membranes, but can act to facilitate reversible solubilisation of hydrophobic membrane proteins. This solubilisation is not believed to be occurring and any conformational change occurring in oleosin is transient, with oleosin not being solubilised out of the membrane into solution. Lacey and co-workers (1998) agree that the major secondary structure elements of oleosin would be expected to reform upon removal of the denaturant and that oleosin is not solubilised out of oil body membranes. Results by Millichip et al

(1996) and Ratnayake (1996) confirm this and show that the physical stability of oil bodies is not affected by urea washing.

Composition of urea-washed oil bodies should reflect the composition of oil bodies in-vivo and contain the fewest proteins and highest lipid content (Table 3-1). Water-washed and salt-washed samples contained residual contaminating proteins and cell debris; this is reflected in the elevated protein content and increased concentration of unidentified mass. It is hypothesised that the contaminating protein originates from the cell debris during seed milling and its presence is further discussed in section 3.1.4.

**Table 3-1 Protein and lipid composition of washed sunflower oil bodies**

| | % Composition (dry weight basis) ± SD | | |
|---|---|---|---|
| Oil Body Preparation | Protein | Total Lipid | Unidentified Mass |
| Water-washed | 6.1 ± 1.6 | 82 ± 3.6 | 11.9 |
| Salt-washed | 3.3 ± 1.1 | 76 ± 2.9 | 20.5 |
| Urea-washed | 1.8 ± 0.9 | 88 ± 2.5 | 9.9 |

Tzen (1993) suggests oil bodies are composed of 94-98% (w/w) neutral lipid, phospholipid at 0.5-2 % (w/w) and protein at 0.5-3.5 % (w/w). The data presented in Table 3-1 does not discriminate between lipid classes and therefore phospholipid and neutral lipid can be included in the total lipid class (94.5-100%) as detailed by Tzen.

Total protein was measured in a range of washed preparations to assess the association of protein to the oil body matrices. Figure 3-1 shows a reduction in protein content with repeat washing with urea (9M) and salt (1M NaCl). The progressive reduction in protein content occurs in the first and second wash, but subsequent washings in urea or salt did not significantly further reduce the protein content.

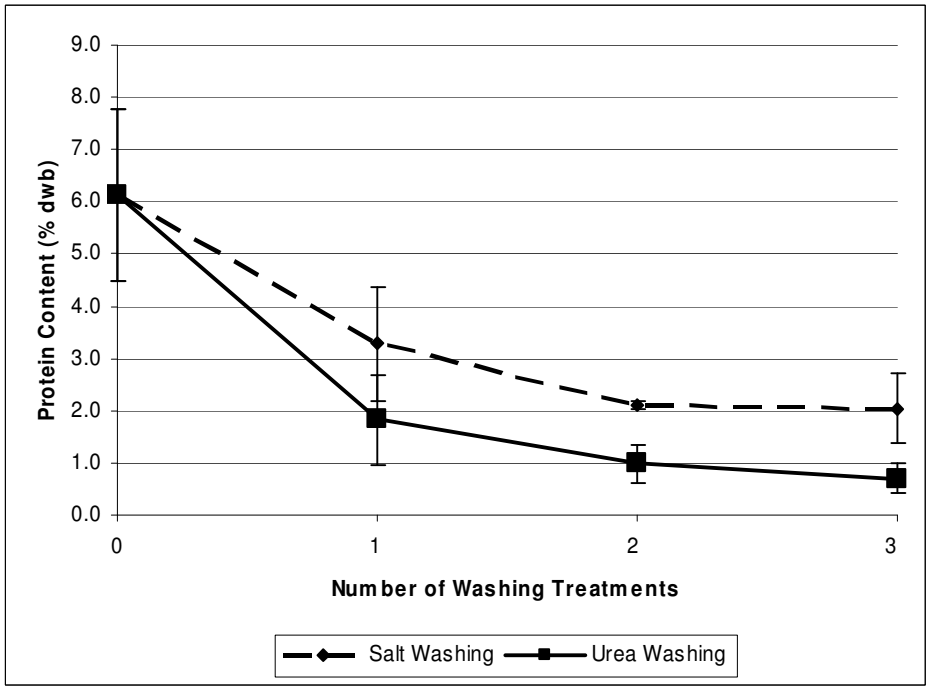



**Figure 3-1 Effect of multiple washing (urea and salt) on protein content of sunflower oil bodies**

### 3.1.1. Neutral Lipid Composition

Sunflower seed lipid content was measured using three independent techniques to assess the robustness of the lipid extraction protocol used hereinafter (diethylether cold solvent extraction). Of the two solvent extraction techniques, soxhlet and cold solvent extraction, soxhlet is the most aggressive and will extract with greatest efficiency as it continuously extracts with clean solvent. Soxhlet is however time consuming, hazardous and not recommended if an alternative method is available. Cold solvent extraction had greater variability, although there was no statistically significant difference between the Soxhlet and cold solvent extraction methods ($P<0.05$). A method developed using pulsed low resolution nuclear magnetic resonance (NMR) was also trialled. NMR although accurate and precise for quantifying lipid content of dehulled sunflower seeds, the NMR technique could not be used when comparing dried oil body preparations against sunflower seed stock as the machine needs to be calibrated with each

matrix. NMR was therefore not used further in this work. Cold solvent extraction with diethyl ether was used as the primary method of assessing lipid content in seeds and dried oil body preparations.

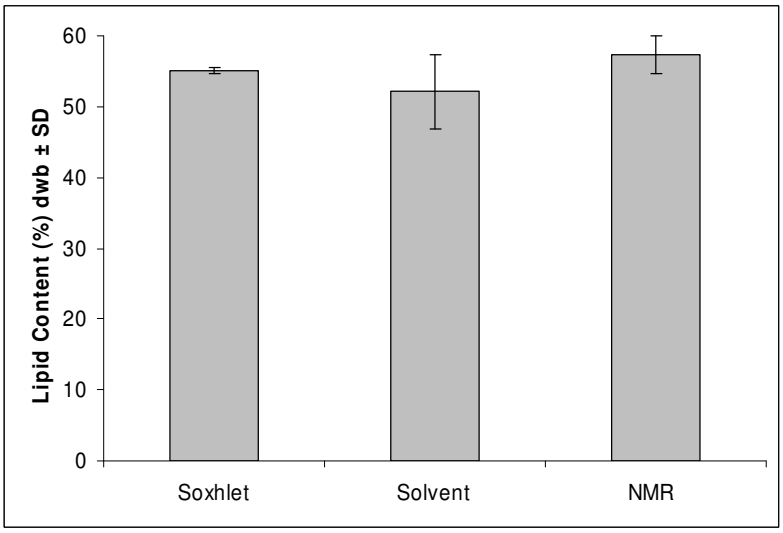



**Figure 3-2 Total lipid composition of sunflower seeds by Soxhlet, cold solvent extraction (diethyl ether) and NMR**

Sunflower seeds have a lipid content of 30-45% dry weight basis (Leissner, Korp et al. 1993). After dehulling (hull accounts for 30-40% w/w of the seed weight and contains 2% lipid (Harrington and D'Arcy-Evans 1985)) the cited lipid content increases to 43-75% (dwb) which correlates well with the three experimental measurements shown in Figure 3-2 and that presented by Carter (1978) (lipid content 52% wwb).

### 3.1.2. Lipid Groups

To further investigate the lipid composition, lipid extracts were separated by thin layer chromatography (TLC) and stained using iodine vapour, thermal charring, and sulfuric acid charring for lipid species identification.

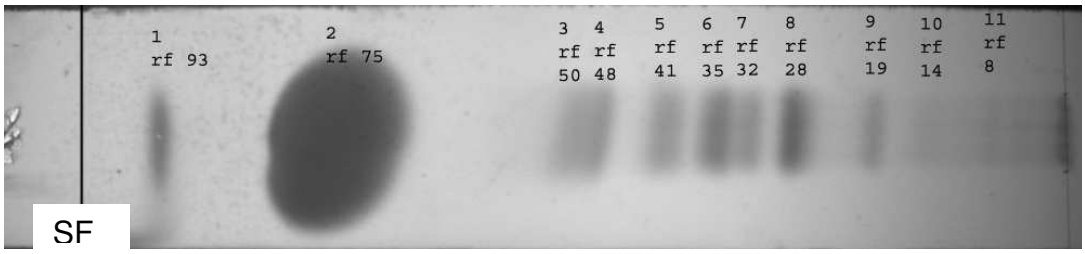



**Figure 3-3 Lipid composition of urea-washed sunflower oil bodies by TLC**

**(rf = retention factor; SF = solvent front. Lipids extracted with diethyl ether and resolved with hexane: diethylether: acetic acid (80:20:2)**

Figure 3-3 shows the TLC separation of lipid classes present in urea-washed sunflower oil bodies. Major peaks are tentatively identified by retention factor as per Christie (2003). These are: 1) unidentified fatty esters; 2) triacylglyceride; 3-8) free fatty acids; 9) diacylglyceride 10-11) monoacylglyceride; phospholipid was not resolved and remained at the origin. The major lipid component is triacylglyceride; this is as expected as the major lipid reserve in oilseeds are storage lipids, of which triglycerides are the most prolific (Miguel and Browse 1995).

Lipid extracts of sunflower oil bodies were derivatized, methylated and fatty acid methyl esters quantified by gas chromatograph mass spectroscopy using retention time and mass spectra to confirm lipid species against authentic standards (Figure 3-4).

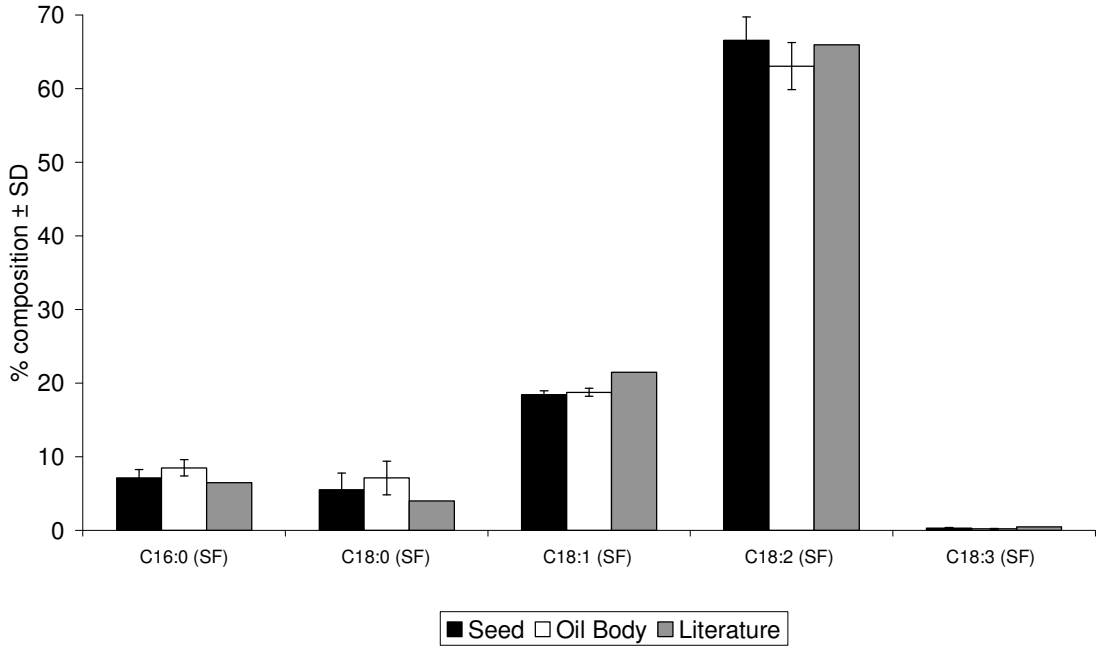



**Figure 3-4 Fatty acid profile of sunflower (SF) seed, sunflower oil bodies and literature values for sunflower oil (Leissner, Korp et al. 1993).**

There was no difference ($P<0.05$) between the fatty acid composition in sunflower oil bodies and sunflower seeds. This is as expected as the bulk of the oil present in the seed will be neutral lipid stored in oil bodies. There may have been some minor differences as additional lipid types, more specialised lipid species that are functional in other seed organelles, may also be present in the whole seed. However, although additional fatty acid peaks were present they could not be identified and no statistical difference could be found between the fatty acid compliment of sunflower oil bodies and sunflower seed extracts ($P<0.05$).

The fatty acid composition correlates well with that from Leissner (ref: 1993), although there are some deviations which probably are due to the cultivar used or the environmental conditions the seed was exposed to during development.

### 3.1.3. Phospholipid Composition

Phospholipids, although a minor component, are fundamental to the stability of sunflower oil bodies due to their interaction with surface proteins (Tzen and Huang 1992). Phospholipids were extracted and separated by TLC, stained by molybdenum blue reagent and resulting plates were quantified by image analysis. (Figure 3-5)

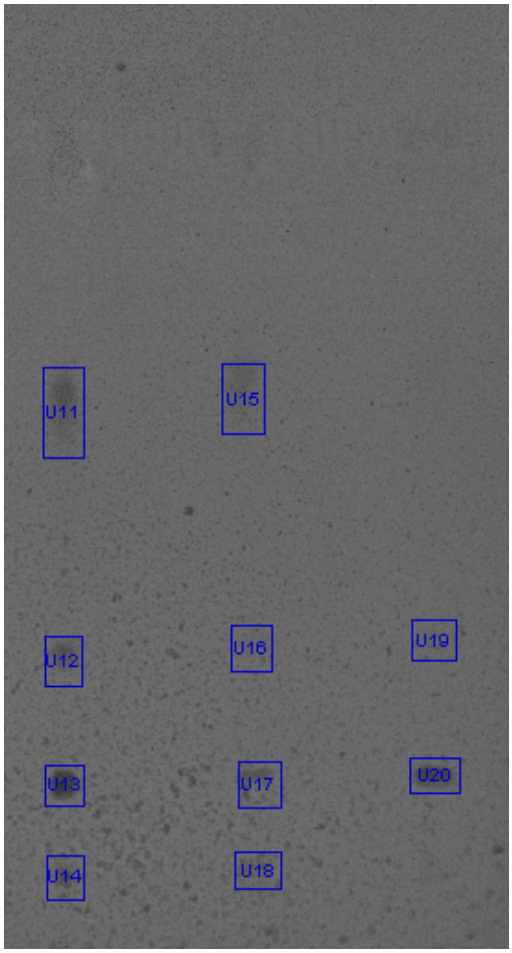



**Figure 3-5 Phospholipid separation by TLC and image analysis of washed sunflower oil bodies.**

**(Runs from left to right: water-washed, salt-washed, urea-washed). Markers indicate phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI)**

Phospholipid groups could be effectively resolved by TLC (Figure 3-5), the major phospholipid species in urea-washed oil bodies is phosphatidylcholine

(PC) with smaller fractions of phosphatidylethanolamine (PE) also present. Less purified preparations of sunflower oil bodies contained, in addition to PE and PC, phosphatidic acid (PA) and phosphatidylinositol (PI). Ratnayake (1996) suggests that urea washing may remove some phospholipid species that are integral to oil bodies, but this work is not supported by other published work (Huang 1992; Millichip, Tatham et al. 1996).

Millichip (1996) concluded in his study on sunflower oil bodies that the major phospholipid class was PC (79% w/w) with a smaller fraction of PE (13%) and PI (8%) also present. Millichip also looked at crude preparations of sunflower oil bodies and showed that they contained an increased fraction of PI. This may be indicative that washing removes the PI, and that, in the data presented in Figure 3-6, the PI has been removed to non-detectable levels. Alternatively, it may be that the TLC densitometry technique is not sensitive enough to detect minor phospholipid components or PI may not be present in sunflower urea-washed oil bodies prepared as described.

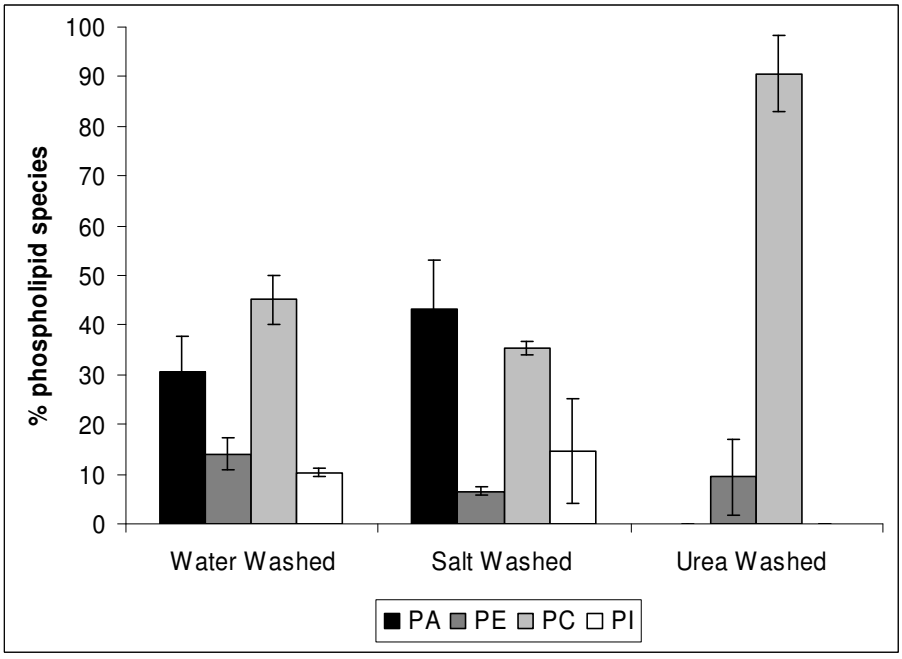



**Figure 3-6 Phospholipid composition of water-washed, salt-washed, urea-washed sunflower oil body preparations**

The lack of any negatively charged (acidic) phospholipid species in the urea-washed oil bodies throws doubt upon work by Huang (1992) who found significant levels of acidic phosphatidylserine (PS) and hypothesised on its contribution to the oil body surface charge, this is further discussed in section 4.3. The results shown in Figure 3-6 suggest that the negative domains of oleosin contribute to the oil body stability and phospholipid serves only to maintain an interfacial environment that is suitable for oleosin to reside on the membrane. Huang (1992) separated the three major components of oil bodies (phospholipid, neutral lipid and protein) and reassembled them to assess their stability concluding that removal of either phospholipid or oleosin renders oil bodies unstable and prone to coalescence, further establishing their importance in oil body stability.

Work by Singleton (1995) for intact peanuts suggests that residual phospholipases may act during periods of thermal stress, degrading PC to PA. Residual enzymes (e.g. phospholipase D) may be present that act to modify the phospholipid profile in the less purified preparations of oil bodies during periods of storage, reflecting in the presence of the uncommon phospholipid species, PA in the water-washed and salt-washed preparations. To test for the presence of phospholipase activity in stored oil body preparations water-washed oil bodies were stored at 5$^{o}$C for 9 days and the phospholipid composition assessed on day 0, 2, 3, 6 and 9. The concentration of PI and PE reduced over the storage period, and on day 6 both phospholipid species were absent (Figure 3-7). Between day 6 and 9 PC reduced in concentration by 22%, and PA reduced only by 11% supporting the hypothesis that intrinsic phospholipase enzymes may be present in the crude preparations converting PC, PI and PE to PA. This may explain why workers have shown conflicting phospholipid compositional data in the past (Huang 1992) (Huang 1992; Millichip, Tatham et al. 1996) (Ratnayake and Huang 1996).

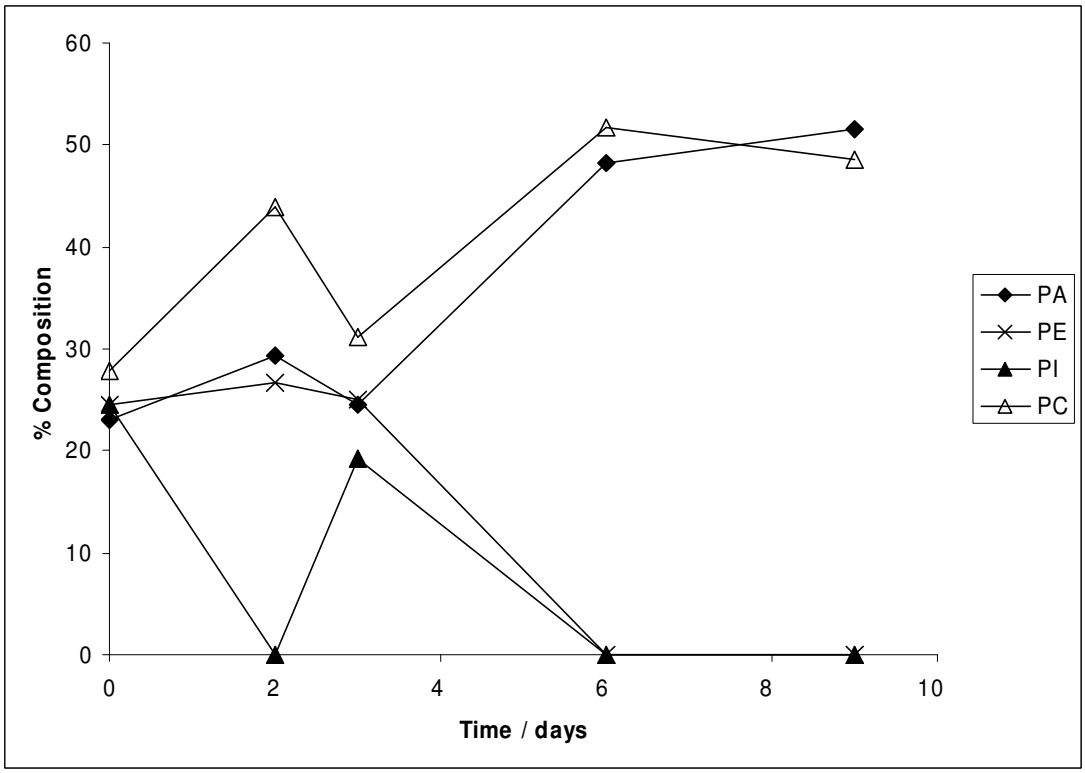



**Figure 3-7 Phospholipid composition of water-washed oil bodies stored at 5°C for 9 days**

Phospholipases, lipases and proteases are all involved in the breakdown of oil bodies for energy release during oilseed germination. Tzen and Huang (1992) showed that the presence of oleosin resisted the activity of phospholipase A2 and C; and that phospholipid breakdown only occurred when the oleosin proteins had been removed. It has also been shown that during TAG mobilisation phospholipase A2 is associated with lipid body membranes in cucumber (May, Preisig-Muller et al. 1998). Current knowledge therefore suggests that proteases enzymatically degrade oleosin subsequently allowing phospholipases to act, this leaves regions of exposed lipids (Noll, May et al. 2000) that creates 80nm holes in the surface of oil bodies through which lipases act to breakdown storage TAG. An alternative explanation could be that lipases bind to oleosin, anchoring them in position close the lipid interface therefore enabling the enzyme to catalyse the degradation of TAG and phospholipid.

Abousalham and co-workers concluded in there 1997 study that phospholipase D may be involved in the degradation of oil bodies. Phospholipase D catalyses the breakdown of phospholipids to a phosphatidic acid and a free head group such as choline (Figure 3-8). The presence of other phospholipases and lipases may explain the elevated concentration of free fatty acids and diacylglycerol within extracted oil bodies.

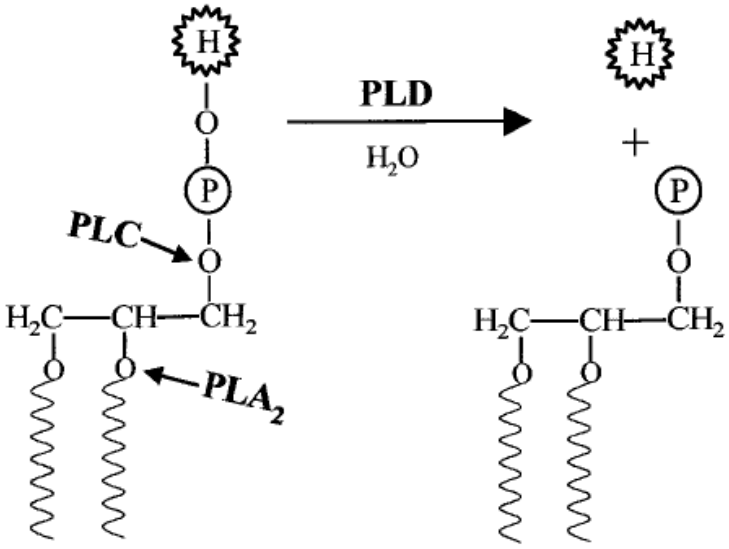



**Figure 3-8 Sites of cleavage of phospholipids by phospholipase D (PLD), C and A2 and the products of PLD cleavage: phosphatidic acid and a head group (H) (Wang 1999)**

The presence of unusual phospholipids (e.g. phosphatidic acid) can therefore be explained by the activity of phospholipases. A recent study by Beisson and co-workers (2001) showed that in almonds some lipases act with combined phospholipase and lipase activity, the reported consequential phospholipid profile was 60% PC, 25% PI, 12% PE and 3% PA, the presence of PA, although at lower concentrations that in our results, supports our hypothesis of enzymatic degradation of phospholipids. Beisson in his final discussion details the similarity of oil bodies to that of chylomicrons and milk fat globules, with respect to lipoprotein lipase activity and the presence of a characteristic lag phase prior to enzymatic breakdown that has been observed in other a range of natural emulsions: chylomicrons (Wieloch,

Borgstrom et al. 1982), Intralipid (Borgstrom 1980) and milk fat globules (Plucinski, Hamosh et al. 1979; Bernback, Blackberg et al. 1989).

### 3.1.4. Protein Composition

Oil body associated proteins are key to the stability of oil bodies, both in-vivo (Leprince, van Aelst et al. 1998) and in-vitro (Tzen and Huang 1992). Work presented in section 4.3.1 demonstrates the importance of protein to the physical stability of sunflower oil bodies through enzymatic digestion of oil body associated proteins with trypsin.

The major oil body associated protein is oleosin, although there has been some reports of lower levels of caleosin and steroleosin associated with oil bodies in specific species (Lin, Tai et al. 2002).

Figure 3-9 shows the profile of proteins associated to sunflower oil bodies; with increasing aggressiveness of washing more passively associated proteins are removed and only intrinsic proteins (oleosin) remain.

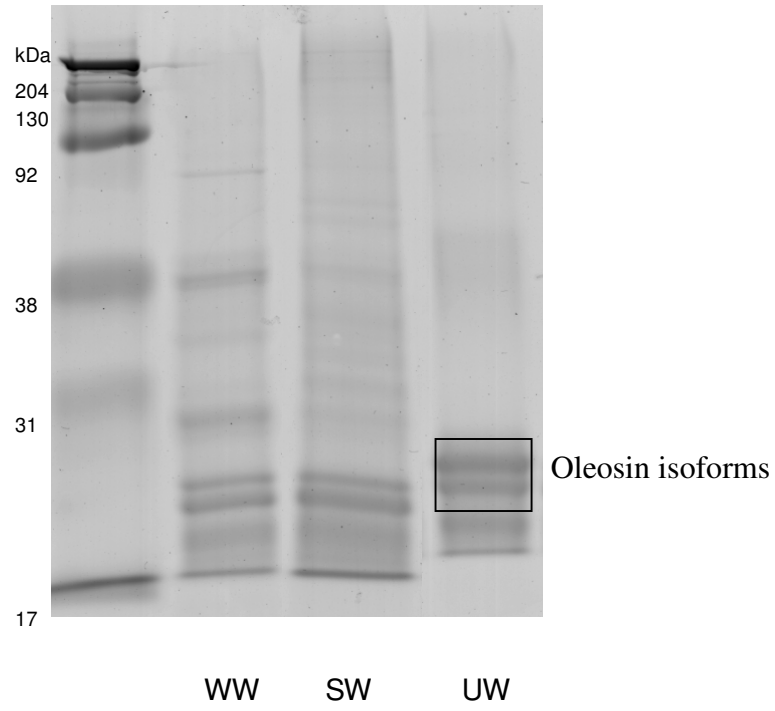



**Figure 3-9 SDS-PAGE of proteins associated with sunflower oil bodies. Protein standards of Mw as indicated.**

**Water-washed oil body preparation; Salt-washed oil body preparation and urea-washed oil body preparation. Oleosin isoforms can be clearly identified in the urea washed sample in the molecular weight range 19<kDa<21 (Millichip, Tatham et al. 1996).**

## *3.2. Associated Phenolics*

Oil bodies are formed by budding of neutral lipid droplets from the endoplasmic reticulum (ER). During this budding phase they passively remove some membrane components of the endoplasmic reticulum membrane. It is clear therefore that in addition to the major structural components commonly associated with oil bodies (protein, phospholipid, neutral lipid) there will also be other compounds passively and intrinsically associated with oil body structures. One key component that is of interest to future applications is the presence of antioxidants in extracted oil bodies.

We hypothesised that tocopherol (the main hydrophobic antioxidant in oilseeds) is present in oil bodies. To test this tocopherol and total phenolic

content were measured in washed oil body preparations. It was expected that if tocopherol was an intrinsic component of oil bodies it would not be lost on washing, and that other phenolic compounds passively associated with the oil bodies, would be lost during washing.

### 3.2.1. Total Phenolic Content

The total phenolic content of oil bodies was assessed using Folin-Ciocalteau reagent (Lowry, Rosebrough et al. 1951). Sunflower seeds contained 2700 µg gallic acid equivalents $.g^{-1}$seed (dwb). After extraction and urea-washing, oil bodies contained a reduced total phenolic content (TPC) (520 ug gallic acid equivalents.$g^{-1}$oil body (dwb)).

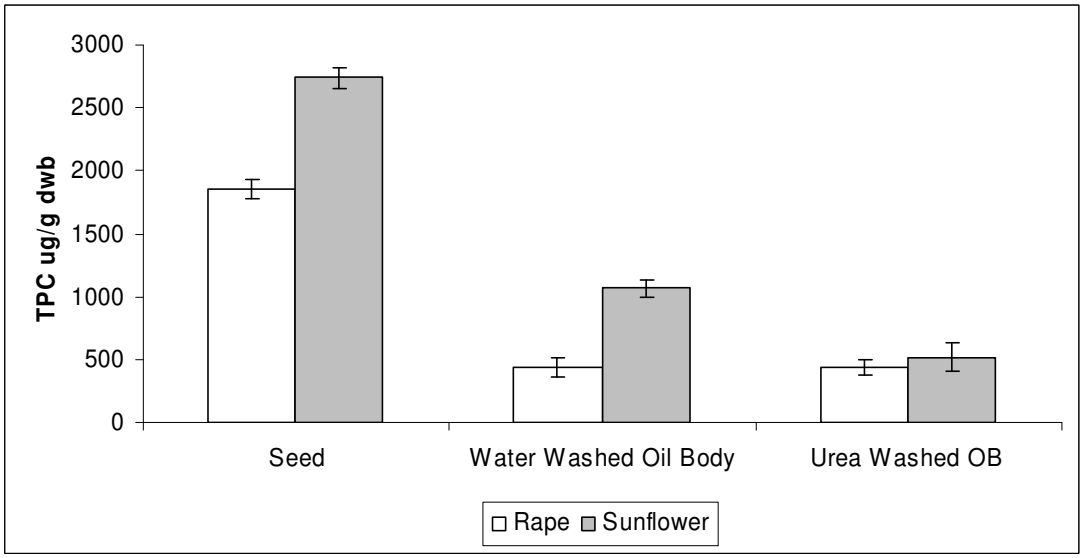



**Figure 3-10 Total Phenolic Content (TPC) of sunflower and oilseed rape, measured in gallic acid equivalents.**

Figure 3-10 clearly illustrates the reduction in phenolic compounds associated with oil bodies after extraction and washing. Further to this overview specific phenolics have been quantified: tocopherol in section 3.2.3 and phenolic acids in section 3.2.2. Two oilseed species were assessed to confirm that the effect was not species dependant and all further quantitative work in this section has been confirmed on rapeseed oil bodies in addition to those of sunflower (not all data shown).

### 3.2.2. Phenolic Acid Composition and Association to Sunflower Oil Bodies.

The major phenolic acid in sunflower seeds is chlorogenic acid (Antonella De Leonardis 2003). Although others are present at lower concentrations, in this study only chlorogenic acid data shall be detailed; nevertheless, the other key phenolic acids followed similar trends.

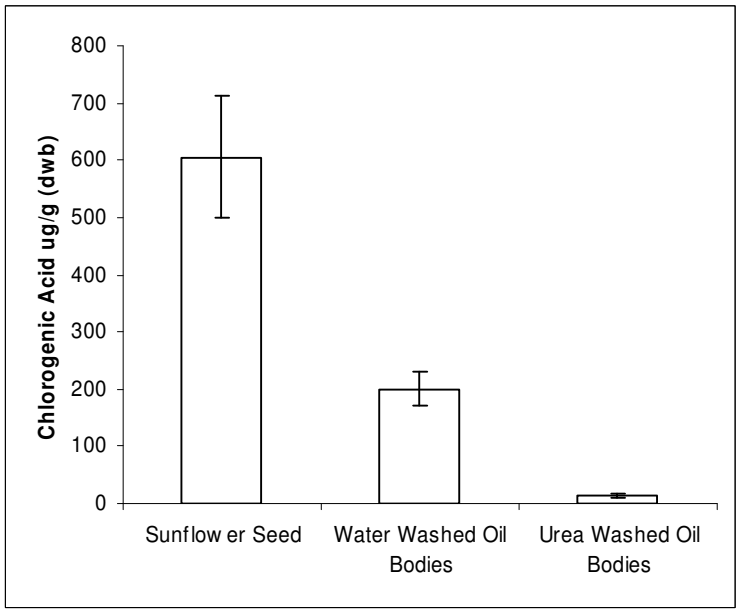



**Figure 3-11 Chlorogenic acid concentration in sunflower seeds, isolated water-washed oil bodies and urea-washed oil bodies**

Chlorogenic acid concentration was 43 times greater in sunflower seeds than in the extracted and purified urea-washed oil bodies (Figure 3-11). This suggests that although freely available in the seed matrix phenolic acids are effectively diluted out of the oil body preparation during extraction and are not strongly associated to oil body structures.

The finding that free phenolic acids are not integral components of sunflower oil bodies is unsurprising as they are hydrophilic and would be present in the highest concentration in aqueous cellular structures (apoplast and vacuole (Beckman 2000)) and not associated with oil bodies. It should be noted that free phenolic acids do have the potential to bind to proteins and charged

compounds, and if this was occurring a significant fraction would be carried over bound to the surface of oil bodies. The presence of residual phenolic acids could impart a significant reduction in quality to the taste and colour of oil bodies if used commercially.

The phytochemical study presented hereinafter illustrates the link between select lipophilic phenolic compounds and oil body structures.

### 3.2.3. Tocopherol Composition and Association to Sunflower Oil Bodies

Tocopherol is a very important lipophilic antioxidant found commonly in oilseeds; tocopherol protects cellular membranes and lipid reservoirs from oxidation by the scavenging of free radical species. Oil bodies were extracted and the levels of associated tocopherol, lipid and protein measured in the seed and extracted water-washed oil bodies, on a yield basis. To further this, urea-washed oil bodies were prepared and concentration of tocopherol isoforms and total phenolic content measured.

Table 3-2 details the yield of total mass, protein, lipid and tocopherol during the extraction of water-washed oil bodies. Water-washed oil bodies contain a significant level of tocopherol (38% of the seed tocopherol). Tocopherol in the residue represented 27% of the seed tocopherol, indicating the total recovery of tocopherol was 65%. This suggests an intrinsic association of tocopherol to water-washed oil bodies.

**Table 3-2: Extraction Yields of Lipid, Protein, Tocopherol and Phenolics in Oil Bodies [a] Relative to Parent Seed**

**[a] The values shown are means ± SD of three replicates. [b] Total phenolic content (TPC) was determined by Folin Ciocalteau method (mg gallic acid equivalent). [c] Total recovery of tocopherol is 65 %. This is calculated from 100 x ([oil body] + [residue])/[seed], where [sample] is total mass of tocopherol in each sample. Residue is the seed meal remaining after oil body extraction**

| | Mass | | Protein | | Lipid | |
|---|---|---|---|---|---|---|
| | Mass (g) | Yield | Mass (g) | Yield | Mass (g) | Yield |
| Seed | 47.5 ± 0.06 | 100% | 11.8 ± 0.94 | 100% | 12.9 ± 2.2 | 100% |
| WWOB[d] | 8.18 ± 2.0 | 17% | 2.04 ± 0.00 | 17% | 3.07 ± 0.32 | 24% |

| | TPC[b] | | Total tocopherol [c] | | |
|---|---|---|---|---|---|
| | Mass (mg) | Yield | Mass (mg) | Yield | Mass (mg/g Lipid) |
| Seed | 130 ± 3.9 | 100% | 4.66 ± 0.42 | 100% | 0.36 |
| WWOB[d] | 9.00 ± 0.56 | 6.9% | 1.75 ± 0.20 | 38% | 0.57 |

Table 3-2 shows that 17% of the seed weight can be extracted via one cycle of this aqueous oil body extraction, which equates to a lipid extraction yield of 24%. Repeat extractions showed a further release of oil bodies (data not shown) but a high extraction yield of oil bodies was not essential for this comparative study.

The total tocopherol extraction yield (38%) is significantly greater than the extraction yield of lipid (24%) and protein (17%). This suggests that tocopherol is either intrinsically associated to oil bodies in vivo, and thus concentrated during oil body extraction, or passively associated with the proteins that bind weakly to the oil body as an artefact of their extraction. This increase in concentration of tocopherol during oil body extraction is further supported if we compare the total tocopherol concentration on a lipid basis which increased from 0.36 $mg.g^{-1}$ lipid in the seed to 0.57 $mg.g^{-1}$ lipid in the

oil body preparations (P>0.1). Nolasco (2004) reported a tocopherol concentration of 0.39 to 1.9 mg tocopherol. g $oil^{-1}$ for intact sunflower seeds.

**Table 3-3: Tocopherol and Total Phenolic Content (TPC) of Sunflower Seed and Extracted Water-washed (WW OB) and Urea-washed Oil Bodies (UW OB)**

**The values shown are means ± SD of three replicates calculated on a dry weight basis. [a] Not detected.**

| | mg Tocopherol.$kg^{-1}$ oil | | |
|---|---|---|---|
| | Seed | WW OB | UW OB |
| α –Tocopherol | 92.7 ± 8.2 | 200 ± 23 | 368 ± 50 |
| β – Tocopherol | 4.64 ± 0.51 | 12.2 ± 1.6 | 21.7 ± 1.1 |
| γ – Tocopherol | 0.53 ± 0.053 | 1.24 ± 0.19 | 1.93 ± 0.23 |
| δ –Tocopherol | 0.30 ± 0.015 | 0.93 ± 0.15 | nd [a] |
| Total tocopherol | 98.2 | 214 | 392 |
| Isoform ratio α:β:γ:δ | 94.4:4.7:0.5:0.3 | 93.3:5.7:0.6:0.4 | 94:5.5:0.5:0 |
| | mg gallic acid equivalent.$kg^{-1}$oil (dwb) | | |
| | Seed | WW OB | UW OB |
| TPC | 2743 ± 83 | 1066 ± 69 | 518.8 ± 110 |

The strength of association of general phenolic compounds and tocopherol, to oil bodies, can be determined through differential washing of oil bodies. The loss of residual (non-intrinsic) proteins with urea-washing (Figure 3-9) was associated with a significant reduction in total phenolics (Table 3-3). All tocopherol isoforms increased in concentration with purification of oil bodies in contrast to TPC values (apart from δ –Tocopherol that was not detected in the urea-washed oil bodies). This supports the hypothesis that oilseed tocopherol is intrinsically bound to oil body structures and that oil body isolation offers a novel route for tocopherol extraction.

What remains unclear at this stage is how tocopherol, known to be synthesised in plastids, becomes an intrinsic component of sunflower oil bodies. Perhaps as suggested by Draper (1980) and Hovarth (2003), an

extra-plastidic location of vitamin E synthesis exists, or an intracellular transport mechanism directs significant amounts of tocopherol to sunflower seed oil bodies.

The presence of tocopherol in sunflower seed oil bodies strongly suggests that it would protect the polyunsaturated fatty acids in oil bodies against oxidation. This would support the suggestion made by Sattler and co-workers (2003) that tocopherol would have to be associated with oil bodies to deliver the required level of antioxidant protection for the seed.

This finding is the first time it has been shown that oil bodies of any plant species contain intrinsic tocopherol (Fisk and Gray 2003; Fisk, Gray et al. 2003; Fisk, White et al. 2004; Fisk, White et al. 2006; White, Fisk et al. 2006). This has significant implications in terms of tocopherol biosynthesis and commercial oil body applications, with benefits including increased natural antioxidant concentration and continued antioxidant protection. Evidence for the increased oxidative stability of these natural emulsions is given in chapter 5.

There are many other examples of natural emulsions; two examples, in addition to oil bodies, are milk fat globules and chylomicrons. Chylomicrons are a by-product of mammalian digestion, once fat is ingested it passes into the small intestine then is emulsified by the gall bladder and forms small protein/phospholipid stabilised droplets that enter the blood, chylomicrons. These chylomicrons then either deliver TAG direct to muscles or are converted in the liver to very low density lipoproteins that deliver TAG to organs and tissues. Milk fat globules (MFG) are lipid droplets formed by budding from the mammary epithelial cell that deliver nutrients and energy to developing offspring.

Both chylomicrons and MFG contain vitamin E in varying levels, reported concentrations of tocopherol in chylomicron and MFG are 1-2mg

tocopherol.mL$^{-1}$ lipid (Couderc, Peynet et al. 1998) and 13-30 µg tocopherol.mL$^{-1}$ lipid (Jensen and Nielsen 1996) respectively.

The presence of tocopherol in MFG delays the onset of lipid autooxidation (Erickson, Dunkley et al. 1964), Stapelfeldt and co-workers (Stapelfeldt, Nielsen et al. 1999) showed a reduction in free radical formation with increasing tocopherol concentration in LDL particles, and other workers have shown that spiking MFG suspensions with additional tocopherol rapidly increases their resistance to autooxidation. MFG associated tocopherol is membrane bound, and contributes to a reduction in the development of oxidation. The concentration of tocopherol in MFG is much lower than that of oil bodies (0.4 mg tocopherol. g$^{-1}$ lipid) which suggests that the intrinsic association of tocopherol to oil bodies will probably contribute to increased oxidative stability.

The concentration of tocopherol in chylomicron is much higher than MFG, in healthy individuals this reflects the tocopherol concentration of ingested lipid rather than a biologically predefined tocopherol level. Chylomicron tocopherol concentrations although dependant on levels of ingested tocopherol over short time periods (10hr) will revert to normal biological levels in fasting individuals as chylomicron tocopherol is mobilised to very low density lipoproteins (vLDL) and low density lipoproteins (LDL), during deposition of lipid to tissues.

In humans, vitamin E originates solely from dietary sources. A normal biological concentration of tocopherol will facilitate in the removal of lipid free radical species and prevent oxidation and subsequent illnesses. Additional supplementation has been shown to reduce the occurrence of second heart attacks. In general, vitamin E is considered to be beneficial in coronary artery disease, however, intervention studies overall show a null effect of vitamin E on atherosclerosis. These results are confounded by the knowledge that under specific conditions the presence of tocopherol in LDL may have a pro-

oxidant effect, through the mechanism of tocopherol mediated peroxidation, although this may be limited by the inclusion of tocopherol co-antioxidants that would reduce the number of tocopherol radicals, examples of such co-oxidants include ubiquinol-10, ascorbate, and bilirubin (Upston, Terentis et al. 1999).

# 4. Physical Characterisation of Sunflower Oil Body Suspensions

## *4.1. Imaging of Sunflower Oil Bodies*

### 4.1.1. In-vivo

Oil bodies are pseudo-spherical neutral lipid storage organelles found commonly across all oilseeds. Their abundance and size is influenced by the density of surface active proteins (principally oleosin), lipid content of the oilseed (Huang 1994) and the environment the seed was exposed to during its life cycle (Berjak and Pammenter 2003). Oil bodies are found in a range of oilseed tissues (root tips, leaves and root nodules), but highest concentrations are located in the cotyledon of dehulled seeds (Murphy, Hernandez-Pinzon et al. 2001).

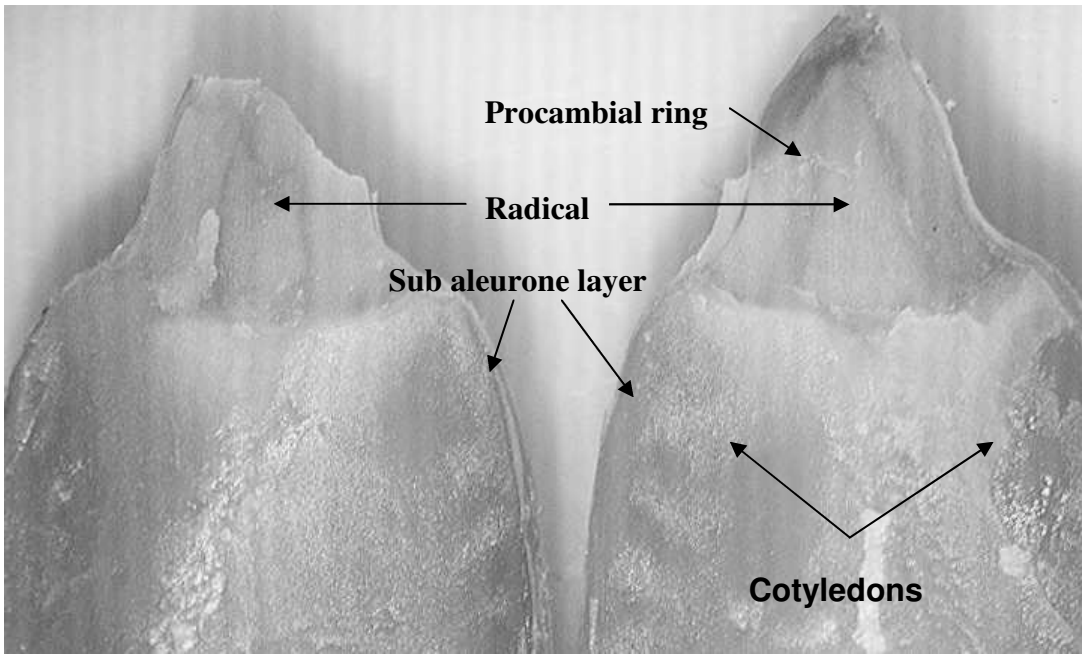



**Figure 4-1 Sunflower seed dehulled and sectioned.**

Oil body density is dependant upon the cell type and location. De-hulled sunflower seeds are used within this study as hull tissue contains a low number of oil bodies and the presence of the hull reduced the efficiency of the extraction process. Sunflower seeds were chosen as they have a high lipid content and stable oil bodies that could be extracted with high efficiency. Dehulled seeds are composed principally of cotyledon tissue (Figure 4-1) containing a high density of oil bodies (Figure 4-2). If we focus on the cotyledon of a sunflower seed, we can see from Figure 4-2 that the dark gray pseudo-spherical organelles (oil bodies) are located ubiquitously across the cell and are present in high density in cotyledon tissue.

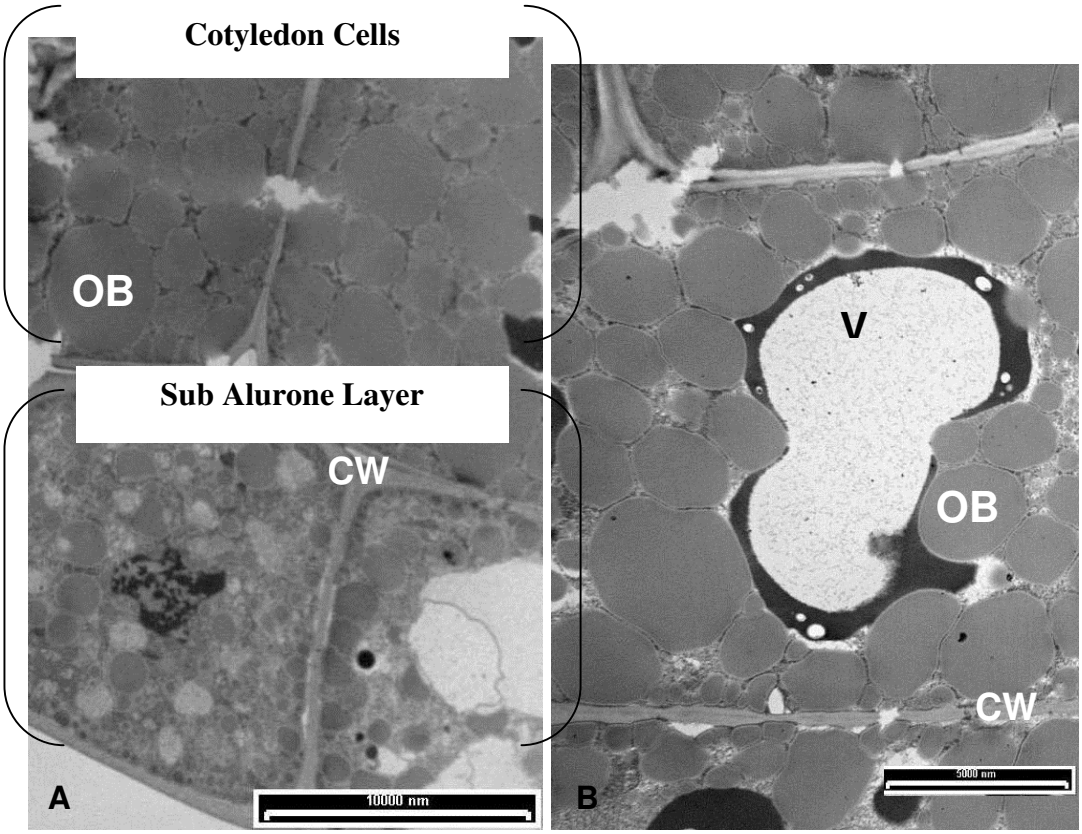



**Figure 4-2 Mature sunflower seed anatomy by TEM**

**A) Sub-alurone layer and inner cotyledon cells illustrating the density and abundance of oil bodies (OB-oil body, CW-cell wall) (Scale bar represents 10µm)**

**B) Cell anatomy of sunflower seed cotyledon cell (OB-oil body, V-vacuole, CW-cell wall) (Scale bar represents 5µm)**

Figure 4-2B illustrates some of the main structural components (vacuole, oil body, cell wall); the size of oil bodies in this transmission electron microscopy (TEM) section (representative of sunflower seeds used herein) corresponds closely to the literature values for a range of oilseeds (0.6-2.0µm) (Tzen, Cao et al. 1993) .

### 4.1.2. Ex-vivo

Due to the presence of surface active proteins, oil bodies are intrinsically stable in-vivo, these functional proteins are retained during extraction and subsequently oil bodies retain their structure, size and resistance to coalescence ex-vivo. As crude oil body preparations contain residual proteins originating from seed cell debris, a range of methods have been proposed for increasing the purity of oil bodies ex-vivo. These include suspension in a range of media to remove residual proteins, lipids, and contaminating cell debris. The three main preparations used hereinafter are water-washed, salt-washed, and urea-washed, although Tzen and co-workers (1997) also cite hexane-washing and detergent-washing as additional methods for increasing the purity of the preparation.

In preparing a food grade product, only water-washed and salt-washed oil bodies can be considered, as the other treatments use chemicals not generally recognised as food grade and would be detrimental to the concept of oil bodies as a natural product. Urea-washed oil bodies, although not classed as food grade were chosen to be investigated as they are a pure system with fewer contaminating proteins than salt and water-washed preparations therefore allowing investigation of the physicochemical properties of oil bodies with less residual cell debris. Figure 4-3 shows a TEM image of urea-washed oil bodies suspended in pH 7.5 buffer. Urea-washed oil bodies are stable intact entities with a diameter of 0.5-2.5 µm, although in

some cases they appear to aggregate (possibly an artefact of the TEM preparation) they do not coalesce or phase separate.

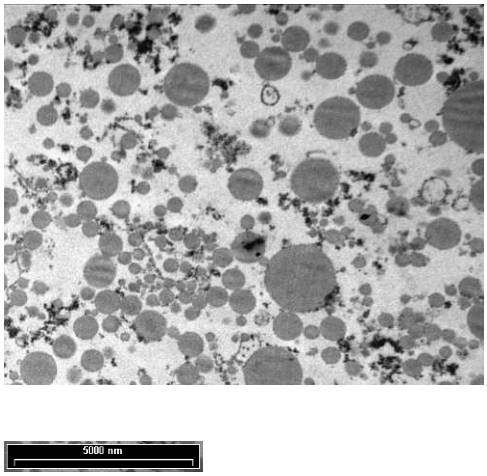



**Figure 4-3 Urea-washed sunflower oil bodies imaged by transmission electron microscopy (Scale bar represents 5µm)**

## *4.2. Effect of Washing on the Physical Stability of Oil Bodies*

Washing treatments have been reported to alter the chemical composition of oil bodies, with varying chemical treatments removing a range of associated proteins and lipids. We speculated that the method of washing will also affect the surface chemistry of oil bodies, and consequentially their physical stability in suspension will be altered. Oil body physical stability as affected by washing treatment was assessed though microscopy, size distribution analysis, surface charge and rheological measurements.

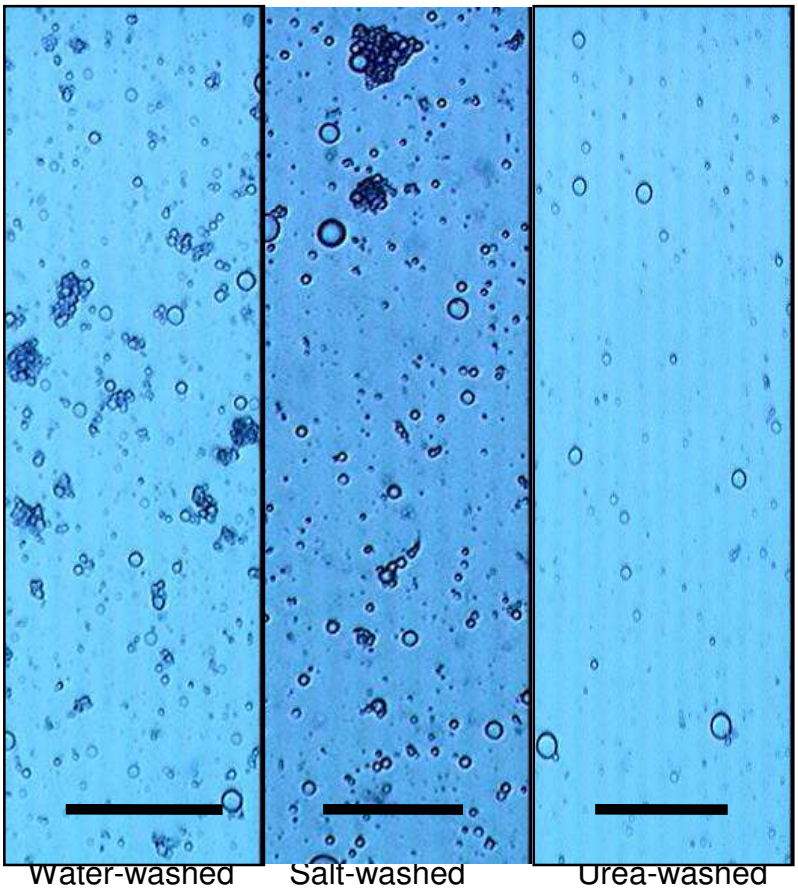



**Figure 4-4 Light microscope images of washed preparations of oil body (water-washed, salt-washed and urea-washed) size bar measures 10µm**

The water-washed preparations are the least pure preparation (as shown in section 3.1) and have a tendency to clump forming aggregates (Figure 4-4 and Figure 4-5). These aggregates can be disturbed and broken up by shear or dilution (data not shown).

Salt-washed oil bodies are of a greater purity than the water-washed oil bodies, but the likely presence of residual salt would affect the surface charge of the oil bodies, the sodium ions mask the natural negative charge of the oil bodies reducing their effective hydrodynamic diameter (through compression of the stern layer) and reduces their effective surface charge, this reduction in effective surface charge decreases their emulsion stability

leading to the formation of aggregates (Figure 4-4 and Figure 4-5) the physical mechanisms behind this are further discussed in section 4.4.

Urea-washed oil bodies are the highest purity preparation of oil bodies used in this study and represent a clean preparation with limited residual proteins (section 3.1.4). The size distribution of urea-washed oil bodies show a monomodal distribution with a numerical mean diameter of about 2µm (Figure 4-5). In salt and water-washed preparations the most frequent particles have a diameter of about 2µm but there are also particles of higher apparent diameter (up to 10µm) due to the presence of aggregated particles. It should be noted that the graph (Figure 4-5) illustrated shows % volume distribution and is extremely sensitive to the presence of high diameter particles. This increased sensitivity is because on a volume basis one 10µm particle would occupy the same volume as 1000 1µm particles, the graph is therefore 1000 x more sensitive to the presence of 10µm particles than it is to those of 1µm diameter.

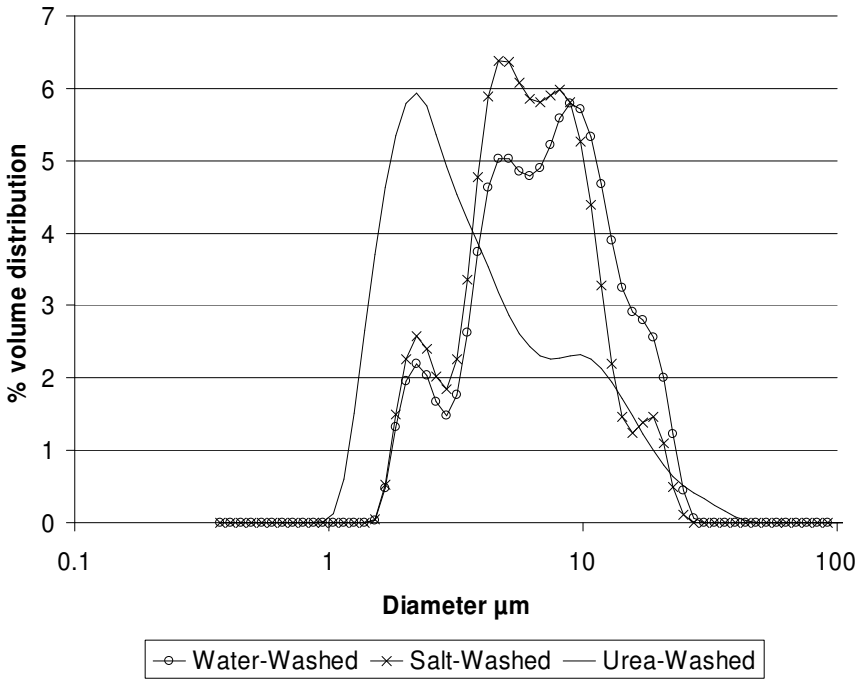



**Figure 4-5 Sunflower oil body apparent diameter size distribution (volume based) for water-washed, salt-washed and urea-washed preparations**

**Results are presented as a curve not a histogram to facilitate ease of comparison.**

## 4.3. Physical Stability

An understanding of the physical stability of oil bodies is critical when commercial applications are to be considered. The most important aspect of oil body physical stability is an appreciation of the conditions that combined would contribute to a loss to stability, for example elevated temperatures, or media pHs that would cause a loss of oil body integrity.

### 4.3.1. Trypsin Digestion

Oil bodies after extraction and washing form a stable suspension that is colloidally stabilised by a combination of electrostatic repulsion produced by the negative surface charge and steric hindrance provided by the oil body associated proteins.

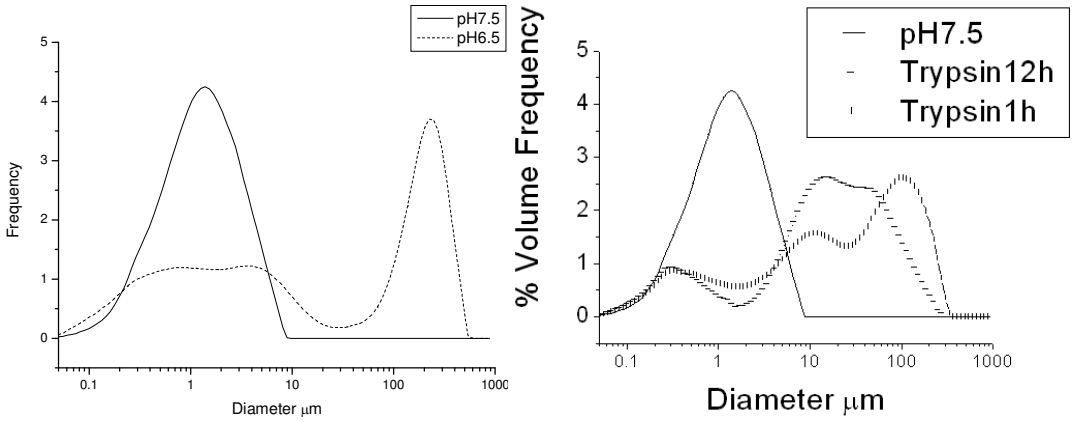



**Figure 4-6 Volume based apparent droplet size distribution analysis.**

**A) urea-washed sunflower oil bodies suspended in pH 7.5 and pH 6.5 10mM sodium phosphate buffer. B) Urea-washed oil bodies digested with trypsin (1hr and 12hr).**

Oil bodies were imaged at a neutral pH (pH 7.5) and again at a reduced pH (pH 6.5) to assess the effect of reduced surface charge and again after enzymatic digestion of surface proteins (pH 7.5) to separate the effect of electrostatic repulsion and steric hindrance. In the reduced pH suspension oil bodies aggregated (Figure 4-7) but did not coalesce. The presence of oil

body associated proteins prevented coalescence. Oil bodies were then digested (pH 7.5) with trypsin (enzymatically degrades proteins) and after 1hr oil bodies enlarged in size through coalescence (Figure 4-6). After extended digestion (12h) the oil bodies had further coalesced and formed flocs of aggregated coalesced oil bodies (Figure 4-7) illustrating the importance of surface proteins in stabilising oil bodies by steric hindrance. This was also shown by White and Fisk (2005) for oil bodies of both sunflower seeds and cereal grains.

Through this fundamental study the effects of electrostatic repulsion and steric hindrance can be separated. Electrostatic repulsion maintains an effective suspension resisting oil body aggregation and steric hindrance prevents oil body coalescence. This highlights the importance and significance of oil body associated proteins.

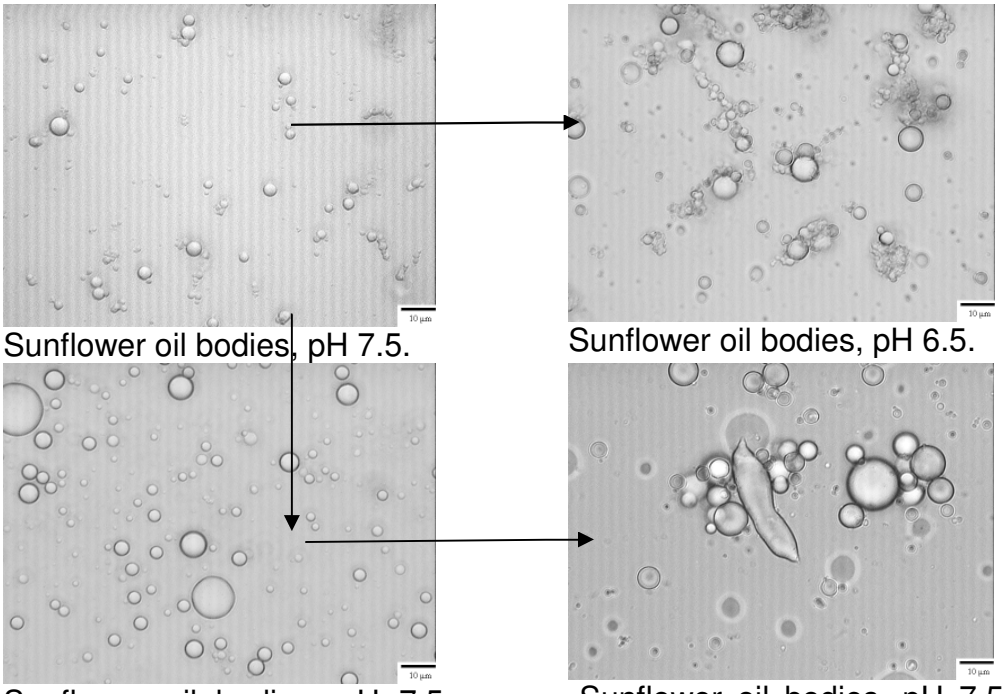



**Figure 4-7 Urea-washed sunflower oil bodies at pH 7.5, pH 6.5 and after trypsin digestion for 1hr and 12hr.**

### 4.3.2. pH Stability

To further understand the electrostatic stabilisation of sunflower oil bodies, three preparations (water-washed, salt-washed and urea-washed) were dispersed in media at a range of pH. Samples were analysed for emulsion stability by creaming index (a measure of the fraction of the emulsion that floats to the top forming a solid layer over a fixed period of time).

Figure 4-8 shows the stability of the three washed preparations of oil bodies to creaming. The point of maximum creaming is proposed to coincide with the isoelectric point of the suspension, for urea-washed oil bodies this point is between pH 4.0 and pH 6.0. The less pure preparations (water-washed and salt-washed) creamed to a greater extent at most pHs when compared to the urea-washed preparations.

The water-washed preparation creamed maximally at pH 3, indicating a lower pI for the least pure water-washed oil body. The pH media of minimum creaming for all preparations was pH 2.

From this data it can be estimated that the isoelectric points of water-washed, salt-washed and urea-washed oil bodies are in the range pH 3.0-6.0; pH 4.0-7.0 and pH 4.0-6.0 respectively.

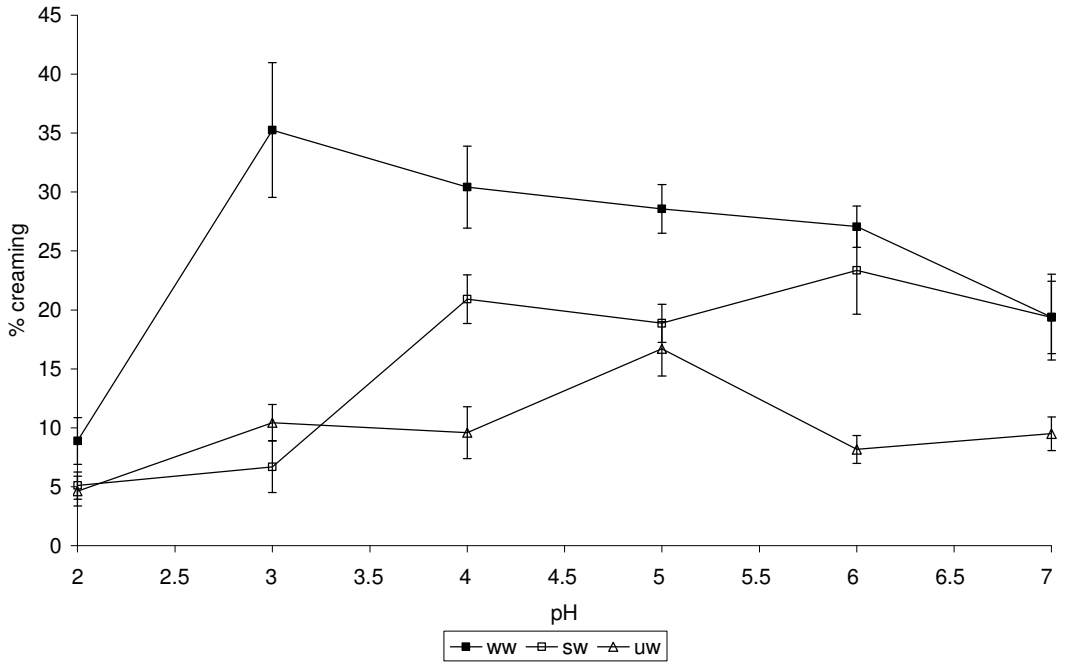



**Figure 4-8 % Creaming Index of three preparations of sunflower oil bodies**

**Oil body preparations (water-washed WW, salt-washed SW and urea-washed UW) suspended in acid to neutral pH buffered solutions (pH 2, pH 3, pH 4, pH 5, pH 6, pH 7).**

Urea-washed oil bodies were prepared and their optical absorbance measured at 680nm (Figure 4-9) it can be seen that between pH 4.5 and pH 5.5 the optical absorbance is the lowest indicating the formation of aggregates (Figure 4-9), unfortunately this data was not repeated with the salt and water-washed samples. This data correlates with the creaming index data that indicated the pH of lowest emulsion stability would be between pH4.0 and pH6.0 for urea-washed oil bodies (Figure 4-8).

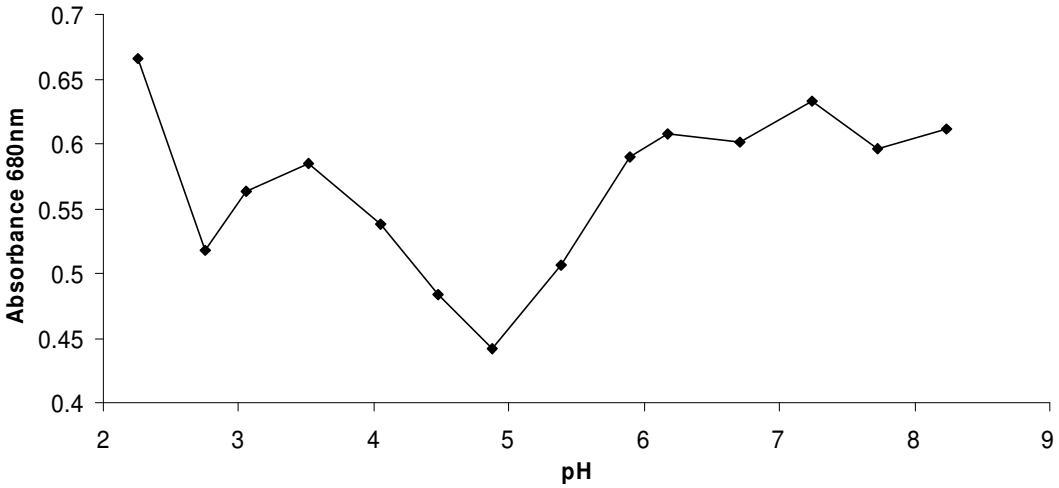



**Figure 4-9 Optical absorbance (680nm) of urea-washed oil bodies over a pH range.**

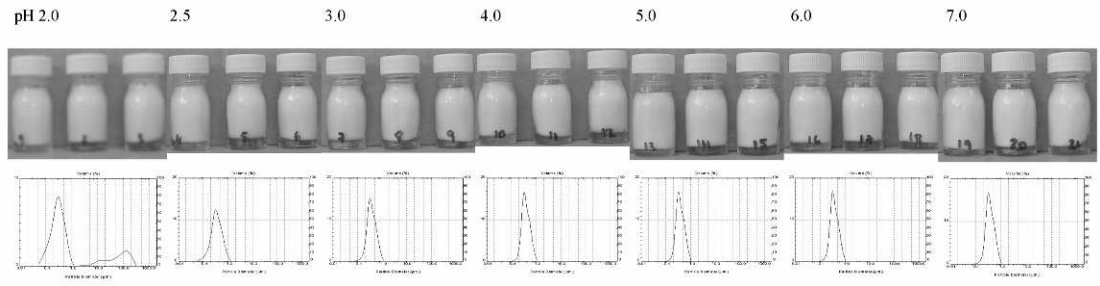



**Figure 4-10 pH stability of Intralipid**

**(Size distribution and physical appearance at pH 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 and 7.0)**

Intralipid is a commercially available medical preparation for intravenous nutrition of patients (Kamei, Hachisuka et al. 2005). Intralipid is composed of purified soya bean oil stabilized by egg phospholipids. Intralipid was studied as it is used as a standard for the zeta-potential data discussed in Section 4.4.1. Intralipid was exposed to media of the same pH as the washed oil bodies. Intralipid was stable across the pH range (Figure 4-10) with the numerical mean diameter stable at 0.4µm. Intralipid was chosen as, although smaller, it can be considered as comparable to an oil body preparation with no stabilizing proteins. The resistance to aggregation or creaming suggests

that the low droplet diameter of Intralipid may be contributing to its stability over the experimental time course.

### 4.3.3. Thermal Stability

Due to the presence of stabilising proteins and phospholipids, oil bodies should be resistant to mild processing forces such as low level shear and thermal stresses. To assess their thermal stability oil body preparations were exposed to elevated temperatures for 2 days and particle size, creaming index and physical appearance (microscopy) measured.

As previously discussed (section 4.3.2), freshly prepared urea-washed oil bodies had a tight size distribution close to 1µm, salt and water-washed preparation had bimodal distributions.

After exposure to thermal stress, urea-washed oil bodies showed limited physical changes over 2 days. The size distribution profile did change slightly (Figure 4-11), but the numerical mean diameter was unchanged at about 1.2µm. Salt-washed oil bodies were also relatively stable, with only slight increases in numerical mean diameter; the size distribution profile showed that although the oil bodies did not coalesce, some aggregated particles did form.

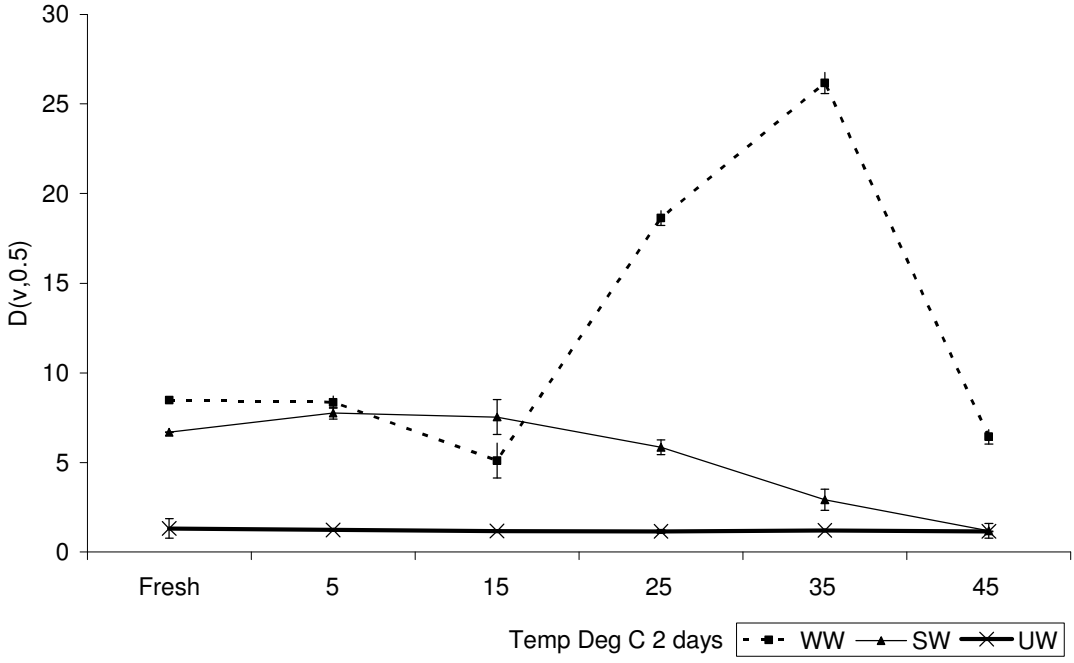



**Figure 4-11 Thermal stability of sunflower oil body preparations washes**

**(Water-washed WW, salt-washed SW and urea-washed UW). Size distribution D(v,0.5) at 5°C; 15°C; 25°C; 35°C and 45°C.**

Water-washed oil bodies were the least stable and showed significant coalescence above 25°C with significant creaming. This could be due to residual proteins masking steric hindrance and surface charge therefore reducing the spatial distance between oil bodies; this would facilitate their coalescence. It should also be noted that some oil droplets in the water-washed preparations may be weakly stabilized by non-oil body associated proteins and when subjected to thermal stress, are not stable enough to retain their structure. This excess oil would then fuse with neighbouring oil bodies increasing their volume and extending the surface area that the oil body associated proteins are required to stabilize, thus reducing the stability of the previously intact oil bodies and destabilizing the entire system. This was confirmed by Peng (2003) who showed that artificial oil bodies formulated with an elevated lipid : protein ratio had reduced thermal stability and coalesced at below 40°C. Peng also showed that purified oil bodies were stable to 50°C; this thermal stability could be increased by the addition of

chemical crosslinking agents such as glutaraldehyde and genipin. Extracted oil bodies stabilized by chemical crosslinking were thermally stable to 90°C.

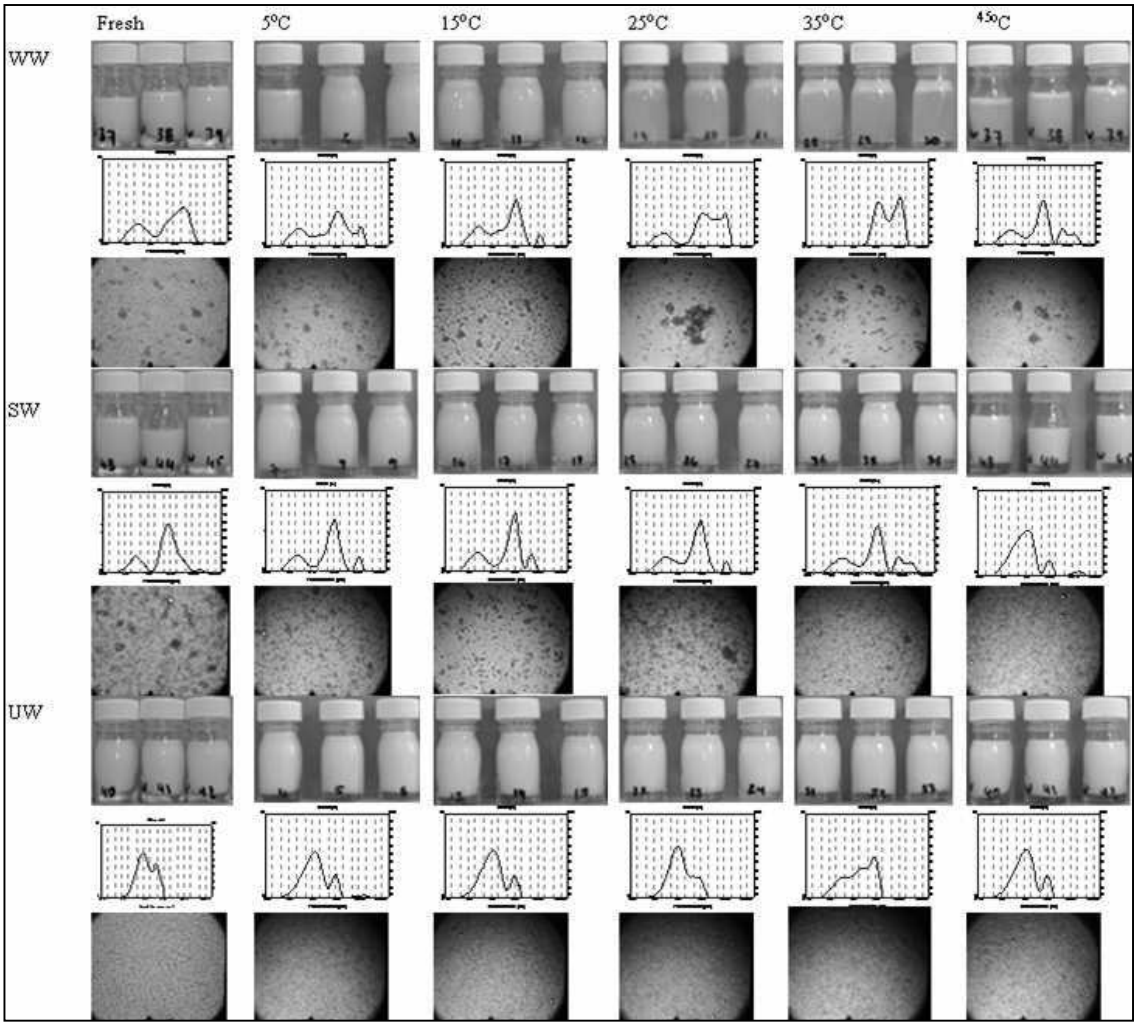



**Figure 4-12 Thermal stability of three sunflower oil bodies**

**(water-washed, salt-washed and urea-washed) Physical stability of vials, size distribution and visual appearance by light microscopy is shown.**

## *4.4. Surface Charge Analysis*

As discussed in Section 4.3.2, the stability of oil body suspensions is significantly pH dependant, this is a consequence of the surface charge imposed by oleosin proteins and charged lipid species varying with media pH. Fundamental knowledge of pH stability is critical to future commercial applications as it offers the ability to predict when oil body will aggregate,

when they will be stable and when their surface charge reverses and the oil bodies become positively charged.

Tzen and co-workers (1993) have presented data on the pH stability and isoelectric point of oil bodies by isoelectric focussing (IEF). The measured isoelectric point of a complex multiphase system is significantly method dependant. IEF uses a system whereby oil bodies are exposed to an electric potential, this forces them to migrate through a series of membrane-separated chambers reducing in pH. When the suspension flocculates it can no longer pass through the 10µm membrane that separates the chambers. The chamber in which aggregation is initiated is designated the isoelectric point of the suspension. Our earlier work (section 4.3.2) has shown that oil bodies aggregate not only at their isoelectric point (pI) but also in media of a range of pH values surrounding the pI casting doubts on the reliability of isoelectric focussing as a method of assessment of oil body isoelectric point. This theory is supported by work by Tzen and co-workers (1992) who found a different pI when the IEF was reversed and the oil bodies were exposed to the electric field at low pH and allowed to migrate from the low pH to the high pH regions.

In an attempt to further probe the pI of oil body suspensions, three separate methods (creaming index, zeta potential and streaming potential) were compared to gain fundamental knowledge, which combined, suggests a more accurate isoelectric point of three oil body suspensions (water-washed, salt-washed and urea-washed).

### 4.4.1. Zeta Potential of Sunflower Oil Bodies

Zeta potential is a measure of the surface potential of a colloidal system measured when exposed to an oscillating voltage across a measurement cell. Any charged species in the measurement cell will move as the voltage changes and the surface potential can be calculated from the measured velocity. It should be noted the zeta potential is a measure of the charge

outside of the hydration radii of a colloidal system, at the stern layer, which is a short distance from the surface of the colloidal suspension (hydration radii and stern layer are discussed in detail in section 1.2.1).

Sunflower oil bodies were prepared at pH 7.5, suspended in a range of pH buffers from pH 2.0 to pH 8.0 and their zeta potential measured. All oil bodies at pH 5.0 - pH 8.0 were electronegative with the water-washed and urea-washed preparations being similar in surface charge, as detailed by Fisk et al. (2003; 2005).

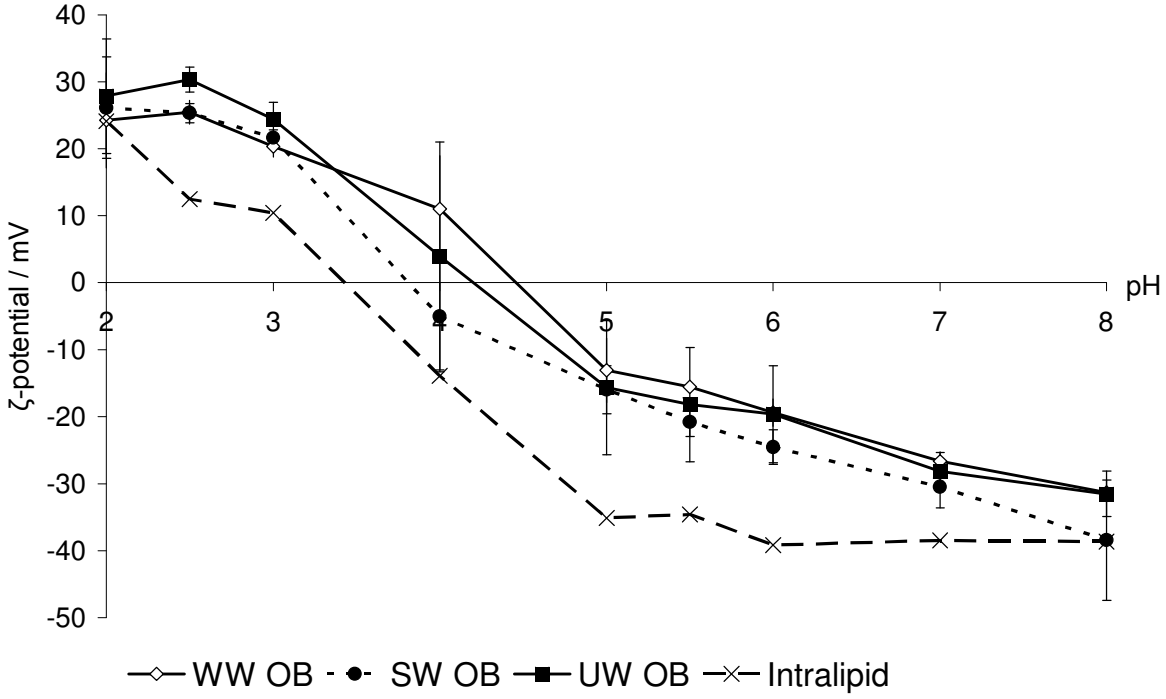



**Figure 4-13 Zeta Potential of three preparations of sunflower oil bodies**

**Water-washed, salt-washed, urea-washed oil bodies and Intralipid (a phospholipid stabilized emulsion) over a pH range 2<pH<8.**

Oil body zeta potential at pH 7.0-8.0 is about -30mV, this charge is sufficient on its own to stabilize a colloidal suspension of a comparable size. As the media pH approaches the isoelectric point (pI) of the oil body preparation the zeta potential reduces, and the oil bodies do not move in the suspension when exposed to an electric field. Although the precise pI cannot be

conclusively derived, the data would suggest that the pI of all oil body suspensions falls between pH 3.0 and pH 5.0. This is very revealing as current literature values for oil body isoelectric points are much higher and suggest the isoelectric point lies between pH 6.0. and pH 6.8 (Vance and Huang 1987; Qu, Vance et al. 1990; Tzen and Huang 1992; Tzen, Cao et al. 1993). A possible reason between this discrepancy is the difference in methodology, also the data from isoelectric focusing experiments uses the point of first aggregation to define pI rather than the point of zero charge. Interestingly, work by Chen and Chyan (2004) on isoelectric focusing of artificial oil bodies reconstituted with caleosin showed that aggregation started to occur at pH 6.0 but maximum aggregation was observed at pH 4.0.

It is difficult to differentiate between the measured pI of the three preparations as the natural experimental variation in the region of an emulsions pI is often greater than that measured above and below the pI, this was observed in our data set and estimates for pI are shown in Table 4-1.

Oil bodies are positively charged on the acidic side of their pI reaching a zeta potential of +30mV at pH 2.5.

The surface potential of the oil body preparation measured over a pH range was compared with the zeta potential profile for an Intralipid preparation. Intralipid is an artificial emulsion formed from soya bean oil and egg phospholipid, the surface charge of the Intralipid emulsion is most likely determined by the phospholipids. The intention of the comparison of Intralipid with washed oil bodies was to evaluate the possible contribution charged lipids have on the surface charge of oil bodies. Zeta potential profiles showed that there are no significant differences in the shape of the zeta potential vs pH curves for Intralipid and the oil body samples. There are however, some differences in the pI values derived from these plots.

The major phospholipid present in Intralipid is phosphatidylcholine (PC) (section 2.5.8). Given the balance of positive and negative charge in this

complex lipid at around neutral pH one must surmise that there is an unidentified acidic element in Intralipid that renders it negatively charged at pH7, only to be neutralized at a low pH. Interestingly when in an emulsion or liposome, PC is negatively charged at neutral pH, this may be due to shielding of the positive charges, or due to its conformational arrangement within the surface membrane. Sabin (2006) has shown that liposomes formulated from PC in a media of pH 6 have a negative zeta potential at neutral pH and an isoelectric point of around pH3-pH4; the isoelectric point varies depending on the pH of the media during liposome formation, this may suggest that the topology of the PC at the interface may vary and subsequently induce a variable pI. The zeta potential data shown in Figure 4-14 shows a strong similarity to urea washed oil bodies and Intralipid (Figure 4-13). This suggests that the phosphatidylcholine may be the controlling factor in the electrophoretic mobility of oil bodies, and any additional charged species may be physically shielded or their charges may be interacting with other charged species on the surface of the oil body.

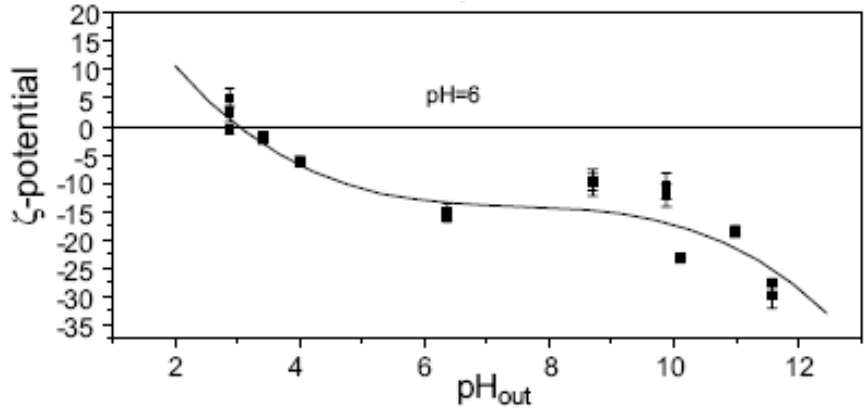



**Figure 4-14 Effect of solution pH on phosphatidylcholine stabilised liposomes formulated at pH 6 (Sabin, Prieto et al. 2006)**

From both zeta potential measurement (Section 4.4.2) and streaming potential measurements (data not shown) the pI of Intralipid was between pH 2.5 and pH 4.0. It is interesting to note that although Intralipid manifests an isoelectric point, there is little evidence of intralipid aggregation, this is

probably due to the small diameter of these entities as shown in section 4.3.2 (0.4μm) compared with oil bodies (over 1μm).

### 4.4.2. Streaming Potential

Streaming potential measurements were used in addition to the zeta potential measurements to further substantiate the isoelectric point measurements. It should be noted that the basis of streaming potential is different to that of zeta potential. Oil bodies are bound to a polystyrene piston that oscillates in an aqueous solution of counter ions, as the piston oscillates the voltage across the solution is measured which is imposed by the charge of the counter ions as they move opposing the movement of the charged oil bodies on the piston. This aqueous solution is then titrated by addition of dilute hydrochloric acid to the point at which no change in the voltage is measured during oscillation of the piston. This point is designated as the isoelectric point by streaming potential. Streaming potential results are given in Table 4-1 and show that the pI by streaming potential is between pH 5.0 and pH 6.1 for all oil body suspensions.

The pI results from streaming potential measurements are different to that obtained by zeta potential and creaming index and suggest that the isoelectric point is higher. The reason for this is unknown but it could be due to the presence of unidentified enzymatic activity modifying the profile of charged lipids during storage, though the breakdown of phospholipids (Section 3.1.3) or formation of free fatty acids (Figure 3-3).

| | Absorbance | Creaming index | Zeta-potential | Streaming potential |
|---|---|---|---|---|
| Water-washed | | 3.0-6.0 | 3.0-5.0 | 5.0 ± 0.1 |
| Salt-washed | | 4.0-7.0 | 3.0-5.0 | 5.5 ± 0.3 |
| Urea-washed | 4.5-5.5 | 4.0-6.0 | 3.0-5.0 | 6.1 ± 0.4 |

**Table 4-1 Isoelectric point estimates by creaming index, absorbance, apparent droplet diameter (D(v,0.5)), streaming potential, and zeta potential**

The values for isoelectric point of the three washed oil body suspensions are given in Table 4-1 and show a marked difference to those obtained from

isoelectric focusing ((pH6.0 < pI < pH6.8) for oilseed rape, sesame, cotton, flax, maize and peanut (Tzen and Huang 1992; Tzen, Lie et al. 1992; Tzen, Cao et al. 1993; Tzen, Peng et al. 1997; Froese, Nowack et al. 2003; Peng, Lin et al. 2003)). This could be explained by one of two reasons, firstly the effect of titrating a solution of oil bodies with acid to reach an isoelectric point may affect the conformation and topology of surface proteins and phospholipids compared to dispersing a suspension directly into media at the pH of interest, this may also explain the differences in the streaming potential and zeta potential results. Secondly, isoelectric focusing only measures the point of first aggregation, which, as discussed previously is not necessarily the point of zero charge, or isoelectric point.

It has been suggested that a minimum surface charge is required to stabilize an emulsion, for oil body suspensions a minimum value of +/-20mV could be hypothesized. If this were to be the case, and the pH of the media was more acidic than pH 5.0, the magnitude of the zeta potential would not be sufficient to stabilize the suspension and the oil bodies would aggregate. In very acidic media, oil bodies are positively charged and again electrostatically stabilised (Section 4.3.2). This is observed to be the case in the urea-washed oil bodies as maximum creaming occurred in the range pH 4.0 - pH 6.0; In addition, minimum creaming occurred in all washed preparations at pH 2.0 indicating the positively charged emulsions are very stable and resistant to creaming.

## *4.5. Rheology*

To the best of the authors knowledge there is only one previous study investigating oil body rheology (Mason and Friis 2001). This study investigated the oscillatory rheometry in the linear viscoelastic region, but does not elaborate further into the rheology of oil body suspensions or how they would be functional as a food ingredient, the paper concludes that oil body creams are weak gels with a delicate structure.

A range of experiments was designed to understand the rheology of oil body suspensions at a range of concentrations and levels of purity. Freshly prepared water-washed oil bodies were diluted to a known solids content and their rheology measured using double gap geometry, rotational rheometry.

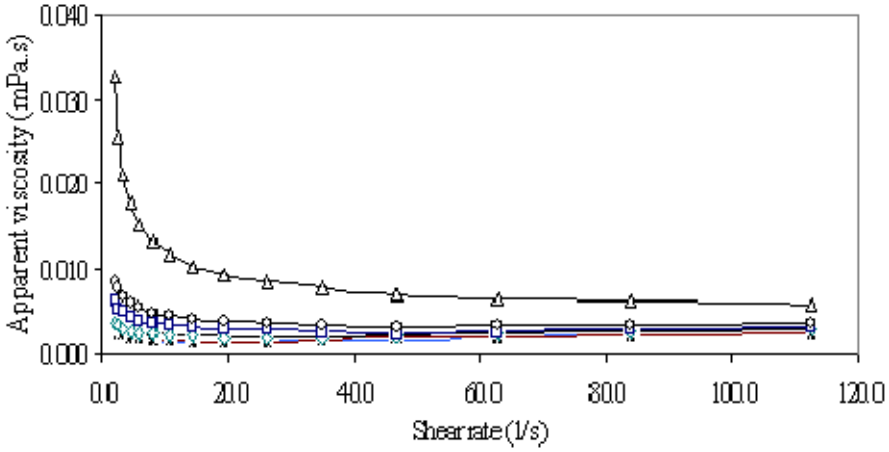



**Figure 4-15 Apparent viscosity at a range of shear rates for fresh water-washed oil body**

**Prepared at a range of dry weight concentrations (+, 1 % (w/v); ∗, 2 %; x, 4%; ◊, 10; □, 20%; o, 30%; Δ, 40).**

All oil body suspensions exhibited shear thinning behavior as is classically expected for a food emulsion; this was detailed by Fisk et al. (2003) through a comparison of washed oil body emulsions to artificial emulsions and concluding that both samples follow similar rheological models. Shear thinning behavior of oil body suspensions is due to two combining factors: the alignment, deformation, and disruption of flocculated particles and the spatial ordering of discrete particles. McClements (2000) suggests that an increase in concentration will increase the apparent viscosity and that the gradient of the apparent viscosity-shear rate graph will be dependant upon this concentration and the shear rate imposed. Experimental results correlated closely with McClements suggestion and the power law equation (Equation 4-1), commonly used to predict emulsion rheology ($R^2$>0.96 for fresh and freeze dried samples).

$$\eta = K\dot{\gamma}^{n-1}$$

Where: $\eta$ = Viscosity, $\dot{\gamma}$ = shear rate, K = consistency index; n = power law index

**Equation 4-1 Power law model**

The effect of water-washed oil body solids concentration is shown more clearly in Figure 4-16. The apparent viscosity increases with increasing concentration, but the actual relationship is heavily dependant upon the method of preparation. Water-washed and urea-washed oil bodies show a comparable viscosity, but the salt-washed oil bodies show a greater apparent viscosity at any given concentration, this may be due to the presence of flocculated oil bodies. When small amounts of salts are present in an oil body system, the salt will disperse into the hydration radii of oil bodies. This will promote aggregation through a reduction in the diameter of the stern layer and a reduction in the effective surface charge. When oil bodies flocculate, part of the continuous phase is entrapped within the flocculated particles; this in turn increases the effective phase volume of the oil bodies in the suspension and therefore increases their viscosity, as is clearly demonstrated in Figure 4-16.

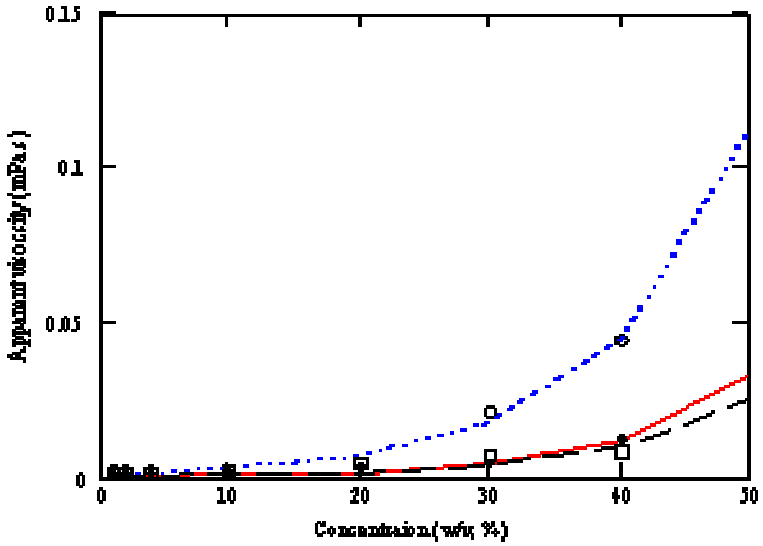



**Figure 4-16 The effect of oil body concentrations on the apparent viscosity of oil body suspensions**

**•, water-washed oil bodies (W-OB); °, sodium chloride-washed oil bodies (S-OB); □, urea-washed oil bodies (U-OB). Apparent viscosity was taken at shear rate 10 $s^{-1}$. Flow curves were obtained from data fitting by non-linear regression by Mathcad professional 2001.**

When oil bodies were freeze dried then rehydrated the viscosity–concentration curves changed significantly (Figure 4-17). Water-washed oil bodies did not appear to be affected by the freeze drying and the rheology was unchanged. The purer preparations (salt-washed and urea-washed) did increase in viscosity, this is possibly due to damage to the oil bodies structure leading to aggregation and an increased effective volume fraction and subsequent increase in viscosity. It is interesting to note that the rheology of the least pure preparation is the most resistant to changes due to drying, this may be due to the presence of residual sugars that reduce the melting temperature of the oil body membranes, leading to an increase in their desiccation resistance (Golovina, Hoekstra et al. 1998). An alternative explanation could be that the residual proteins and cell debris are surface active and act to stabilized the oil bodies in addition to the closely associated

oil body proteins and phospholipids (Burnett, Rigby et al. 2002; Betecz, Mackie et al. 2005).

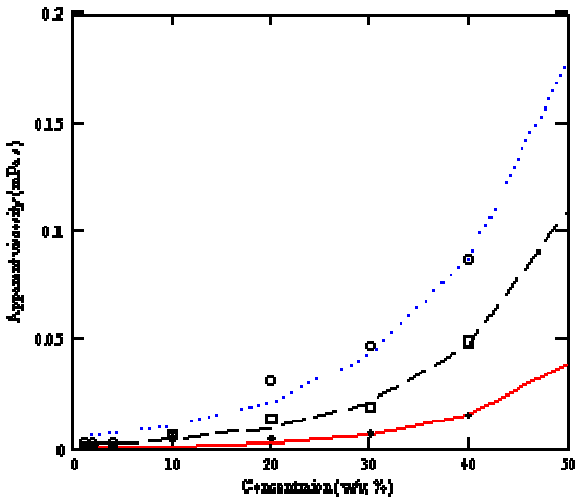



**Figure 4-17 The effect of oil body concentrations on the apparent viscosity of oil body suspensions**

**•, freeze-dried water wash oil bodies (FD-WOB); °, freeze-dried sodium chloride washed oil bodies (FD-SOB); □, freeze-dried urea-washed oil bodies (FD-UOB) Flow curves were obtained from data fitting by non-linear regression by Mathcad professional 2001.**

Oil bodies were prepared at a higher level of purity to further understand the physical and structural reasons for their emulsion stability. The oil body preparation was water-washed, then urea-washed four times and finally water-washed twice to remove residual urea. It should be noted that hereinafter concentrations refer to lipid content rather than solids content.

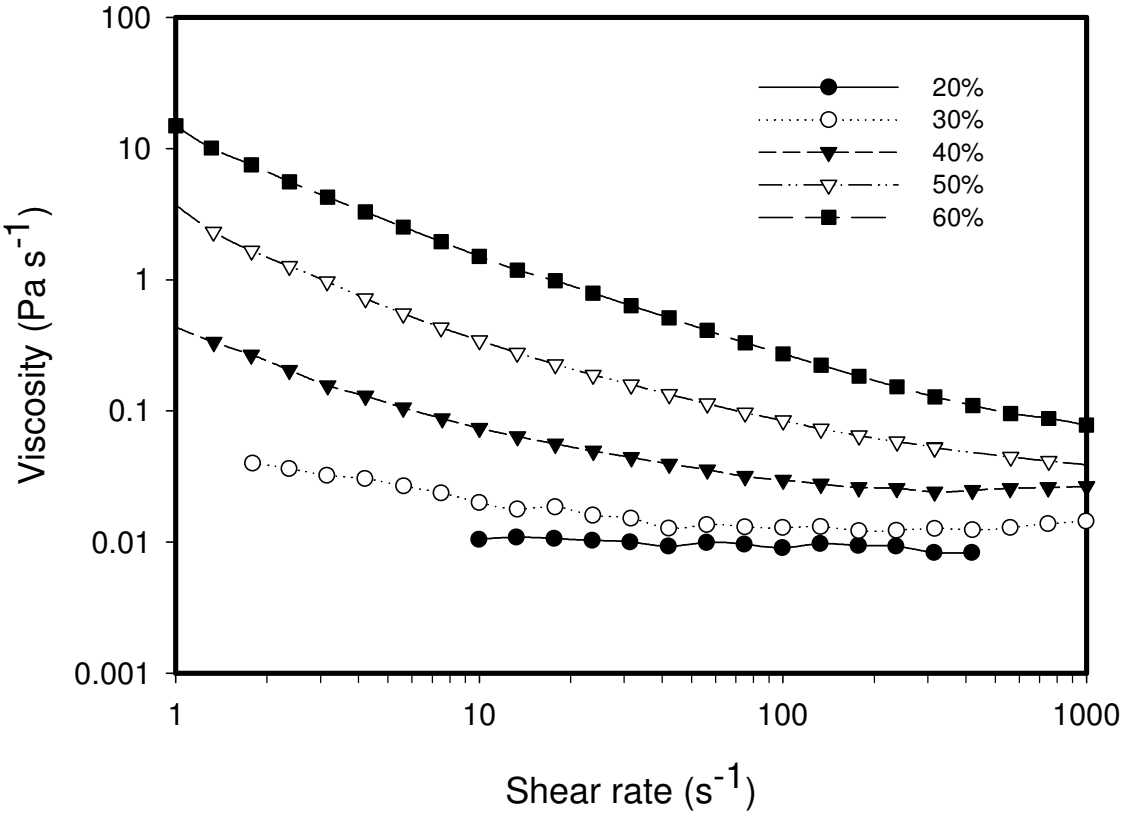



**Figure 4-18 Effect of lipid concentration (% wwb) on viscosity of oil body emulsions (pH 7).**

4 x urea-washed oil bodies were subjected to a dynamic stress sweep analysis (Figure 4-19) to allow greater understanding of their rheology in a food system. At a stress amplitude of 1-10Pa the samples show viscoelastic behavior as defined by the elastic modulus (G') being greater than the viscous modulus (G''). This linear viscoelastic domain extends to a stress amplitude of 10 Pa.

Figure 4-20 shows the dynamic frequency sweep at controlled stress at a stress amplitude of 1Pa. The elastic modulus is greater than the viscous modulus which indicates that the oil body suspension can be classed as a gel structure. This is indicative of interactions occurring between suspended oil bodies. In addition both modulii show a frequency dependence that indicates the sample is fundamentally a gel-like liquid. These intraparticulate

interactions will have implications on end product rheology for any oil body food applications.

Prior work by Mason (2001) on oilseed rape oil bodies showed similar results, but with different magnitudes of G' and G'' is presumed to be due to differences in extraction protocol and sample preparation.

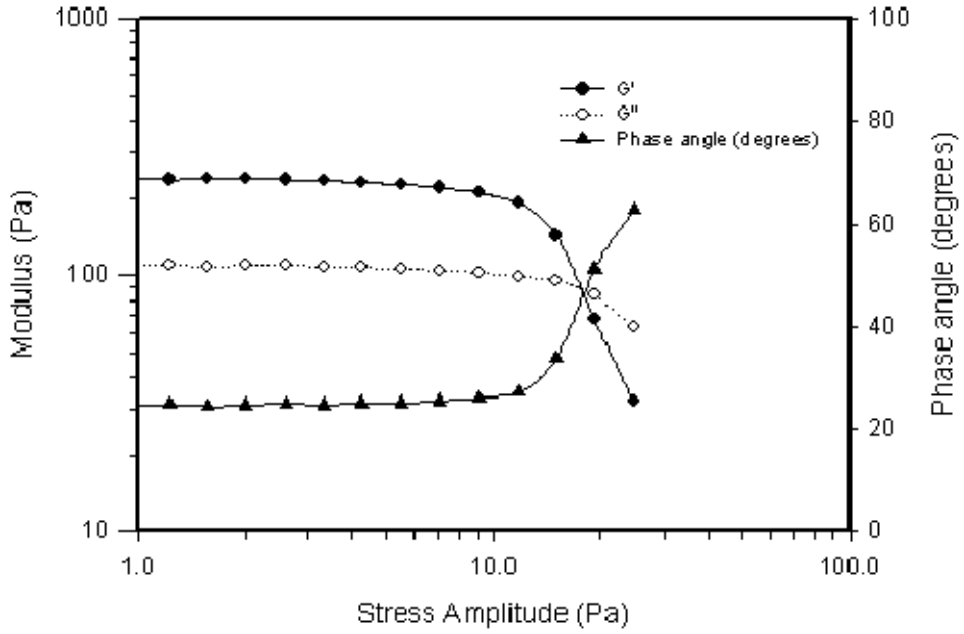



**Figure 4-19 Dynamic stress sweep of urea-washed oil bodies**

**(68% lipid; 5% protein; 25% moisture)**

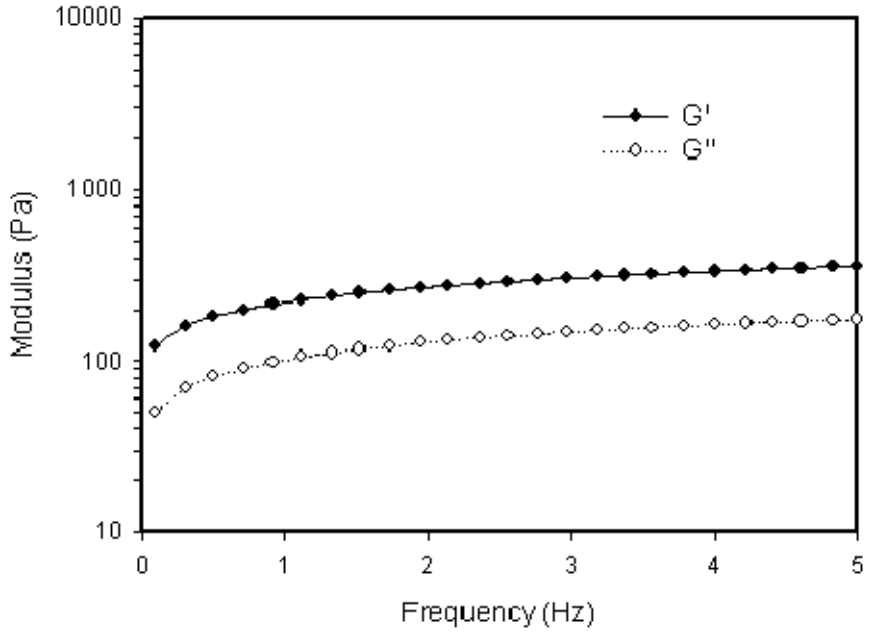



**Figure 4-20 Frequency sweep of urea-washed oil bodies (1 Pa stress) G'(-●-), G''(-○-) shown.**

# 5. Oxidative Stability of Sunflower Oil Bodies

Oxidation of lipids in food can result in the production of anti-nutritional factors and undesirable flavour and aroma compounds (Let, Jacobsen et al. 2005). The process of oxidation may also generate genotoxic and cytotoxic oxygenated aldehydes (Guillen M, N. et al. 2005). Knowledge of lipid oxidation and the ability to control its development is therefore fundamental to the preservation of a food's nutritional and sensory quality; the ability to limit or delay the onset of oxidation through a choice of ingredients would offer significant benefits to the food industry.

To assess oxidative stability, sunflower oil bodies were stored at elevated temperatures for 8 days and the development of oxidation measured. Three sunflower oil body suspensions were prepared (water-washed, salt-washed and urea-washed) and compared to three emulsions formulated with additional surfactants. The overall aim was to understand how oil body associated proteins, levels of contaminating proteins and surface charge would affect oxidative stability. This was achieved by comparing thermally induced oxidation of sunflower oil body suspensions against conventional oil-in-water emulsions.

Oxidative stability of sunflower oil body suspensions was compared with three oil-in-water emulsions stabilized by sodium dodecyl sulfate (SDS), polyoxyethylenesorbitan monolaurate (tween 20) and dodecyltrimethylammonium bromide (DTAB). The three emulsifiers (SDS, tween 20 and DTAB) were chosen on the basis of their surface charge as per Manusco (2000). SDS is an anionic emulsifier (negative surface charge), tween 20 is non-ionic (neutral surface charge) and DTAB is cationic (positive surface charge).

Droplet size, lipid content, lipid source and initial lipid hydroperoxide concentration were measured and controlled to minimize inter-sample

variation. The sunflower oil for the surfactant stabilised emulsions was extracted from the same seed batch as the oil bodies.

The change in concentration of lipid hydroperoxides and presence of hexanal in the headspace above the emulsions were used as markers for the state of oxidation. Oil bodies were prepared by wet-milling, centrifugation and subsequent water washing; “water-washed” preparations contained residual proteins not directly associated with oil bodies. Further washing with salt, followed by its removal, produced a “salt-washed” preparation with fewer contaminating proteins.

“Urea-washed” oil bodies were also prepared but probably contained residual urea. Urea-washed sunflower oil bodies appeared to oxidise rapidly producing an increase in lipid hydroperoxide concentration and elevated headspace hexanal concentrations. It is not known if this is true oxidation or an effect of urea on the lipid hydroperoxide assay. Potentially the presence of urea may accelerate the formation of lipid hydroperoxides or the urea may change the physical conformation of oil body associated surface proteins allowing easier access to the surface lipids for the development of oxidation. A direct effect of urea on the hydroperoxide concentration is unlikely as the hexanal concentration was also elevated in the urea-washed oil bodies; the latter (conformational change on the surface proteins) is unlikely as urea-washed oil bodies do not coalesce or phase separate, which would be characteristic of damage to the surface proteins. Urea may however propagate or initiate oxidation of oil body lipids, an idea suggested by Hatefi (1969). Hatefi looked at lipid oxidation in mitochondria and microsomes and found that the presence of urea accelerated oxidation of membrane lipids. Urea is not recognised as food grade and therefore urea-washed oil bodies cannot be used as a commercial food ingredient. Urea-washed oil bodies are only used in this study as a model of pure oil bodies allowing oil bodies to be tested in isolation from contaminating proteins; therefore it was not felt

necessary to further pursue the measurement of oxidative stability of urea-washed oil bodies.

## *5.1. Physical Stability*

At the start of the experiment and at day 8 the oil body and emulsion droplet diameters were measured. Emulsions formulated from mixing sunflower oil and a surfactant were stable over the experimental time course and did not phase separate, although DTAB stabilized emulsions did increase in droplet diameter slightly (21% increase in D(v, 0.5) over 8 days) when exposed to elevated temperatures (Table 2). Apparent oil body droplet diameter also increased (Table 2) due to aggregation, as revealed by light microscopy (data not shown).

**Table 2 Droplet diameter (numerical mean) of sunflower oil bodies (water-washed and salt-washed) and sunflower oil emulsions (stabilised by SDS, tween-20 or DTAB) over 8 days stored at 5°C, 25°C and 45°C.**

| | D(v,0.5) μm | | | |
|---|---|---|---|---|
| | Day 0 | Day 8 (5°C) | Day 8 (25°C) | Day 8 (45°C) |
| Water Washed | 0.91 | 4.95 ± 1.75 | 17.42 ± 4.97 | 9.72 ± 2.00 |
| Salt Washed | 0.90 | 6.15 ± 0.26 | 9.72 ± 2.00 | 16.06 ± 13.52 |
| SDS | 0.78 | 0.31 ± 0.01 | 0.32 ± 0.01 | 0.31 ± 0.01 |
| Tween-20 | 0.32 | 0.33 ± 0.01 | 0.33 ± 0.02 | 0.32 ± 0.01 |
| DTAB | 0.29 | 0.29 ± 0.01 | 0.32 ± 0.02 | 0.35 ± 0.02 |

Increased emulsion droplet size is theorized by McClements and Decker (2000) to reduce oxidation rate, as for a fixed lipid content the surface area on which oxidation chemistry can occur is reduced. Oxidation is often initiated at lipid-water interfaces in a lipid emulsion and then propagates into the bulk oil of the emulsion droplets.

A recent paper by Osborn and Akah (2004) found no significant effect of surface area on rapeseed emulsions stabilised with whey protein isolate or ryoto sugar ester with low lipid hydroperoxide concentrations (~0.5 mmol lipid hydroperoxides/kg oil). Osborn's results support McClements' and Decker's

(2000) theory that droplet size is only influential when reactant concentration (lipid hydroperoxides) at the surface of oil droplets is high, although McClements does not specify a value. In systems of a high lipid hydroperoxide concentration a doubling of the surface area would double the number of available reactants at the surface. In an emulsion with low lipid hydroperoxide concentration this would not be true as all reactants would be available at the droplet surface.

Average surface area per unit volume of emulsion was calculated as per McClements (2000) and showed that although variation in surface area was occurring in some cases, the difference in most cases was not significant or did not correlate with the order in which the samples oxidised (oxidation data is detailed in section 5.2).

**Table 3 Surface area per unit volume of sunflower oil bodies (water-washed and salt-washed) and sunflower oil emulsions (stabilised by SDS, tween-20 or DTAB) over 8 days stored at 5°C, 25°C and 45°C.**

| | Emulsion Surface Area per unit volume ( $\mu m^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 0 | 5°C | | 25°C | | 45°C | |
| Water Washed | 1.30 | 0.97 ± | 0.14 | 0.53 ± | 0.09 | 0.61 ± | 0.13 |
| Salt Washed | 1.43 | 0.87 ± | 0.02 | 0.76 ± | 0.11 | 0.68 ± | 0.09 |
| SDS | 0.95 | 2.43 ± | 0.06 | 2.40 ± | 0.00 | 2.50 ± | 0.00 |
| Tween-20 | 2.31 | 2.22 ± | 0.08 | 2.43 ± | 0.06 | 2.34 ± | 0.05 |
| DTAB | 2.61 | 2.65 ± | 0.07 | 2.34 ± | 0.19 | 2.07 ± | 0.14 |

In most cases the samples with elevated lipid hydroperoxide concentrations were not the same as the samples with increased surface area (Table 3). The lipid hydroperoxide concentrations were low at the start of the experiment (1.14 - 2.96 mmol lipid hydroperoxides/kg oil), although not as low as Obsorn (2004) (0.5 mmol lipid hydroperoxides/kg oil). We can therefore conclude that droplet size (at the start of the experiment) will not influence oxidation as samples are compared at low hydroperoxide concentrations and any measured increase in particle size does not correlate with the small increases in lipid hydroperoxide concentration (Linear regression, XLSTAT, $P<0.05$).

## 5.2. Lipid Hydroperoxide Formation

Lipid hydroperoxides are a bioproduct of the propagation phase of lipid oxidation; they are typically formed when peroxyl radicals react with lipid bound hydrogen forming lipid radicals and hydroperoxides. Lipid hydroperoxides are an intermediate product of lipid oxidation and are only transiently present as they break down to form a wide range of secondary products, including hexanal (Frankel 1998).

Lipid hydroperoxide concentration was measured daily in the oil body suspensions and lipid emulsions. Significant formation of lipid hydroperoxides did not occur in either of the oil body isolates at most temperatures, although lipid hydroperoxide concentration did increase slightly in the 25$^{o}$C salt-washed suspension and more significantly in the 45$^{o}$C water-washed oil body suspension (Figure 5-1). The concentration of lipid hydroperoxides in most oil body samples, is below the level usually associated with poor oil quality and significantly lower than those found in the surfactant stabilised emulsions. Frankel (1998) states that a lipid hydroperoxide concentration of 2.5 mmol lipid hydroperoxides.kg$^{-1}$ oil is the limit of acceptability for polyunsaturated vegetable bulk oils.

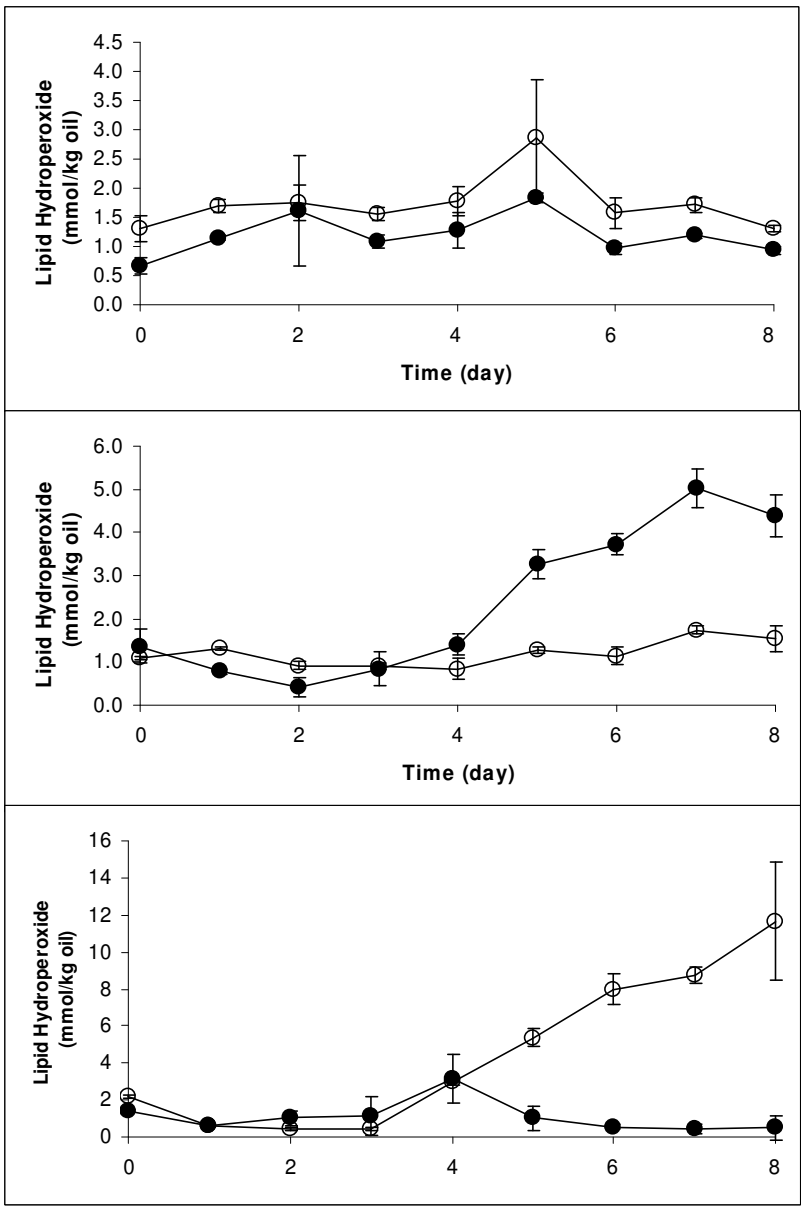



**Figure 5-1 : Lipid hydroperoxide concentration mmol.kg$^{-1}$oil for water-washed (empty circles) and salt-washed oil bodies (filled circles).**

**Samples were analyzed at (a) 5$^{o}$C (b) 25$^{o}$C and (c) 45$^{o}$C. All data points represent means (n=3) ± Standard Deviation.**

Water-washed oil bodies contain a number of residual proteins originating from the seed cell debris; upon salt washing this number is selectively reduced, and only the more strongly associated proteins remain. Protein oxidation is known to occur concurrently with lipid oxidation in many foods

and is proposed to initially retard lipid oxidation by preferentially oxidizing over lipid species or through the chelation of free metal ions (Faraji, McClements et al. 2004; Elias, McClements et al. 2005). Proteins may also initiate lipid oxidation through the formation of peroxyl radicals or reactive carbonyls. The balance between pro-oxidant and antioxidant effects of proteins is both concentration and matrix dependant as demonstrated by Villiere and Viau (2005). In the water-washed oil body suspension at 45$^{o}$C residual contaminating proteins may have contributed to the increased lipid hydroperoxide concentration relative to the salt-washed suspension through this pro-oxidant effect (Figure 5-1). However, it should be noted that the difference in lipid hydroperoxide concentration between the two oil body preparations is minor when compared with the lipid hydroperoxide concentration in the surfactant stabilized emulsions after storage.

The surfactant stabilised emulsions were formulated from sunflower seed oil extracted from the same seed batch as the oil bodies, and in all cases the starting lipid hydroperoxide concentrations were similar (1.9±0.75 mmol lipid hydroperoxides.kg$^{-1}$ lipid). Figure 5-1 and Figure 5-2 allow a comparison of the oxidative stability of oil bodies and the surfactant stabilised emulsions. In all cases, except tween 20 at 25$^{o}$C, natural oil bodies were significantly more stable ($P<0.05$) against oxidation than the surfactant stabilised emulsions made from oil from the same seeds. The extent of oxidation was dependant on the time and the nature of the emulsifier in the order DTAB > SDS > tween 20 (based on the maximum lipid hydroperoxide concentration over the experimental time course) (Figure 5-1).

Development of oxidation was dependant upon temperature in the order (45$^{o}$C>25$^{o}$C>5$^{o}$C). Thermal dependence concurs with classical models of oxidation, and the concentration of lipid hydroperoxides is in line with literature values during early stages of oxidation (Shantha and Decker 1994).

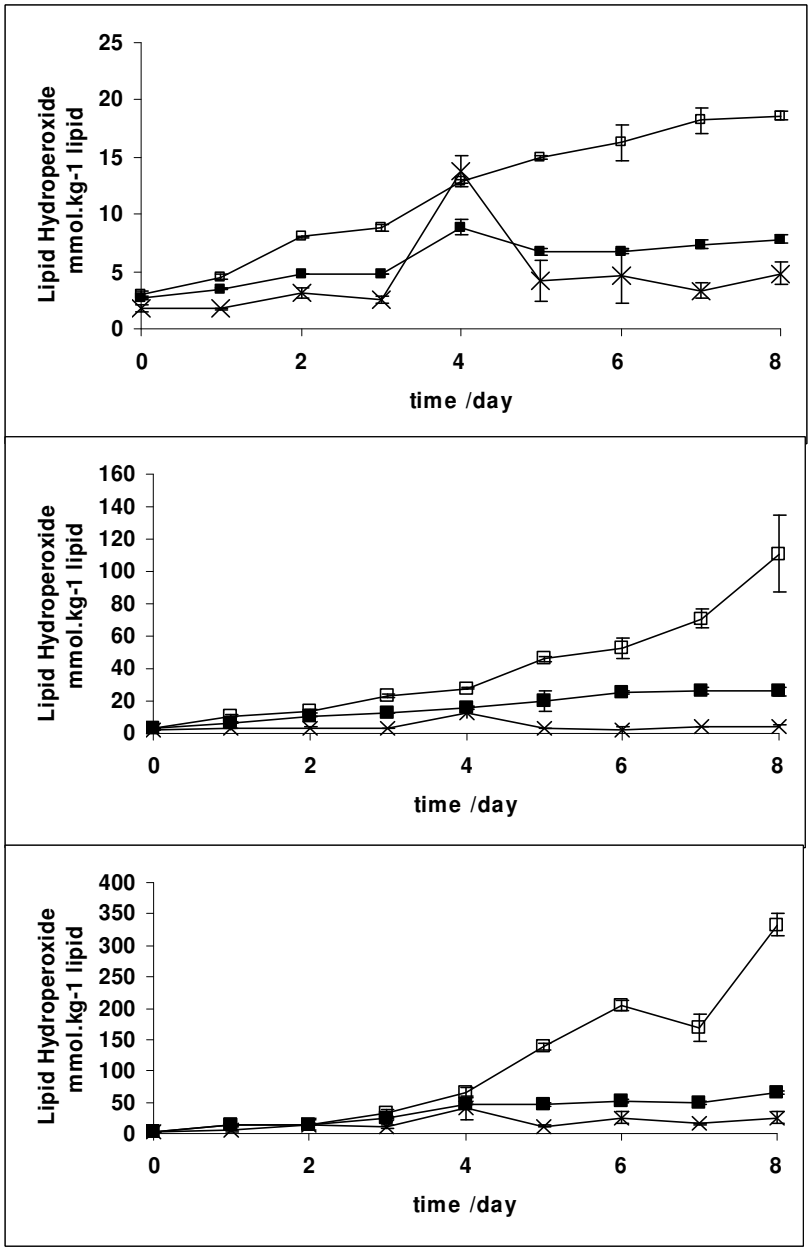



**Figure 5-2 : Lipid hydroperoxide concentration mmol.kg$^{-1}$ oil for 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square).**

**Emulsions were analyzed at (a) 5$^{o}$C (b) 25$^{o}$C and (c) 45$^{o}$C. All data points represent means (n=3) ± Standard Deviation.**

## *5.3. Secondary Product Formation*

No increase in hexanal concentration could be detected in any of the oil body samples (Figure 5-3) indicating that lipid hydroperoxide break down was minimal during the time course of the experiment. Oil body samples did contain endogenous hexanal at the start of the experiment at relatively high levels. Other potential volatile secondary products of linoleic acid oxidation were also followed but did not increase in concentration (data not shown). The presence of endogenous hexanal has been noted previously in the hydrophobic sub-compartments of carnation petals by Hudak and Thompson (1997), who suggest that these structures resemble oil bodies and contain a number of volatile compounds including hexanal that originated as byproducts of the lipoxygenase pathway.

Lipid hydroperoxides were formed in the surfactant stabilized emulsions increasing to significant concentrations (DTAB, 45$^{o}$C, day 8, lipid hydroperoxide concentration > 300mmol.kg$^{-1}$ lipid). Hexanal, 2-Heptanal (data not shown) and 1-octen-3-one (data not shown) headspace data supported this and their concentrations increased in DTAB samples at all temperatures. SDS stabilised emulsions also demonstrated an increase in all three characteristic volatile secondary products but only at 45$^{o}$C (Figure 5-2 and Figure 5-4).

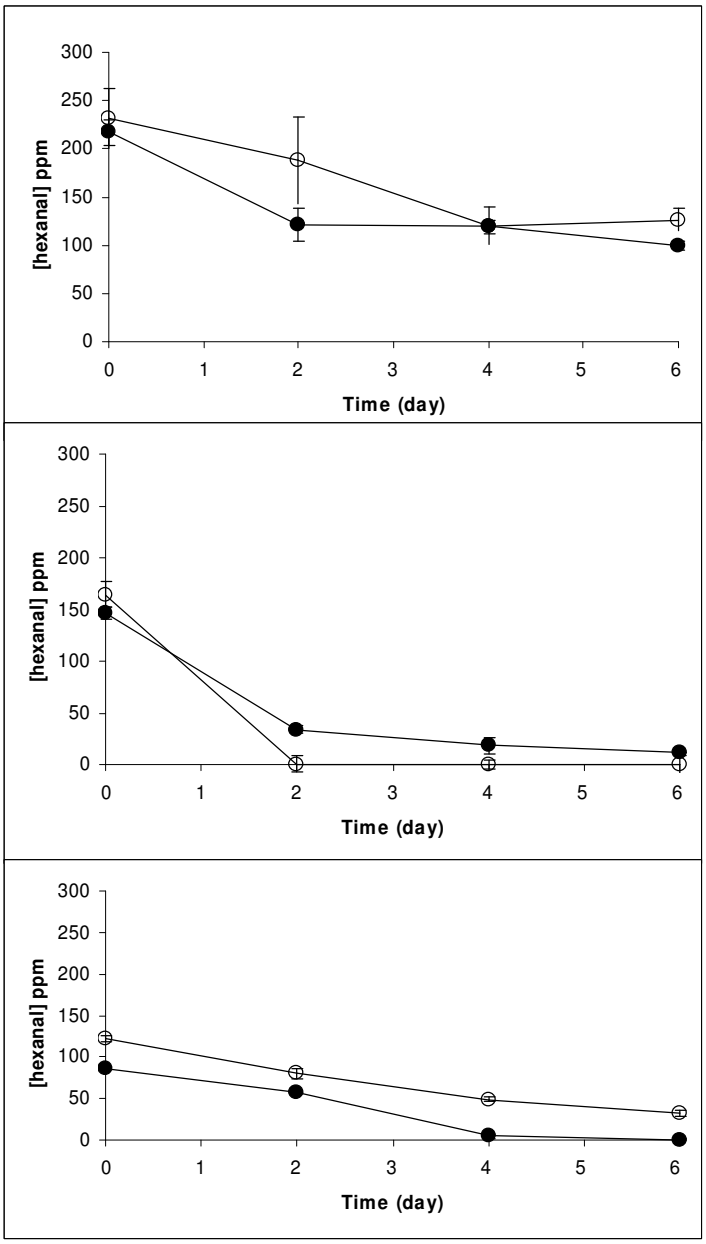



**Figure 5-3 : Influence of temperature on hexanal production in water-washed (empty circles) and salt-washed oil bodies (filled circles).**

**Extraction by solid phase microextraction and quantification by GC-MS. Samples were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent means (n=3) ± Standard Deviation.**

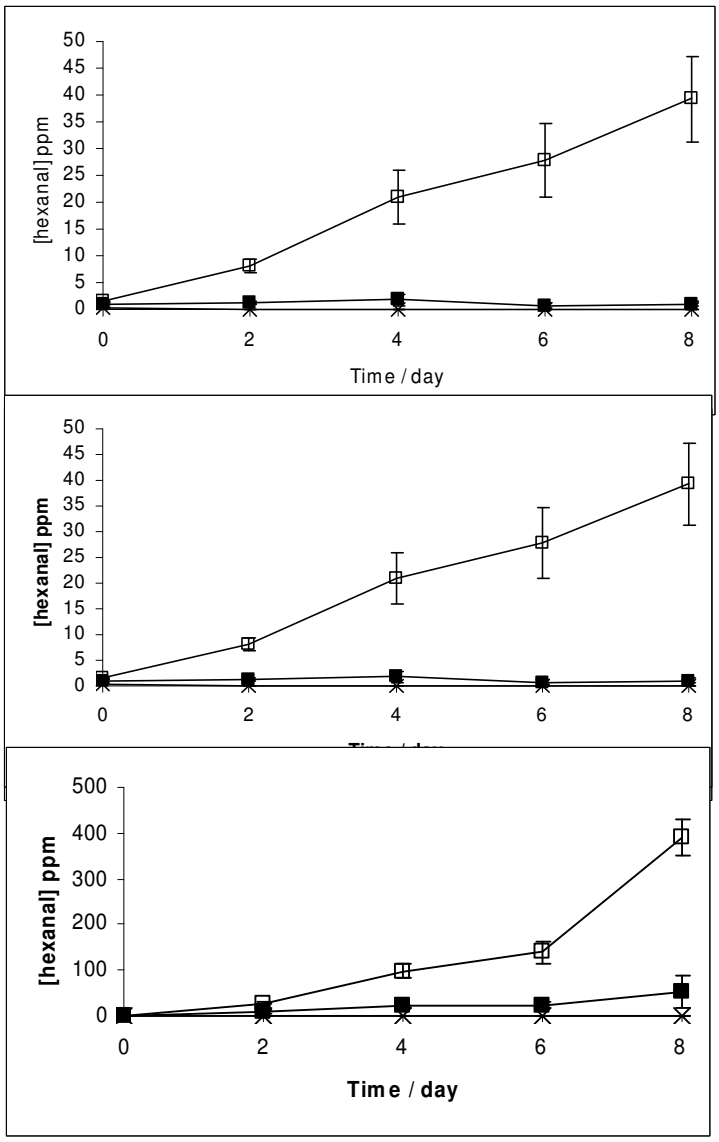



**Figure 5-4 : Influence of temperature on hexanal production in 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square).**

**Extraction by solid phase microextraction and quantification by GC-MS. Emulsions were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent means (n=3) ± Standard Deviation.**

Oil bodies are negatively charged (zeta potential of water-washed and salt-washed oil bodies at pH 7.5 are -27mV ± 1.4mV and -30mV ± 3.1mV respectively) but despite this potential to attract oxidation-promoting cations they are significantly more resistant to oxidation than emulsions formulated from synthetic emulsifiers.

The three surfactants were chosen to test the effect that surfactant chemistry has on the development of oxidation. It would be expected that the most negatively charged emulsion (SDS) would oxidize more rapidly than the emulsion formed from the non-ionic surfactant (tween 20) as the SDS emulsion would attract cationic ions (e.g. $Fe^{2+}$, $Cu^{2+}$) that may facilitate the initiation of lipid oxidation (Frankel 1998; Mancuso, McClements et al. 1999). This can be seen clearly when we compare the lipid hydroperoxide concentration in the SDS and the tween 20 stabilized emulsions at all temperatures (Figure 5-2).

Surprisingly, lipid hydroperoxides were formed most rapidly in emulsions stabilized by cationic DTAB. DTAB stabilized emulsions had the highest lipid hydroperoxide concentration at the start of the experiment (lipid hydroperoxide concentration of DTAB, SDS and tween 20 stabilized emulsions prior to storage were 2.96±0.31, 2.66±0.18 and 1.77±0.27 mmol lipid hydroperoxides.$kg^{-1}$ lipid respectively). Although the difference is not statistically significant (P=0.05) it may indicate DTAB naturally contained lipid hydroperoxides that could have promoted the development of oxidation. The presence of residual lipid hydroperoxides may have promoted the initiation of oxidation, and resulted in the rapid production of further lipid hydroperoxides.

In conclusion, oil bodies are stable against oxidation at 5°C, and when stored at 25°C and 45°C lipid hydroperoxide formation rate is significantly less than within emulsions formulated with synthetic surfactants. The level of purity of oil bodies also effects oxidation rate; the less purified preparations (washed once with buffer) showed a small increase in lipid hydroperoxide formation rate at 45°C and the further purified preparation (salt-washed; washed once in salt and once in buffer) showed only negligible changes in lipid hydroperoxide concentration at all temperatures. The oxidative stability of sunflower oil bodies ex-vivo demonstrates protection of the naturally encapsulated polyunsaturated oil.

The reasons for the increased oxidative stability in sunflower oil bodies cannot be conclusively derived. Residual enzymes may stabilise the system protecting the surface of oil bodies from oxidation, the protein and phospholipid layer may act as a protective barrier from oxidation propagating between suspended oil bodies, residual proteins may be preferentially oxidised over the oil body lipids, or the associated antioxidants (Fisk, White et al. 2006) may contribute to their stability.

Tocopherol is successfully extracted using diethyl ether as an extraction solvent (AOCS 2004), tocopherol concentrations should therefore not differ between the surfactant stabilised emulsions and the oil body suspensions. This suggests that tocopherol, the major antioxidant associated with oilseed lipids would not be the major reason behind the differential oxidative stability of the samples.

Nonetheless, it is still clear that a food product formulated with sunflower oil bodies could offer significant benefits over artificial emulsions through extended shelf life and the removal of the need for additional antioxidants and surfactants.

# 6. Flavour Release from Wet and Dry Oil Body Systems

## 6.1. Static headspace

Prior to the addition of oil bodies into food systems, it is important to know, in addition to their physico-chemical properties, how their presence will effect the release, retention, and perception of flavour compounds. In this chapter such a study is presented assessing how oil bodies affect the physical release of flavour compounds, their potential as flavour carriers, and how consumers perceive oil bodies as an ingredient in a model food system (Fisk and Gray 2004).

$$\%RHI = 100 \times \frac{C_e}{C_w}$$

where %RHI = percentage relative headspace intensity
$C_e$ = Headspace concentration of flavour volatile above emulsion
$C_w$ = Headspace concentration of flavour volatile above water

**Equation 6-1 Percentage relative headspace intensity (%RHI)**

The ability of urea-washed sunflower oil bodies to modify the headspace concentration of a range of flavour volatiles was assessed. This was achieved by combining a suspension of urea-washed oil bodies and flavour compounds in an aqueous buffer then measurement of the concentration of flavour volatile in the headspace at equilibrium (

Table 6-1). Headspace concentrations above sunflower urea-washed oil body suspensions (OB) were compared to an artificial emulsion (AE) formed by mixing sunflower oil with phosphatidylcholine (14% dry volume basis) at the same lipid content (10% w/w) and the same droplet size distribution (1µm). Relative headspace intensities (%RHI) were calculated as per Carey (2002) (see Equation 6-1).

**Table 6-1 Static headspace equilibrium %RHI ± SD for 8 flavour volatiles in 10% lipid emulsions (AE) and 10% lipid urea-washed oil body suspensions (OB) (n=3)**

| %RHI | Carvone | 2-nonanone | Methyl hexanoate | 2,5 Dimethyl pyrazine |
|---|---|---|---|---|
| OB | 42 | 10 | 43 | 97 |
| AM | 41 | 11 | 52 | 94 |
| Model | 52.0 | 2 | 10 | 82 |
| log P | 1.0 | 2.8 | 2.0 | 0.5 |
| %RHI | Ethyl butyrate | Menthol | Ethyl octanoate | (+)-Limonene |
| OB | 61 | 12 | 1.6 | 17 |
| AM | 72 | 14 | 1.7 | 8.9 |
| Model | 27 | 0.81 | 0.61 | 0.25 |
| log P | 1.4 | 3.1 | 3.2 | 3.6 |

The static headspace results (

Table 6-1) for all 8 flavour compounds show a close correlation between the relative headspace intensity above the urea-washed oil body and artificial emulsion (correlation coefficient = 0.98). This is consistent with work reported by Carrey (2002) who concluded that the major determining factors effecting %RHI in a cloud emulsion were the oil fraction and the molecular characteristics of the volatile. As the lipid content was fixed at 10%, a model was tested to predict the %RHI values found based on the molecular characteristic of the flavour volatile.

Headspace %RHI data was compared with %RHI values produced from the Buttery model (Buttery, Guadagni et al. 1973). For most volatiles the model %RHI value correlated well with the experimental data (correlation coefficient = 0.86). Deviation from the model was probably due to errors incurred during estimation of octanal-water partition coefficient (Log P) (ChemicalComputingGroup 2006).

The results suggest that a urea-washed oil body suspension is effectively comparable to a phospholipid stabilised emulsion at partitioning volatiles in a static headspace situation. This suggests that the proteins associated with urea-washed oil bodies do not affect the partitioning of flavour compounds in a static state.

## 6.2. Dynamic headspace dilution

Static headspace volatile concentrations above urea-washed oil body suspensions were comparable to phospholipid stabilised emulsions as detailed in section 6.1. The simple partitioning of flavour volatiles between a solution and its headspace at equilibrium does not encompass all the facets of flavour release. To start to explore the more complex dynamic association of flavour volatiles to the emulsion and the associated proteins and phospholipids, the rate at which an oil body suspension and an artificial emulsion containing nonanone could maintain their headspace volatile concentration with constant headspace dilution was investigated.

Urea-washed oil body suspensions were able to maintain the headspace nonanone concentration, with constant headspace dilution, at a higher normalised headspace intensity (nHI see Equation 6-2) than both the artificial emulsion and buffer samples. This was observed over the entire experimental time course (see Figure 6-1).

$$nHI_{t=i} = 100 \times \frac{C_{t=i}}{C_{t=0}}$$

Where: $C_{t=i}$ is concentration at time, i
nHI = normalised headspace intensity

**Equation 6-2 Normalised headspace intensity**

The normalised headspace intensity of nonanone above the buffer sample (nonanone and buffer) rapidly reduced to below 50% of its initial concentration (Figure 6-1); in less than 2 minutes the concentration plateaus at 40%. The urea-washed oil body suspension maintained the headspace nonanone concentration at a higher normalised headspace intensity over the 10 minutes of sampling. The artificial emulsion sample had an intermediate normalised headspace intensity.

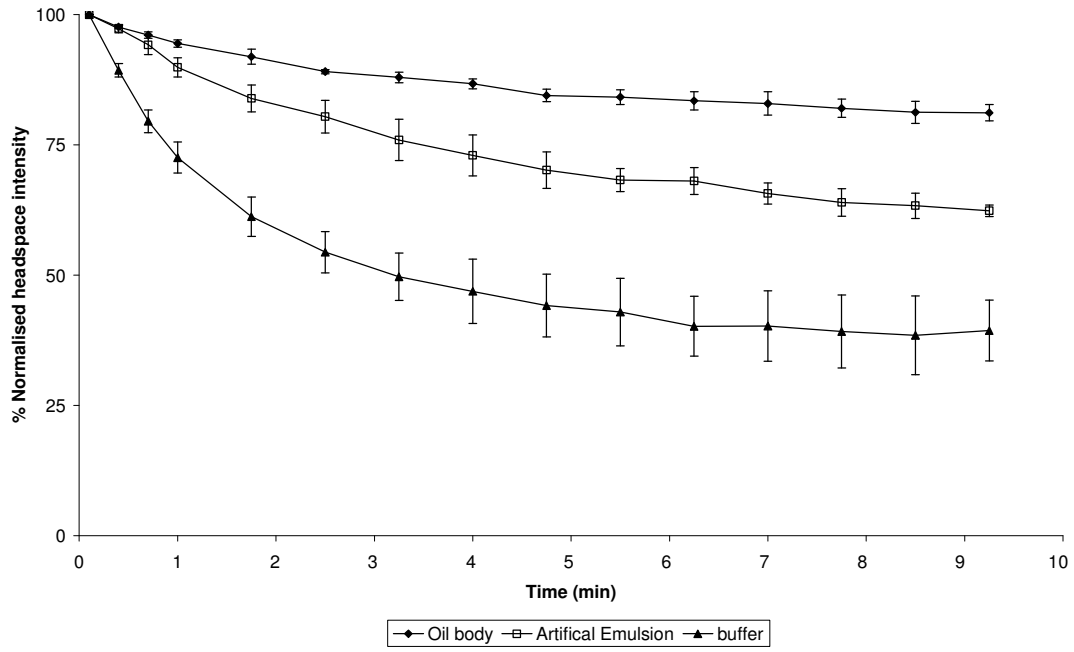



**Figure 6-1 Normalised time-course profiles for dynamic headspace dilution measurements of nonanone in an aqueous suspension of urea-washed oil bodies, artificial emulsion and buffer**

**(PTR-TOF-MS, $N_2$ flow 70mL.min$^{-1}$ sampling flow 20ml.min$^{-1}$) nHI of a 10% lipid sample**

Doyen's (2001) study on the volatile release of esters dissolved in lipid emulsions of a range of concentrations (0-19 g lipid.L$^{-1}$) concluded for ethyl octanoate the normalised headspace intensity above lipid emulsions had a greater resistance to dilution than in a comparable system containing only water and volatile. Doyen also suggests that in addition to maintaining volatiles headspace concentration at a higher normalised headspace intensity the time to reach equilibrium was shorter in a lipid emulsion than above a purely aqueous system; this was consistent with data presented in Figure 6-1.

The experiment was repeated with limonene at two lipid fractions (0.015% and 1%). Limonene was chosen as it is a common flavour ingredient, is strongly lipophilic (Log P = 3.6) and has commercial relevance to cloud emulsions. Both limonene concentrations showed similar results to nonanone (Figure 6-2 and Figure 6-3) with volatiles above the oil body suspension

showing a higher normalised headspace intensity than those above the artificial emulsion or the buffer solution. The extent of deviation from the buffer curve was significantly affected by the lipid content confirming earlier work by Doyen (2001).

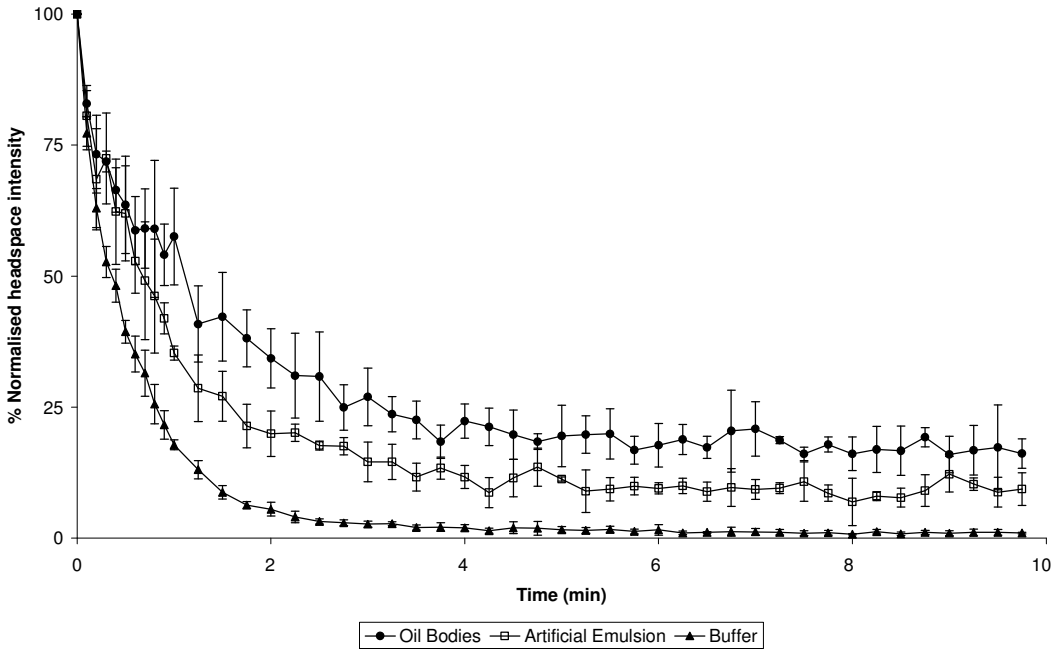



**Figure 6-2 Normalised time-course profiles for dynamic headspace dilution measurements of limonene in an aqueous suspension of urea-washed oil bodies, artificial emulsion and buffer**

**(PTR-TOF-MS, $N_2$ flow 70mL.min$^{-1}$ sampling flow 20ml.min$^{-1}$) nHI of a 1% lipid sample**

In an aqueous system, the rate of headspace volatile replenishment is primarily dependant upon the rate of movement of flavour molecules across the air-water interface. If the headspace concentration is low compared to that of the solution (as is the case for a volatile of $K_{aw}= 10^{-5}$) (Equation 6-3) the headspace can be rapidly replenished as only a limited number of flavour molecules are required to volatilise and transfer into the headspace to restore equilibrium. In a system of higher headspace concentration relative to the solution concentration (as is the case for a volatile of $K_{aw}= 10^{-2}$) a larger number of flavour molecules will be required to transfer into the headspace from the solution to restore equilibrium, thus reducing the rate at which the

volatile concentration in the headspace can be replenished with dilution, resulting in a lower normalised headspace intensity.

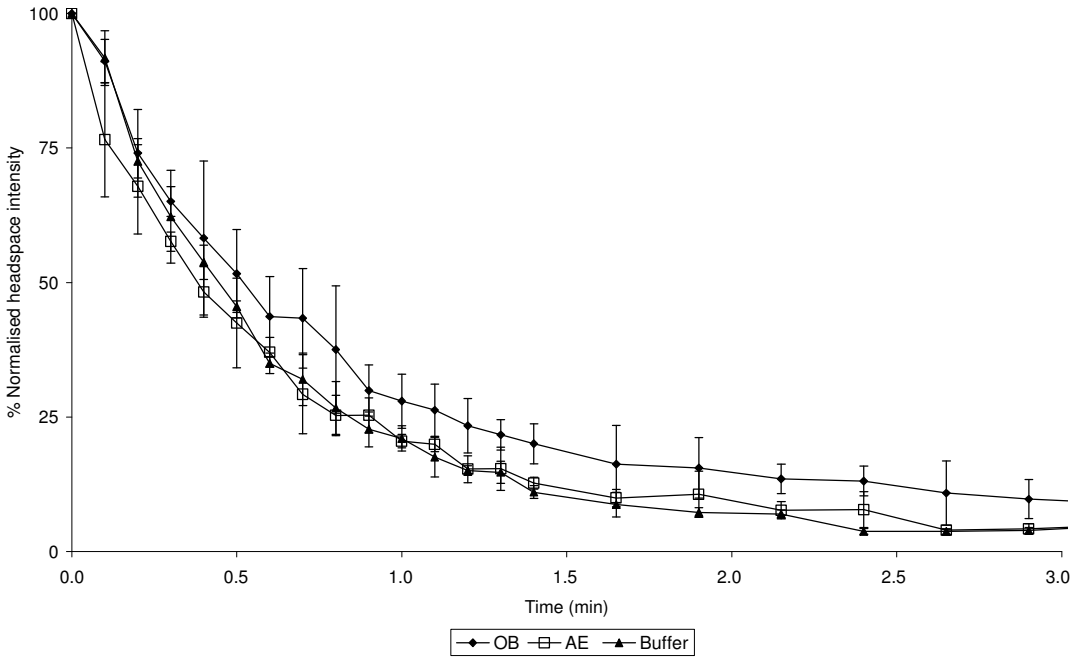



**Figure 6-3 Normalised time-course profiles for dynamic headspace dilution measurements of limonene in an aqueous suspension of urea-washed oil bodies, artificial emulsion and buffer**

**(PTR-TOF-MS, $N_2$ flow 70mL.min$^{-1}$ sampling flow 20ml.min$^{-1}$) nHI of a 0.015% lipid sample**

When lipid is suspended in a solution producing an emulsion, the solubility of any lipophilic flavour volatile increases. This increase in solubility reduces the original $K_{aw}$ to a lower $K_{ae}$ (Equation 6-3). This reduction in the partition coefficient results in a reduced headspace concentration and a volatile headspace concentration that can be replenished more rapidly giving flavour volatiles in the emulsion system a higher normalised headspace intensity.

$K_{ae}$ = volatile headspace concentration/ volatile concentration in emulsion

$K_{aw}$ = volatile headspace concentration/ volatile concentration in water

**Equation 6-3 Partition coefficients (K) for air water and air-emulsion interfaces**

This increase in normalised headspace intensity was observed for limonene and nonanone in our emulsion and oil body suspensions. The reasons why volatiles in an oil body suspension have a greater normalised headspace intensity than those in an artificial emulsion are unknown. For an oil-in-water emulsion to have a greater normalised headspace intensity it is generally accepted that the rate of mass transfer across the air-emulsion interface must be greater.

One explanation for this proposed increased mass transfer rate would be that oil bodies and their associated proteins are present at a higher concentration at the surface forming a boundary layer. This boundary layer would act as a reservoir for flavour volatiles and reduce the effective air-emulsion partition coefficient between this boundary layer and the headspace (during headspace dilution). The layer therefore allows a more rapid transfer of flavour molecules into the headspace and subsequently maintaining the headspace concentration of the flavour volatile at a higher normalised headspace intensity. In a static system the boundary layer may still form but the effect of the apparent increased interfacial lipid content may not be observed since the static situation reflects bulk phase properties. This would require the oil body associated proteins to be surface active and be more thermodynamically stable at the air-emulsion interface than in the bulk solution, this was recently proved by Roux (2004), who showed that the interaction of oleosin with phospholipid stabilised the air-water interface.

The implication of this finding is that oil body suspensions would have a greater headspace concentration of volatile compounds compared with a comparable phospholipid stabilised emulsion when a flow of air is passing over the surface. This characteristic ‘controlled release’ from urea-washed oil body emulsions could improve flavour retention in a food product and retronasal flavour perception on consumption. If oil bodies are surface active this would contribute to a further increase in retronasal flavour delivery, as oil

bodies would adsorb to the buccal epithelia and contribute an elevated flavour volatile concentration retronasally.

Cook (2003) looked at the effect of physical properties on flavour release and has shown that the viscosity of a consumed solution did not affect retronasal flavour delivery. Cook therefore hypothesised that it was the interfacial properties of the solution that are most significant in controlling flavour concentration retronasally. This was also documented by Linforth and co-workers (2002) who showed that a reduction in partition coefficient increases efficiency of volatile delivery, resulting in a volatile concentration in-nose greater than expected on a basis of $K_{ae}$.

## *6.3. Spray Drying*

Oil bodies in-vivo are exposed to extended periods of desiccation with oilseed moisture content reducing significantly during periods of seed dormancy (5%-10% (Murphy 2001)). Oil bodies were tested for their resistance to desiccation by spray drying and their ability to act as carrier agents for limonene, a lipophilic flavour compound.

Spray drying operates by atomising a sample into a flow of heated air (>100$^{o}$C) into the cylinder. Because of the small particles size and elevated air temperatures any moisture in the sample rapidly evaporates. Dry samples then flow with the exit air into a separating device (cyclone) which operates at a lower temperature (<100$^{o}$C) and fractionates the exit air into clean air and dry product which can be collected.

Initial trials showed that urea-washed sunflower oil bodies when suspended in buffered deionised water (10% solids), could produce a powder that was not greasy to touch and when rehydrated produced a stable resuspension of oil bodies.

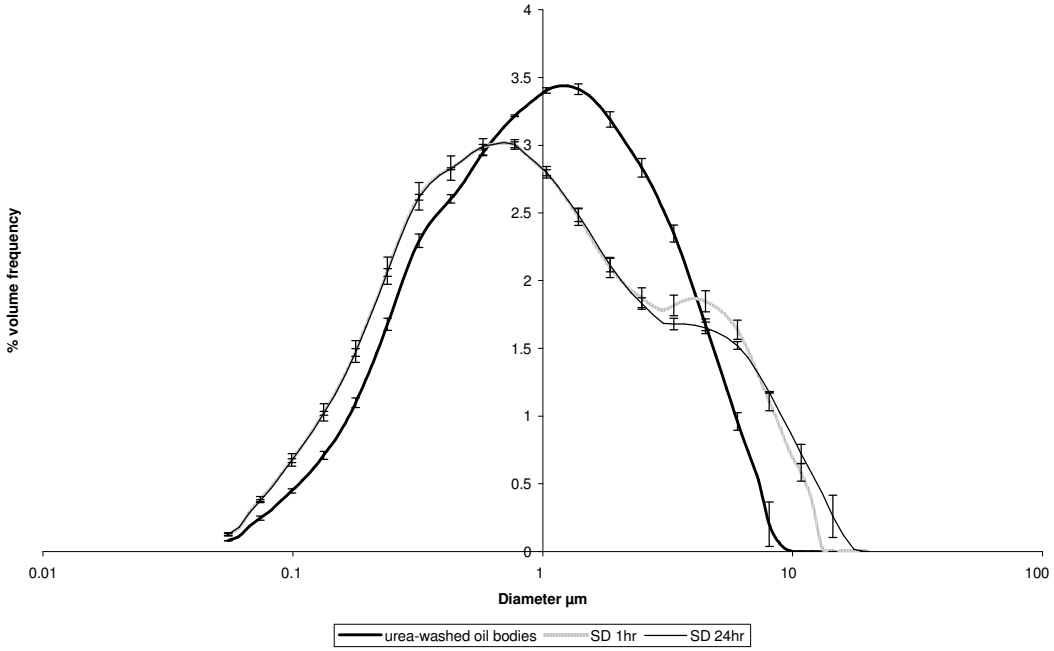



**Figure 6-4 Volume based size distribution of urea-washed sunflower oil bodies (UW) before and after spray drying and rehydration**

**(1 hour (S1) and 24 hours (S24) after rehydration)**

Urea-washed oil bodies prior to spray drying were stable and had a tight size distribution profile around 1µm (Figure 6-4). After spray drying and rehydration some clumping/aggregation had occurred and a small shoulder can been observed on the volume based size distribution plot at about 4µm (Figure 6-4). The dispersed sample was then held for a further 23 hours and measured again; the number of 4µm particles measured reduced over the 23hr storage period ($P<0.05$). This illustrates for the first time that oil bodies are stable to spray drying and are physically intact after rehydration.

Further to the preliminary study at 10% solids content, water-washed oil bodies were spray dried at 40% solids content. Spray drying of low solids content solution is more expensive than using concentrated stock solutions and throughput can be greatly increased at higher solids contents; in addition a greater flavour retention can be achieved with a higher infeed solids content (Reineccuis 1989; Reineccuis 2004). Water-washed oil body suspensions (40% solids content) were spray dried in a Niro-mobile minor

spray dryer. The partially dried product adhered to the drying chamber wall and due to the high temperatures the oil from the oil bodies leached out producing an unworkable caked powder. To further refine this work an intermediate feed stock concentration of 30% was chosen and a maltodextrin carrier (10-19DE) was used to reduce adhesion to the chamber wall and facilitate the formation of a dry powder.

The maltodextrin carrier (Morex 10-10DE) was chosen for its intermediate DE (dextrose equivalent) value and current use in the food industry as a carrier in spray drying. Samples of Morex (20% w/w) in a water-washed oil body suspension (7% w/w) were sonicated twice for two minute pulses to test the effect of Morex on the stability of oil bodies. The sample containing Morex had a significantly lower particle size than the sample without. This confirmed that Morex was not detrimental to the physical stability of water-washed oil bodies, and actually stabilised the suspension; this is probably due to the increase in viscosity of the continuous phase.

**Table 6-2 Composition of spray dried products**

| | Total composition (wet weight basis) | | |
|---|---|---|---|
| | Oil body | Lecithin | Capsul |
| Moisture | 70% | 70% | 70% |
| Total Solids | 30% | 30% | 30% |

| | Solids fraction (dry weight basis) | | |
|---|---|---|---|
| | Oil body | Lecithin | Capsul |
| Limonene | 11% | 11% | 11% |
| Morex | 66% | 53% | 53% |
| Oil body | 22% | | |
| Sunflower oil | | 22% | 22% |
| Lecithin | | 14% | |
| Capsul | | | 14% |

The stability of water-washed sunflower oil body suspensions to spray drying was assessed by mixing water-washed oil bodies with Morex (maltodextrin) and a flavour compound (limonene). The suspension was spray dried and the resulting product analysed for moisture, lipid and limonene content. The aim of this trial was to elucidate if spray drying is an effective technique for the

preparation of dry oil bodies and if oil bodies could be used as carrier agents for limonene.

Samples were compared against a sunflower oil emulsion stabilised by lecithin and a sunflower oil emulsion stabilised by a modified starch (Capsul) (Table 6-2).

All three samples were mixed, homogenised, then spray dried. To check the effectiveness of the homogenisation process and to make sure that the oil bodies were not being damaged excessively, two levels of homogenisation were used (high and low). Low homogenisation level was low shear mixing that would not damage the oil body integrity. The high homogenisation level was high shear mixing that may damage the oil bodies, but would reduce the particle size and distribute, more evenly, the limonene in the in-feed stock suspension.

Capsul is an octenyl succinylated starch which has been chemically modified to increase flavour retention on spray drying (Porzio, A et al. 2002). Its effectiveness is fundamentally due to its stability in the glassy state which is reached rapidly during the early stages of spray drying. It subsequently confers physical and chemical stability to entrapped flavour compounds. Capsul was chosen as a commercial benchmark to compare against spray dried oil bodies. The lecithin stabilised emulsion was used to assess the effectiveness of a purely phospholipid stabilised system.

Limonene is a lipophilic (Log P = 3.6) terpene. At room temperature it is a clear, colourless liquid with a lemon-orange aroma. In a water-washed oil body suspension (1% lipid, d(4,3)= 1µm) it has a percentage relative headspace intensity (%RHI ± %cv) of 13.5% ± 9% and in a phospholipid stabilised emulsion (1% lipid, d(4,3)= 1µm) it has a %RHI ± %cv of 14.1% ± 1%. The %RHI values for water-washed oil body suspensions deviates slightly from the urea-washed oil body suspensions %RHI values presented in

Table 6-1. The similarities of the relative headspace intensities for limonene in a non-spray dried product allows direct comparisons of water-washed oil body suspensions with phospholipid and Capsul stabilised emulsions.

All products spray dried successfully and formed dry, white-yellow powders. Figure 6-5 shows the physical appearance of the six products. The Capsul powders were white, powdery, free flowing and felt crunchy when compressed. Lecithin powders were a yellow-white colour and tended to clump in aggregates and felt slightly oily; however, the high homogenisation lecithin sample appeared to clump less and felt less oily. The oil body powders were white, free flowing and appeared denser than the Capsul samples. The variation in the products apparent density was probably due to variation in lipid content between the samples. The variation in perceived oiliness was probably due to the location of lipid in the spray dried particles, indicating that the Capsul stabilised samples had the lowest oil content and that the lecithin had the greatest free surface lipid. The variation in lipid content was later confirmed by solvent extraction and is discussed later Table 6-3.

| | Oil Body | Lecithin | Capsul |
|---|---|---|---|
| Low homogenisation |  |  |  |
| High homogenisation |  |  |  |

**Figure 6-5 Images of spray dried samples**

A Buchi B-191 small scale spray drier was used to spray dry the six samples. It has a small drying chamber (cylinder) with a high surface area to volume ratio. This will affect total yields and the way in which the products are spray dried, but it should not affect the products themselves. In this small laboratory spray dryer the spray dried product was more likely to come into contact with the cylinder wall which will increase the likelihood of losses by adhesion to the wall and oiling out due to extended time at high temperatures.

$$\%Total\_Yield = 100 \times \frac{W_{product}}{W_{raw\_material}}$$

Where: $W_{product}$ and $W_{raw\_material}$ are weight of product and raw material respectively

**Equation 6-4 Percentage total yield**

Percentage total yield (Equation 6-4) after spray drying varied between the samples. This was principally due to adhesion of the wet or oily product on the wall of the drying chamber as discussed previously. The total yield of product (Equation 6-4) in the water-washed oil body sample at high homogenisation level (24% dwb) was higher than the other five samples (12%-16%) (Figure 6-3). This may be for several reasons; the lecithin product is believed to have a higher amount of free lipid on the surface of the powder making it more adhesive and harder to transport to the cyclone and the Capsul product was a very fine powder which may increase losses via the waste air (see Figure 6-5). To compensate for variation in losses, the samples shall be compared on a % retention basis only and not on an absolute total yield basis.

For all three carrier matrices, the high homogenisation samples produced a greater proportion of sample in the cyclone compared with that collected at the base of the drying cylinder (adhered unworkable product). The high homogenisation water-washed oil body sample had the highest fraction with 45% of the total powder collected being from the cyclone (data not shown). It should be noted that all analysis hereafter concerns the cyclone product only.

**Table 6-3 Composition and limonene headspace concentration of spray dried product**

| Sample | OB | | Lecithin | | Capsul | |
|---|---|---|---|---|---|---|
| Homogenisation level | Low | High | Low | High | Low | High |
| Moisture Content (wwb) | **3.86** ± *0.04* | **2.76** ± *0.03* | **3.45** ± *0.04* | **1.86** ± *0.05* | **3.69** ± *0.05* | **3.94** ± *0.01* |
| Limonene Content (dwb) | **2.68** ± *0.1* | **2.95** ± *0.04* | **0.85** ± *0.01* | **1.00** ± *0.02* | **6.00** ± *0.05* | **6.52** ± *0.2* |
| Lipid Content % (dwb) | **20.5** ± *1* | **19.6** ± *2* | **29.6** ± *0.9* | **32.0** ± *1* | **10.9** ± *0.6* | **11** ± *1* |
| % Total yield (dwb) | **15.8** | **23.8** | **12.2** | **17.5** | **15.4** | **15.8** |
| | | | Headspace Concentration | | | |
| | OB | | Lecithin | | Capsul | |
| | Low | High | Low | High | Low | High |
| | | | Above dry powder | | | |
| ppmv[a] | **103** ± *10* | **148** ± *10* | **15.2** ± *1* | **24.4** ± *1* | **107** ± *23* | **116** ± *9* |
| | | | Above resuspended powders | | | |
| Imax[b] | **247** ± *57* | **230** ± *20* | **37.5** ± *4* | **56.8** ± *2* | **1491** ± *514* | **1095** ± *157* |

**[a]Sample (0.1g) in 2L container headspace measured by APcI-MS with internal standards.**

**[b]Sample (0.015% Lipid (w/w) predisolved in 250mL distilled water measured by APcI-MS.**

The lecithin samples had the highest lipid content of all six samples. When the water-washed oil body spray dried product (high homogenisation) was compared with the Capsul spray dried product (high homogenisation) the lipid content of the end product (dwb) is markedly different: 20% and 11% respectively (Table 6-3). This indicates a loss of lipid during the spray drying process in the Capsul product.

The % lipid retention was calculated for limonene and lipid for each spray dried sample (Soottitantawat, Yoshii et al. 2003) (Table 6-4). This calculation removes the inefficiencies of the spray dryer as it calculates on a dry weight basis per gram of product and raw material and not on a total yield basis.

Water-washed oil bodies combined with Morex is a very effective carrier to spray dry lipid. The % lipid retention was 89-93% indicating that only 7-11% of lipid was lost during spray drying. The % lipid retention of the Capsul stabilised emulsion was 48-50% and indicates that for each 10% of lipid (dwb) introduced to the process in the infeed stock solution a lipid concentration of 4.8-5% (dwb) would be achieved in the spray dried product. This shows a marked benefit of using water-washed oil bodies as a method of drying and storing lipid as a dry powder. Additional benefits include the antioxidant protection offered by Morex (Reineccuis 2004) as free reducing groups in the maltodextrin act as antioxidants.

**Table 6-4 % Lipid retention of spray dried oil bodies, and lecithin and capsul stabilised emulsions.**

| | Oil body | | Lecithin | | Capsul | |
|---|---|---|---|---|---|---|
| | Low | High | Low | High | Low | High |
| Lipid | 93 ± *1.0* | 89 ± *2* | 82 ± *0.9* | 89 ± *1* | 50 ± *1* | 48 ± *1* |
| Limonene | 24 ± *0.1* | 27 ± *0.04* | 7.7 ± *0.01* | 9.1 ± *0.02* | 55 ± *0.05* | 59 ± *0.24* |

**Lipid content of raw material = 22%**

**In 1g of dry raw material there is 0.22g of lipid**

**Lipid content of dry product = 20.5% (Table 6-3)**

**In 1g of product there is 0.205g of lipid**

**Yield Calculation = 100* (0.205/0.2) = 93% yield**

Capsul was the most effective carrier at encapsulating limonene and oil bodies are significantly better carriers for limonene than lecithin (Table 6-3). indicates that the lecithin product contained 0.85-1.0% limonene, whereas the water-washed oil body product contained 2.7-3.0% limonene and the Capsul product 6-6.5% limonene (dwb). This was reflected in the % retention of limonene (a measure of the % limonene not lost per gram of dry infeed material) (Table 6-4). This data shows that whilst Capsul retained 55-59% of the limonene, water-washed oil bodies retained 24-27% and the lecithin system retained only 8-9% of the limonene.

To assess long term stability of entrapped lipid, spray dried water-washed oil bodies (6 months storage at 5$^{o}$C) were resuspended at 30% (wwb) in buffered water, allowed to disperse, and the particle size of the resulting oil emulsion measured (Table 6-5). In all samples the low homogenisation preparation produced bimodal distributions with peaks at 1µm and at 10µm (data not shown). The original numerical mean particle diameter was 1µm and therefore it can be presumed that the 10µm are either aggregates or coalesced emulsion droplets. For each carrier matrix the high

homogenisation samples showed a bimodal distribution but the 10µm peak was significantly smaller than in the samples subjected to low homogenisation prior to spray drying. In conclusion the three high homogenisation samples produced emulsion droplets of comparable size distribution to the original sample (Table 6-5) and all spray dried samples retained their physical stability over a 6 month storage period.

**Table 6-5 Particle size of resuspended spray dried powders after storage ($5^{o}C$, 6 months) 30% solid content (numerical mean diameter, D(v,0.5))**

| | Oil body | | Lecithin | | Capsul | |
|---|---|---|---|---|---|---|
| | Low | High | Low | High | Low | High |
| D(v,0.5) | 3.18 | 2.02 | 2.91 | 2.24 | 3.39 | 1.92 |

In all systems the high homogenisation samples showed a smaller droplet diameter and greater % limonene retention. It is generally accepted that small emulsion droplets in the in-feed solution have a higher flavour retention (Reineccuis 2004). Soottitantawat (2003) suggests that a reduction in particle size diameter increases retention and reduces flavour loss (limonene); this is proposed to be due to shearing of the large droplets by the spray dryer atomiser, their rupture allowing a selective loss of volatile through volatilisation from the large disrupted emulsion droplets.

Table 6-4 shows that water-washed oil bodies are more effective than the Capsul and lecithin samples at retaining encapsulated oil. Water-washed oil bodies were also more effective than the lecithin sample at retaining limonene, but the commercial product (Capsul) retained the greatest amount of limonene. This may be for several reasons; the primary reason is that Morex has no emulsification properties (Reineccuis 2004) and the system relies on the emulsifier present in the water-washed oil bodies. Most of the emulsifier in the water-washed oil body suspension is actively involved in stabilising the neutral lipid in the water-washed oil bodies. This may lead to only a proportion of the limonene being stabilised and the remaining lost

through volatilisation. One solution to this would be to partially defat the oil bodies prior to spray drying making available more surface active agents (protein and phospholipid) to stabilise the flavour volatile.

The headspace concentration of limonene above dry and resuspended spray dried products when compared at equal lipid contents and normalised by limonene content is shown in Table 6-6. Capsul stabilised samples after high homogenisation had a significantly lower limonene headspace concentration when in a dry system compared to water-washed oil bodies (oil body stabilised suspension 27 ppmv; capsul stabilised suspension 18 ppmv ($P<0.05$)(Table 6-6). A reduced limonene headspace concentration above the dry product indicates a greater entrapment of limonene and subsequent reduced loss of limonene during storage.

**Table 6-6 Headspace limonene concentrations above dry and wet spray dried samples per gram of limonene on an equal lipid basis**

| Sample | Limonene headpace concentration.g$^{-1}$ limonene on an equal lipid basis | | | | | |
|---|---|---|---|---|---|---|
| | OB | | Lecithin | | Capsul | |
| Homogenisation | Low | High | Low | High | Low | High |
| | | | Above dry powder | | | |
| ppmv[a] | **20.3** ± *4* | **27.0** ± *5* | **6.51** ± *0.7* | **7.92** ± *1.0* | **17.6** ± *5* | **18.1** ± *4* |
| | | | Above resuspended powders | | | |
| Imax[b] | **83.9** ± *27* | **67.7** ± *12* | **58.0** ± *9* | **80.9** ± *8* | **121** ± *49* | **79.5** ± *23* |

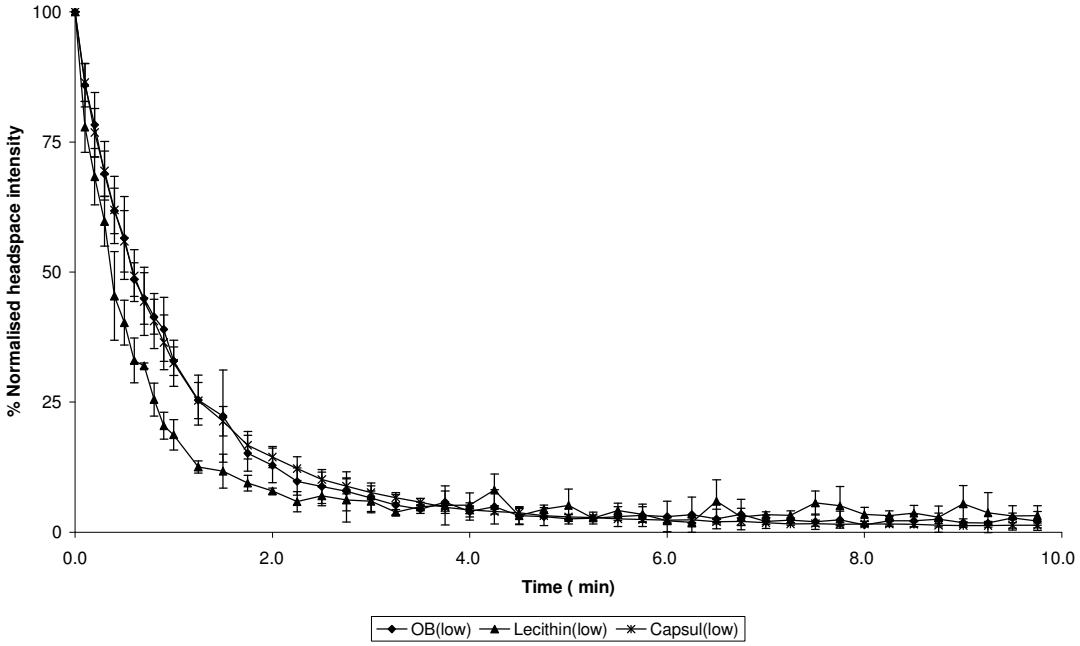
**[a]Limonene headspace concentration.g$^{-1}$Limonene on an equal lipid basis above dry powder (0.1g) in 2L container headspace measured by APcI-MS with internal standards.**

**[b]Limonene headspace concentration.g$^{-1}$Limonene on an equal lipid basis above sample (0.015% Lipid (w/w) predisolved in 250mL distilled water measured by APcI-MS.**

When suspended in water (0.015% lipid) a similar calculation was carried out and showed that although the Capsul product has a lower limonene headspace concentration during dry storage, when hydrated it released limonene into the headspace to a similar extent as the water-washed oil body system (Table 6-6). There were no significant differences in limonene headspace concentration between the Capsul and the water-washed oil body product (compared between homogenisation levels at $P<0.05$) (Table 6-3).

This indicates that in this system, water-washed oil bodies maintained a similar limonene headspace concentration, when compared on an equal lipid and limonene basis, but struggled to encapsulate enough limonene to be an effective competitor to Capsul.

Further to the static headspace measurements of limonene, the spray dried rehydrated water-washed oil bodies were assessed by a dynamic headspace dilution method that was comparable to the method used for the 0.015% lipid limonene sample presented in Figure 6-3. Figure 6-6 shows that there were no major differences between the six samples, when assessed by a dynamic headspace dilution method, statistical analysis was performed (ANOVA, $P<0.05$) and showed that there is no statistically significant differences between the data sets after 2.5 minutes. It can be noted that the samples subjected to low shear mixing had higher %nHI than those that were subjected to high shear mixing. It can also be noted that in both cases the %nHI of the oil body suspension was higher in the early phases of headspace dilution than the lecithin sample. This corroborates our earlier work on higher lipid content oil body suspensions, which showed that oil body samples had a higher normalised headspace intensity than phospholipid stabilised emulsions but due to the low lipid contents in this system this difference was less apparent.



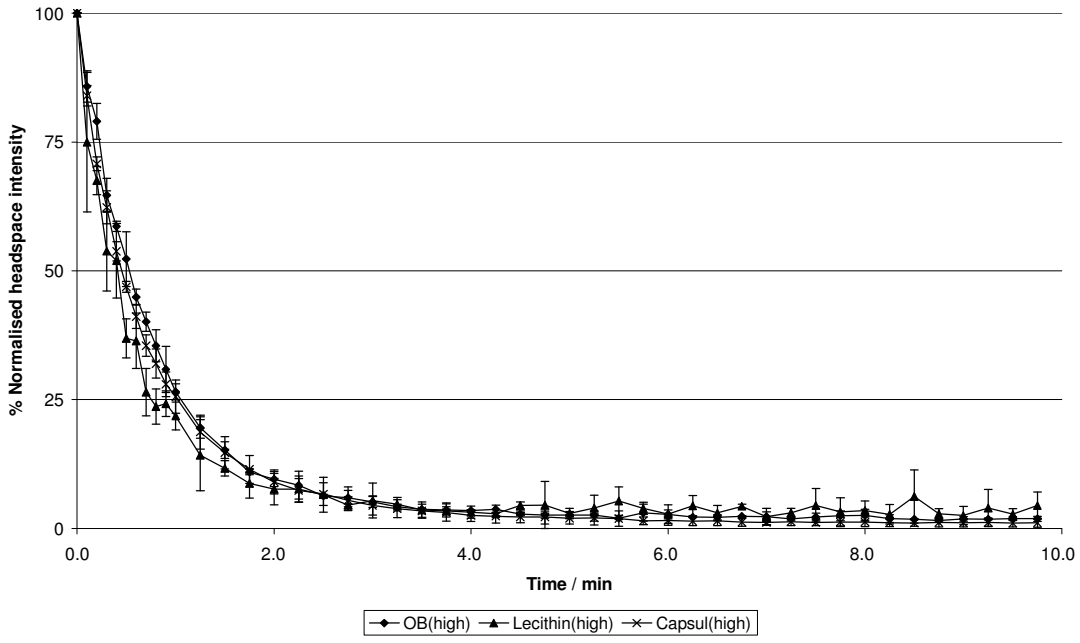




**Figure 6-6 Normalised time-course profiles for dynamic headspace dilution measurements of limonene in an aqueous suspension of spray dried samples stabilised by oil bodies, lecithin and Capsul**

**(PTR-TOF-MS, $N_2$ flow 70mL.min$^{-1}$ sampling flow 20ml.min$^{-1}$) nHI of a 0.015% lipid sample.**

## *6.4. Sensory Assessment*

To assess consumers' perception of water-washed oil bodies as a food ingredient, a likeability trial was designed using a basic food product, salad dressing, as a model system. In this study a water-washed oil body formulated salad dressing (25% lipid) was compared against a product made in-house with a comparable ingredient composition (25% lipid and 75% lipid) and a commercial product that was sourced as the most comparable on the market. Water-washed oil bodies were chosen over urea-washed as urea is not food grade and any food application therefore could only use water-washed or salt-washed oil bodies.

**Table 6-7 Likeability of four salad dressings: a commercial product, model salad dressings produced at 25% and 75% lipid, and an oil body dressing (25% lipid)**

| Product | Panellists | Likeability Rating | Statistical Group |
|---|---|---|---|
| Commercial Salad Dressing | 59 | 6.24 ± 2.14 Like slightly | A |
| Salad Dressing 25% Lipid | 59 | 5.34 ± 2.14 Neither like nor dislike | B |
| Salad Dressing 75% Lipid | 59 | 5.19 ± 2.19 Neither like nor dislike | B |
| OB Salad Dressing 25% Lipid | 59 | 4.78 ± 2.24 Neither like nor dislike | B |

Panellists were asked to rate the samples (consumed on a bread matrix) on a hedonic nine point scale which was converted to ordinal data (1=dislike extremely; 5= neither like nor dislike; 9=like extremely). Likeability ratings for the four products were similar and showed that across the panellists, the average consumer did not dislike any of the products. Statistical analysis by ANOVA (post hoc L.S.D test, $P<0.05$) showed that the commercial product had a significantly higher likeability rating, but that neither of the other samples were significantly different on this likeability scale (Table 6-7).

**Table 6-8 Likeability of four salad dressings [subset of panellists based on their consumption of cream salad dressings]**

**A commercial product, model salad dressings produced at 25% and 75% lipid, and an oil body dressing (25% lipid)**

| Product | Panellists | Likeability Rating | Statistical Group |
|---|---|---|---|
| Commercial Salad Dressing | 22 | 5.38 ± 2.46 Neither like nor dislike | A |
| Salad Dressing 25% Lipid | 22 | 4.95 ± 2.40 Neither like nor dislike | A |
| Salad Dressing 75% Lipid | 22 | 5.18 ± 2.56 Neither like nor dislike | A |
| OB Salad Dressing 25% Lipid | 22 | 5.09 ± 2.52 Neither like nor dislike | A |

In addition to assessment on the likeability scale, panellists were asked to add comments on their normal eating habits and their reasoning behind their choice of rating. The variation within the products likeability was highlighted within the comments sections that indicated that consumers had a varied preference to a number of products. Some consumers preferred creamy dressings (ceasar style) over vinagettes (French or Italian style) and when the panel was filtered to just panellists that stated that they consumed creamy dressings, there was no significant difference between the four samples (ANOVA with L.S.D at 5%) (as shown in Table 6-8).

The commercial product contained additional ingredients to the three model dressings, which may have contributed to a higher likeability rating, a theory supported by the additional comments. Comments included details on the preferred thickness and taste of the commercial product. It should be noted that the comments were extremely varied and as with all the products the average likeability rating was “neither like nor dislike”

Data is represented graphically below (Figure 6-7) for the entire data set illustrating numerical mean, 1 standard deviation from the mean (shaded

box) and 2 standard deviations from the mean (extended line). Graphically the variation between the samples is much smaller than the residual variation which was confirmed by ANOVA.

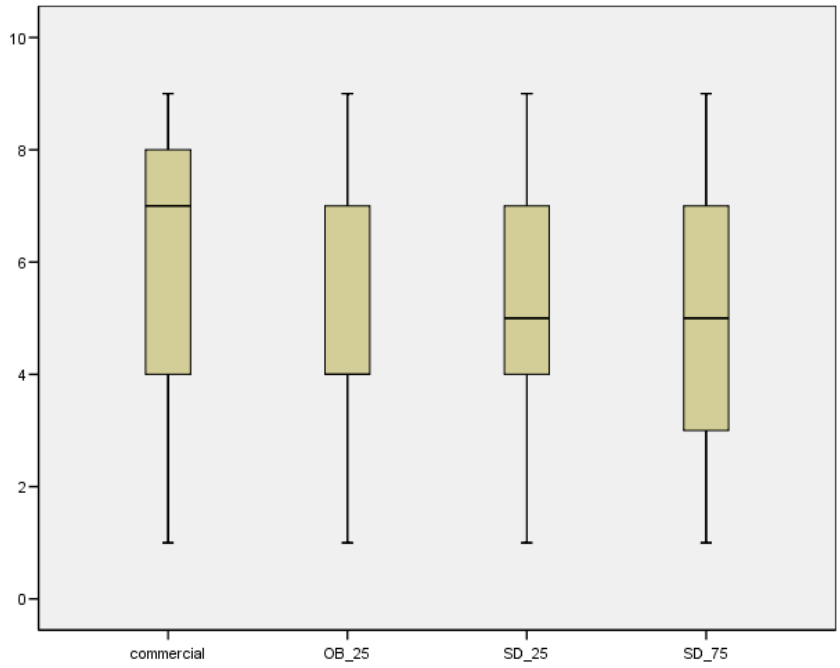
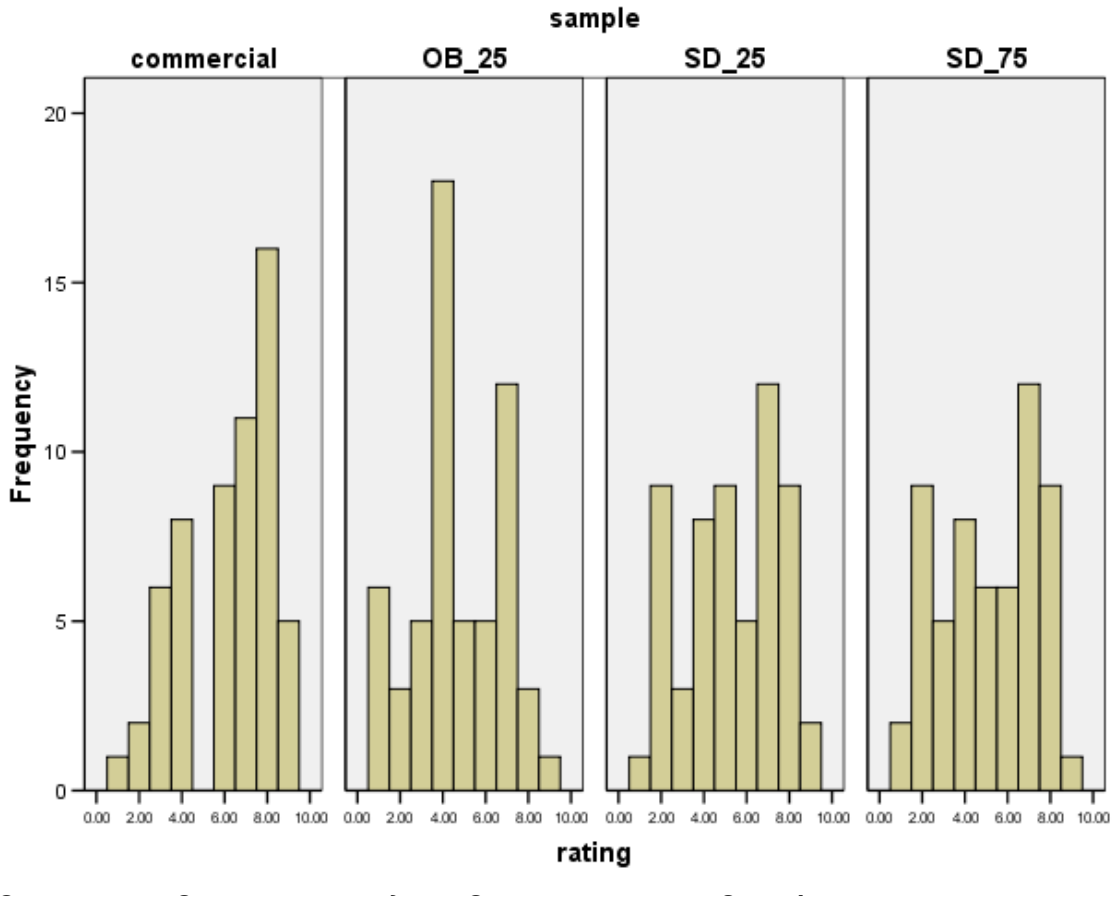



**Figure 6-7 Average likeability of 9 salad dressings**

**(1=dislike extremely; 5= neither like nor dislike; 9=like extremely) 1 s.d. = shaded box.**




**(1=dislike extremely; 5= neither like or dislike; 9=like extremely)**

Friedman's two factor ranked analysis of variance was carried out on the data set, after each panellist's data had been ranked (ties were taken as average values between ranks). Friedman's test proved the commercial product had a higher likeability than the 75% lipid salad dressing and the oil body dressing at 25% lipid, but no other significant differences could be established.

This clearly illustrated that oil body based salad dressings have a comparable likeability to the model salad dressings at equivalent and 3 times lipid content. A number of comments indicated that the 25% lipid dressing was "too watery" and "thin", whereas the 25% lipid oil body dressing was often quoted as being "creamy" and having a "nice mouthfeel". This has clear

benefits to the commercial market in terms of reducing fat content but maintaining structural and textural attributes.

Some negative comments on the oil body dressing included the dressing having a bitter aftertaste. This may be due to the presence of phenolic acids, principally sinapic acid, which could contribute a bitter astringency to the product. Earlier work has shown that total phenolic acid content of this water-washed preparation could have reached 40ppm, the taste threshold for phenolics in oilseed meals is 40-500ppm (Xu and Diosady 2002). This may account for the comments regarding bitterness, astringency and a strange aftertaste. The presence of associated phenolics would have to be addressed prior to the commercial use of sunflower water-washed oil bodies. This could be achieved using techniques such as isoelectric precipitation, filtration, or dialysis (Xu and Diosady 2002). Alternatively, other oilseeds could be used, including safflower or rapeseed, which contain fewer phenolic acids or seeds with phenolic acids that are less strongly associated to the oil body structures and associated proteins.

# 7. Conclusions

The research presented here has provided an insight into the structure and functionality of sunflower oil bodies. Oil bodies were extracted by wet milling, filtration, centrifugation and isolation of the boyant fraction. This fraction was then purified by washing in sodium chloride or urea to produce preparations of varying levels of purity. Purity was assessed by the concentration of contaminating proteins, and physical stability.

Urea-washed oil bodies are composed of 1.8% protein and 88% lipid (dwb). The lipid component was principally triacylglyceride, with smaller fractions of phospholipid also present. The phospholipid fraction of urea-washed oil bodies was principally phosphatidylcholine (91%) and phosphatidylethanolamine (9%). Less aggressively purified preparations of oil bodies also contained other phospholipid species (phosphatylinosiol and phosphatidic acid). These additional phospholipids may be present as contaminants due to the extraction process or be formed as a bi-product of the enzymatic degradation of phospholipids due to carry over of contaminating proteins in the purification steps, for example phosphatidic acid can be produced through the activity of phospholipase D.

One intrinsic component of oil bodies that was first identified through this work is vitamin E (tocopherol). Tocopherol was concentrated during the purification of oil bodies; urea-washed oil bodies contained 392 mg total tocopherol.kg$^{-1}$ oil. It was hypothesised that the intrinsic association of tocopherol to oil bodies may contribute to an increased oxidative stability.

To this end, oxidative stability of sunflower oil bodies was assessed over 8 days at elevated temperatures (5$^{o}$C, 25$^{o}$C and 45$^{o}$C). The progression of oxidation was quantified though assessment of headspace hexanal and lipid hydroperoxide concentration. Washed oil bodies were stable to oxidation showing only minor variations in hydroperoxide and headspace hexanal

concentration. Oxidation was compared to that of three comparable formulated emulsions; all formulated emulsions showed significant increases in hydroperoxide and headspace hexanal concentration over the oxidation trial. These novel findings suggest significant commercial benefits in the use of oil bodies as food ingredients.

The physical stability of washed oil bodies was also tested. Urea-washed and salt-washed oil bodies were physically stable when subjected to thermal stresses up to 45$^{o}$C for 48 hours, although less purified preparation showed loss of physical stability above 15$^{o}$C; this is believed to be due to the presence of residual seed cell debris. Washed oil bodies maintained their physical integrity during spray drying when carbohydrate was added as a carrier matrix, and subsequently could be used as a stable rehydrateable emulsion.

Oil bodies are thought to be stabilised by a combination of steric hindrance and electrostatic repulsion. Through this work we have separated the two phenomena and assessed oil body surface charge through the use of three techniques: streaming potential, zeta potential and physical resistance to creaming. All techniques indicated that oil bodies are electronegative at neutral pH with an isoelectric point (pI) of between pH3 and pH6. The absolute pI is dependant on the assessment methodology and method of preparation.

Oil bodies were also tested for their palatability and ability to carry and deliver flavour molecules. Oil body bulk phase flavour delivery properties comparable to artificially formulated emulsions; although the dynamic association of flavour molecules with the solution was significantly different. Oil bodies probably concentrate at the air-water interface forming a concentrated boundary layer; this would contribute to an increased ability of oil bodies to maintain the concentration of aroma above the solution during headspace dilution (headspace flavour concentration).

Through this study sunflower oil bodies were found to be: extremely physically stable, resist thermal and shear stresses; have elevated levels of resistance to thermally induced oxidation; contain high levels of naturally occurring antioxidants and efficiently deliver flavour compounds. This work provides a broad knowledge base of the physicochemical properties of sunflower oil bodies most relevant to the use of these natural emulsions in food applications.

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