Lipid Based Interfacial Design of Food Emulsion for controlling thelipolysis progress of GI-track

Guanghua Chen, MSc

Thesis submitted to the University of Nottingham for the degree of Master of Philosophy

June,2020

Division of Food, Nutrition and Dietetics

School of Biosciences

University of Nottingham

Table of Content

Chapter One:Introduction..... 1

1.1 Overview..... 1

1.2 Overall aims..... 4

Chapter Two:Literature Review..... 6

2.1 Emulsions..... 6

2.1.1 Definition..... 6

2.1.2 Emulsion instability..... 6

2.1.2.1 Flocculation..... 8

2.1.2.2 Creaming..... 8

2.1.2.3 Coalescence..... 9

2.1.2.4 Ostwald ripening..... 9

2.1.3 Emulsification processes..... 10

2.1.4 Characterisation of emulsion properties..... 11

2.1.4.1 Visual observation..... 12

2.1.4.2 Droplet size distribution..... 12

2.2 Emulsion interfaces..... 14

2.2.1 Overview..... 14

2.2.2 Lipid surfactants..... 17

2.2.2.1 Phospholipids..... 18

2.2.2.2 Galactolipids..... 19

2.2.2.3 Practical users of surfactant..... 23

2.3 Galactolipid and satiety studies..... 24

2.4 Controlling lipid digestion..... 26

2.4.1 Matrix of lipid components..... 25

2.4.2 Modulating droplet sizes..... 25

2.4.3 Interfacial layer..... 27

2.4.4 Inhibition of by-products..... 27

2.4.5 Deactivation of lipase..... 28

Chapter Three:Materials and Methods..... 29

3.1. Materials..... 29

3.2. Preparation and preservation of spinach crude Lipid..... 30

3.3 SPLIP Analysis..... 31

3.3.1 Thin Layer Chromatography (TLC)..... 32

3.3.2 Gas Chromatography- Flame Ionisation detector (GC-FID) 33

3.4. Oil-in-water emulsion preparation..... 34

3.4.1 Purification of sunflower oil..... 35

3.4.2 Vesicles solution..... 35

3.4.2.1 Vesicle Solution Preparation..... 36

3.4.2.2 Vesicle Size Measurement..... 36

3.4.3 Emulsion preparation.....37

3.5. Emulsion Characterisation.....39

3.5.1 Visual observation.....39

3.5.2 Optical Microscopy.....39

3.5.3 Particle Size Distribution.....39

3.6. Human digestion simulation with in-vitro digestion model.....40

3.6.1. Preparation.....40

3.6.2 Mouth.....41

3.6.3 Stomach.....41

3.6.4 Small Intestine.....41

3.7. pH-Stat Method to characterize lipolysis rates.....42

3.8. In-vitro digestion interfacial model.....44

3.9. Statistical Analysis.....45

Chapter Four:Results and Discussions.....47

4.1. Comparation of lipolysis rates of different common types of food emulsifiers.....47

4.1.1 Characterization of emulsions.....47

4.1.2 Lipolysis rates comparation of emulsions prepared with whey protein, sucrose ester, lecithin and cocoa particle.....50

4.1.2.1 Microscopy and droplet size analysis.....51

4.1.2.2 Free fatty acids analysis..... 55

4.2 Characterization of spinach crude lipid extract..... 59

4.2.1 Composition Analysis of spinach crude lipid extract..... 59

4.2.1.1 Thin Layer Chromatography analysis(TLC)..... 60

4.2.1.2 Gas Chromatography analysis(GC)..... 62

4.3 lipolysis rates of spinach crude lipid extract..... 66

4.3.1 Vesicle solutions..... 67

4.3.2 Characterization of emulsions..... 67

4.3.3 Droplet size distribution and mean diameter analysis..... 70

4.3.4 Free fatty acids analysis..... 75

4.3.5 in-vitro digestion interfacial model analysis..... 79

4.4 Overall Summary..... 82

Chapter Five:Discussion..... 85

References..... 88

CHAPTER ONE:INTRODUCTION

1.1 Overview

Obesity is currently a world-wide issue that a large number of people have to fight against today. A Lipolysis delay study is highly relevant to combat obesity, because the delays in lipid digestion is considered to have an effective impact on reducing appetite and promote satiety to combat obesity(Wilde and Chu, 2010). Lipolysis is an interfacial reaction, it is possible through modifying interfacial properties and structures, to control the digestibility of lipid droplet.

Since an emulsion is formed during the majority of lipid digestion, the emulsifiers play an important role to impact on the rate of digestion. There are lots of studies based on digestion of oil in water emulsion, which have been conducted by an *in-vitro* digestion model system(McClement and Li, 2010). Thus to figure out and compare the properties from different common types of food emulsifiers, such as protein, sugar, low molecular weight surfactants and particulate emulsifiers, based on oil in water emulsion by *in-vitro* digestion model is necessary.

A recent assessment shows that purified digalactosyldiacylglycerols(DGDG) have significant lipolysis delay properties at the interface of oil-in-water (o/w) emulsions with an *in-vitro* digestion model(Wilde and Chu, 2010) as well as increasing

satiety (Maljaars et al., 2007). DGDG is a kind of galactolipids with two acyl chains bound to glycerol backbone(Benson, 1964). The main function of galactolipids is to enhance membrane stability of an emulsion(Chapman et al., 1983), more than stabilizing membrane, it is one of the main polar lipids in plant photosynthetic membranes and as such a main structural component. DGDG could delay lipolysis by its galactose moieties groups, which could provide steric hindrance to slow the small intestine enzyme's ability to absorb to the emulsion oil-water interfaces, and thus impedes the rate at which lipolysis occurs (Chu et al., 2009).

However, purified DGDG is extremely expensive, so it is difficult to use in food product. It is reported that spinach is abundant in DGDG, and easy to obtain with low cost(Nakea et al., 2000). Spinach not only contains a significant amount of MGDG and DGDG (Nakea et al., 2000), but also contains a significant amount of essential nutrients such as proteins, vitamins and minerals(Hanif et al., 2006). Mieke et al.(2007) reported that spinach provides a significant contribution of micronutrients namely iron, vitamin A and riboflavin as it is rich in them. It is good for health if consumed spinach more frequently and in larger amounts, especially in children. To obtain the spinach crude lipid extract(SPLIP),which is rich in DGDG with low cost, and figure out whether SPLIP has an similar impact on delaying lipolysis compared to purified DGDG is meaningful and relevant.

SPLIP is not only abundant in DGDG, but also contains a promising amount of TGDG. TGDG has three galactose moieties, which might investigate more stearic hinderance than two galactose moieties, to defend bile salts attack, which is the first step of lipid digestion(Chu et al., 2009). As a result of that, SPLIP therefore be more efficient in delaying lipid digestion.

Within this thesis, whey protein, sucrose ester, sunflower lecithin and cocoa particle were selected, standing for protein, low molecular weight surfactants and particulate emulsifiers, to compare their lipolysis rates based on oil in water emulsion by *in-vitro* digestion model.

With respect to spinach crude lipid extract, SPLIP was used as surfactant in oil in water emulsions to compare with the commercial purified DGDG. There ability to delay lipid digestion was investigated by using an *in-vitro* digestion model and then explored with an *in-vitro* interfacial model. The first model was based on pH-stat method, which could directly display the percentage of free fatty acid(FFA%) released; and the second experiment was based on double-capillary technique of a Tensiometer, which investigated the changes at interface during lipolysis process by a sub-phase exchange. The results of *in-vitro* interfacial model can validate the results of the *in-vitro* digestion model.

1.2 Overall aims

On the whole, this MPhil thesis is aimed to:

- Firstly, to compare the lipolysis rates of four different types of food emulsifiers: whey protein, sucrose ester, sunflower lecithin and cocoa particles, based on oil in water emulsion by *in-vitro* digestion model.

    - To examine the lipolysis progress by using optical microscope and particle size distribution analysis during the experiment in mouth, stomach and small intestine phase.

    - Determine lipolysis rates by using pH-stat measurement in small intestine phase.

- Moreover, DGDG will be tested as an alternative emulsifier to evaluate the efficacy of spinach crude lipid extracts(SPLIP) to delay in lipolysis, compared with commercial purified DGDG, by using *in-vitro* digestion model and *in-vitro* digestion interfacial model.

    - To obtain SPLIP from spinach leaf.

- To use Thin Layer Chromatography analysis to carry out the separation of the lipid classes to confirm the presence of DGDG and phospholipid components, and then through Gas Chromatography analysis to quantify the amount of each lipid class.

- To use particle size analysis, pH-stat measurement to evaluate the lipolysis rates of SPLIP.

- To investigate the changes at the interfaces during the small intestine lipolysis when submitted to the sub-phase exchange through interfacial tension.

CHAPTER TWO:LITERATURE REVIEW

2.1 Emulsions

2.1.1 Definition

An emulsion is a mixture of two immiscible liquids which can be classified as oil-in-water or water-in-oil emulsions. These two types of emulsion are defined based upon the liquid which forms the continuous phase. Oil-in-water emulsion refers to instances where oil is the dispersed phase and water is the continuous phase; consequently, oil is dispersed in water (McClements, 2005). In contrast, when water is the dispersed phase and oil is the continuous phase, the water is dispersed in an oil and is therefore called water-in-oil emulsion. The process of mixing oil and water together to prepare emulsions is known as emulsification. However, when oil and water are mixed together, they tend to separate into two layers; this is known as emulsion instability. Emulsions are unstable thermodynamically systems, which are high in free energy. Thus to lower the free energy in the system, surfactants are added. Reducing the free energy in emulsion system can prolong the emulsion's stability.

2.1.2 Emulsion instability

Emulsion stability can be defined as the ability of the emulsion to resist any changes during a certain period of time. Several factors can affect the emulsion properties and stability, including the type of emulsifiers and the phase volume of the dispersed phase and continuous phase. Other

processing parameters, such as pH, viscosity, heat and enzymatic hydrolysis, can also impact upon the stability of the emulsion (Dickinson, 2003;

McClements,2005).

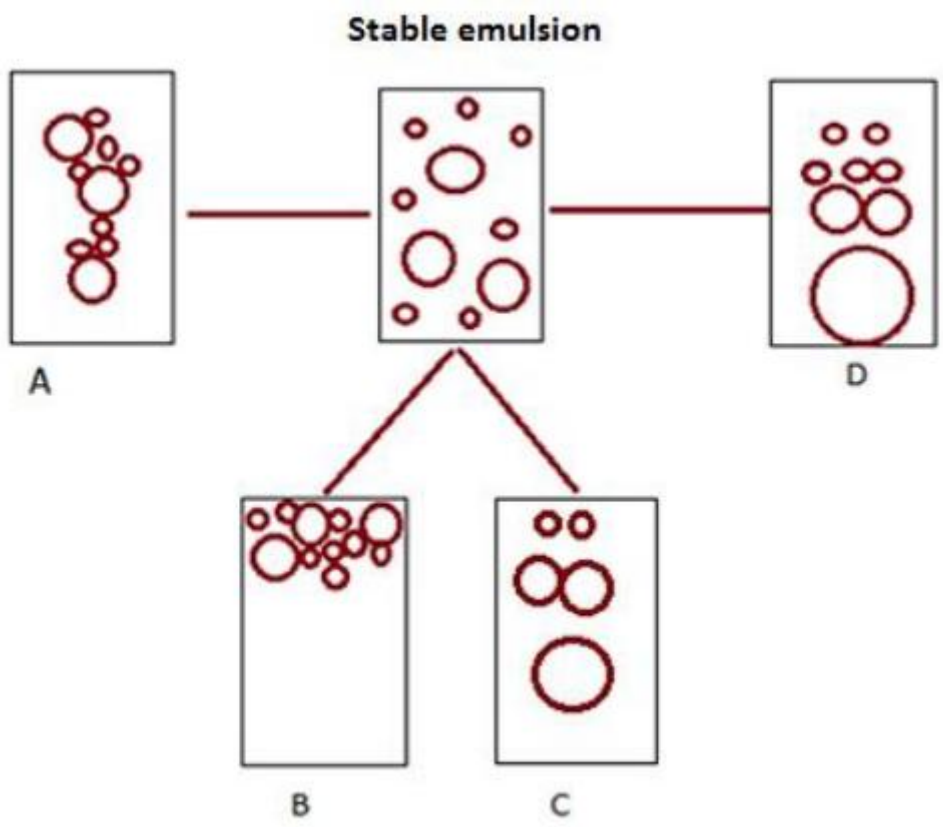


Figure 2.1: The process involved in emulsion breakdown (A) flocculation (B)Creaming (C) Coalescence and (D) Ostwald ripening

The instability of emulsions can be divided into two: physical instability and chemical instability. The physical instability which occurs in the emulsion can be referred to as the changes in structures, distribution and properties of droplets in the emulsion; whereas, the chemical instability refers to the chemical reaction such as oxidation and degradation which may cause the emulsion droplets splitting into small ones (McClements

and Decker, 2000; McClements, 2005). The various ways in which physical instability can occur are shown in Figure 2.1, each of which will now be explained in more detail.

2.1.2.1 Flocculation

Flocculation refers to the aggregation of droplets, without changing primary droplet's size and no rupture of interfacial layer. And flocculation can be reversed by agitation (Tadros, 2009). Image analysis techniques can be used to determine the size of the droplets or flocks (Jokela et al., 1990). Particle sizing instruments can also be used to measure the size of the flocks.

2.1.2.2 Creaming

Creaming refers to the gravitational separation of emulsified droplets to form a densely packed phase of the emulsion, the creaming velocity depends on the density difference between the two liquids; thus, reducing the density difference between the two phases will minimise the creaming problem. As the density of oil is lower than the density of the water, droplets of oil tend to move up to form an upper layer of emulsion. The rate of creaming can be predicted by Stoke's equation (McClements, 2005, Singh et al., 2009). Two factors are reported to reduce creaming: firstly, the production of small droplets during emulsification; and, secondly, the increasing of the viscosity of the continuous phase (McClements, 2004; Dickinson, 2003; Chanamai and McClements, 2000). In practice, creaming

is often assessed through observation and the measuring of the volume of the cream layer over time. It is a reversible process by agitation.

2.1.2.3 Coalescence

Coalescence is defined as the merging of two or more droplets into a larger one; which involve rupture of the interfacial layer. This disruption encourages two or more droplets to fuse together as a result of the driving forces from the intermolecular interaction(s) (Tadros, 2009). This phenomenon is directly related to the properties of the interfacial layer.

Coalescence is an irreversible process; which lead to complete separation of two immiscible phases. Coalescence is the also another instability observed in the emulsions, which is can easily identified when using microscopy.

2.1.2.4 Ostwald ripening

Ostwald ripening is the process by which components of the discontinuous phase diffuse from smaller to larger droplets through the continuous phase. The diffusion of liquid through continuous phase is due to Laplace pressure; this pressure arises from the interfacial tension of a mixture of two liquids at a curved interface when one liquid is dispersed as droplets into another liquid. During fusion of smaller to bigger droplets, the interfacial layer is not ruptured. The Laplace equation is as follows;

$\Delta P = 2\gamma / R$

where Υ = interfacial tension, R = particle radius

The larger droplets formed can be observed using microscopy; however, measurement of the droplet sizes with time is required to differentiate between coalescence and the Ostwald ripening phenomenon (Bremer et al., 1996). In a case for Ostwald ripening alone, this can be done through the observation of sharp distribution, along with the increase in droplet size which then will progressively disappear (Fernando et al., 2007). Ostwald ripening is an irreversible process.

2.1.3 Emulsification processes

As mentioned before, emulsification is a process where two immiscible liquids are mixed together. Generally, two steps are involved in the emulsification process. In the first step of the process, the oil is mixed in the water – this can often be achieved through the use of a high mixer. As a result a coarse emulsion is obtained. In the next step of emulsification, the larger droplets in the primary emulsion are converted into smaller droplets by using a mechanical force to create agitation (McClements, 2005) during the homogenisation process.

As in this study, the emulsification process is referring to the oil-in-water emulsion; thus, emulsification is defined as dispersing an oil-in-water through the presence of energy which is supplied to break down the large oil droplets into smaller droplets which are evenly distributed in the water. During this emulsification process, these small droplets are constantly

moving and colliding with each other; it is therefore likely that they could reform to the primary large droplets due to the difference in density. As such, in order to prevent this reforming, and to make sure that the emulsification process is achieved, the surfaces of the small droplets need to be resized. This can be done, for example, by adding a surface agent; this surface agent will act by coating the surface of the created small droplets in order to prevent the small droplets from merging or allowing coalescence to occur when they are close to each other (McClements, 2005).

The process of emulsification, as mention above, can be achieved using a range of devices which can be chosen in accordance with the droplet size required. The most common devices used are high shear mixers, ultrasonicators and high pressure homogenisers. High pressure homogenisers are the most commonly used devices for

producing emulsion as they are more effective in reducing droplet size than high shear mixers (Stang et al., 2001). Based on the applications, the droplet sizes in the emulsification process may vary from 0.1 μm to 100 μm (Singh et al., 2009). High pressure homogenisers were used during the emulsification work for this research.

2.1.4 Characterisation of emulsion properties

In this study, physical chemical characterisation was performed to investigate the properties of the prepared emulsions. Several parameters

were used to characterise the emulsion properties, each of these will now be discussed in turn.

2.1.4.1 Visual observation

This technique provides direct information on the stability of the emulsion based on their appearance. Common instability based on physical assessment is indicated by the separation of water phase at the bottom and/or showing a sign of total breakdown between the surfactant (Herrera 2012) .

2.1.4.2 Droplet size distribution

Optical measurement has been widely used and established to gain more information about biological samples; it has also become a powerful tool for obtaining information from emulsion samples (Latimer, 1982). In this study, laser light scattering was used to measure the droplet size distribution of the prepared emulsions. This method is based on the measurement of intensity versus the scattering angles. The scattering patterns were created to provide additional information about the droplet sizes as the scattered angle was inversely proportional to the droplet sizes (Allen et al., 1992; Lambourne and Strivens, 1993). The intensity of the scattered light is measured using an array of photosensitive detectors, this was then calculated by comparing the sample's scattering pattern with the Fraunhofer optical model. This model relies on the assumption that particles diffract light at a narrow angle; in practice, the droplet size

distribution is based on a log-normal distribution (Packer and Rees, 1972; Kiokias et al., 2004). The droplet size of an emulsion is defined as a diameter of the internal phase globule (Harrison and Cunningham, 1985). Generally, a stable emulsion is associated with a fine and uniform droplet size. Measuring droplet size in an emulsion is one of the crucial factors which provides information about the properties of the emulsion. When measuring the droplet size distributions, the data obtained can be used to estimate the properties of the way in which an emulsion resists to the instability phenomena (McClements, 1999; Mason et al., 1996) as well as how it resists to chemical activity and/or reaction; for example, in the presence of lipase catalysed reactions (Knezevic et al., 1998). Examples of instability phenomenon that could be predicted when measuring droplet size distribution include coalescence and Ostwald ripening.

Various terms could be employed to calculate the droplet size distribution of emulsions, including Sauter diameter, d 32 (μm) (Sajjadi et al., 2002) and volume mean-droplet size d 43 (μm) (Ruiz et al., 2002). The Sauter diameter or average surface diameter, d 32 (μm) refers to the drop diameter of the drop of average surface area (total surface area/number of droplets) while the volume mean-droplet size d 43 (μm) refers to the total volume of oil or number of droplet. However, the terms chosen can be dependent on the one reporting their results. In this study, the sauter diameter has been chosen for the reporting of the results because it represents the surface area of the droplets during the measurements.

2.2 Emulsion interfaces

2.2.1 Overview

An interface is defined as the contact region between oil and water (two immiscible region). In order to obtain an emulsion, this interface needs energy to facilitate the change. As mentioned before, during emulsification, the breakdown of the large droplets to a smaller droplet of emulsion increases the interaction of the oil droplets in the water phase. The different interactions occur between molecules at the interfaces and molecules in the bulk system which generate interfacial tension. This can be explained due to a high attractive force which is generated when one molecule of oil and one molecule of water interact at the interface; this induces tension across the interface, rather than a low attractive force which is generated when the same two molecules of water interact at the interface as well as in the bulk system (Kaufman, 2002). The total amount of attractive forces at the interfaces is higher than in the bulk system; these forces are defined as free energy per unit area.

Due to the thermodynamic equilibrium, these new interfaces are likely to minimise their free energy. To extend the shelf life of an emulsion, surfactant can be added. Surfactant will be adsorbed at the interface and will subsequently alter the structural interfacial area, by displacing the water and oil molecules of the original interface. Surfactants do so by orienting themselves in such a manner that their headgroups point into water while there nonpolar tails point into the oil, in O/W emulsion and

vice versa for W/O emulsion. The orientation of surfactant is shown in Figure 2.2. The alteration increases the interaction of water and oil with the molecules in surfactant which will prompt the lowering of the interfacial tension (Dembitsky, 2004).

The surfactant used in an emulsion will be categorised based on its solubility in oil or water. Due to the Bancroft rule (Bancroft, 1913), when a surfactant dissolves in oil, it tends to stabilise the water/oil (W/O) emulsion, whereas when a surfactant dissolves in water it tends to stabilise the oil/water (O/W) emulsion. Selection of surfactants in order to prepare a stable emulsion is also depends on the ratio of hydrophilic head group to the lipophilic tail.

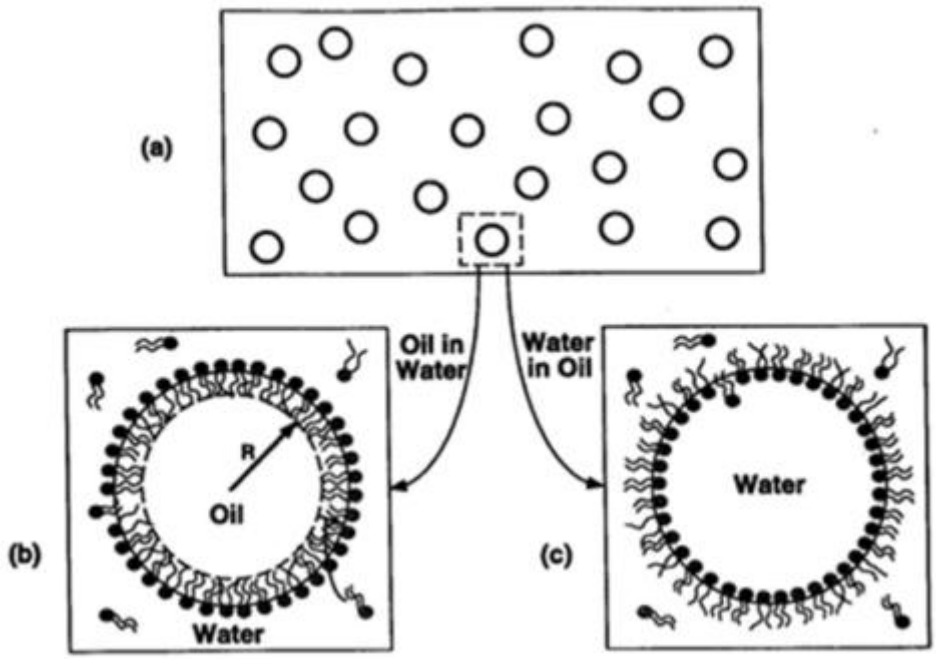


Figure 2.2. Emulsion with surfactants (a); orientation of surfactant when in O/W emulsion (b) and action of surfactant when placed in W/O emulsion (c).

Source: Evans, Fennell and Stokes (1997)

A surfactant is defined as surface active agents which tend to reduce the surface tension of a liquid in which it is dissolved. As the solvent used in this study is water, the terms hydrophobic and hydrophilic will be used. Surfactants in general possess a hydrophobic part and a hydrophilic part, this allows both parts to adsorb onto the interfaces and tends to lower the free energy of these interfaces. Because surfactants possess both hydrophobic and hydrophilic parts, the structure of the surfactant is said to be amphiphilic. Note that the term interfaces is used to refer to

the liquid/liquid interfaces.

Surfactants are available from natural and synthetic sources and have been used in many applications such as in detergents as the foaming agent, as well as in food and pharmaceutical industry applications. In food industries, food grade surfactants have been used; however, natural sources for food grade surfactants still need to be established further. Figure 2.3 shows some common materials used as surfactants.

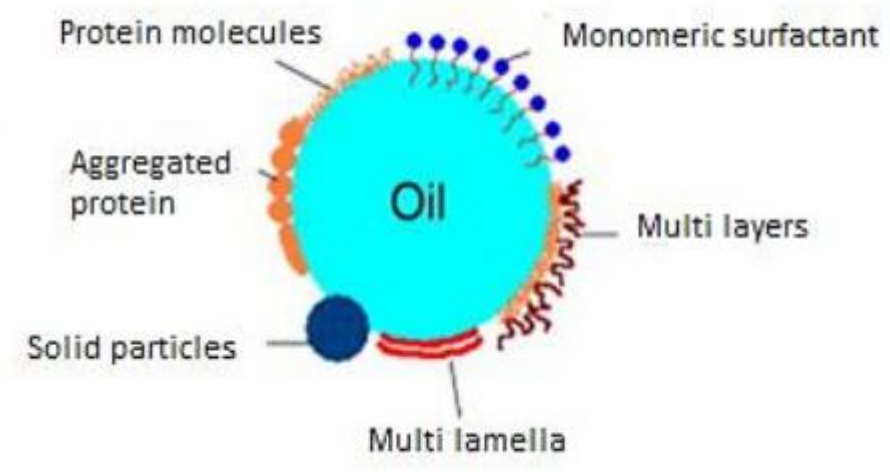


Figure 2.3: Types of surfactant that can be used to stabilise emulsions by forming an adsorbed layer.

Source: Harjinger et al. (2009)

2.2.2 Lipid surfactants

Lipids surfactants contain both hydrophilic and hydrophobic segments. In fact, lipids with amphiphilic characters can be claimed to be natural surfactants. In general, natural surfactants can be defined as surfactants obtained from natural sources via extraction, distillation or precipitation as natural surfactants don't require further treatment or chemical synthesis. Natural sources may include plants, yeast, bacteria or animals (Holmberg, 2001). Furthermore, lipids are easy to extract and can therefore be economically obtained (Kralova and Sjoblom, 2009). Recently, the extraction of lipids from plants has gained much attention as they often hold dense nutrients that can be used to promote health (Vibhute et al., 2013). In this study phospholipid and galactolipid were used as lipid

surfactant because both of phospholipid and galactolipid are amphiphilic molecules.

2.2.2.1 Phospholipids

Phospholipid shows amphiphilic characters as it contains both hydrophobic and hydrophilic parts; the phosphate polar headgroup is the hydrophilic part whereas the non-polar tail group, which is composed of fatty acids, is the hydrophobic part as shown in Figure 2.4.

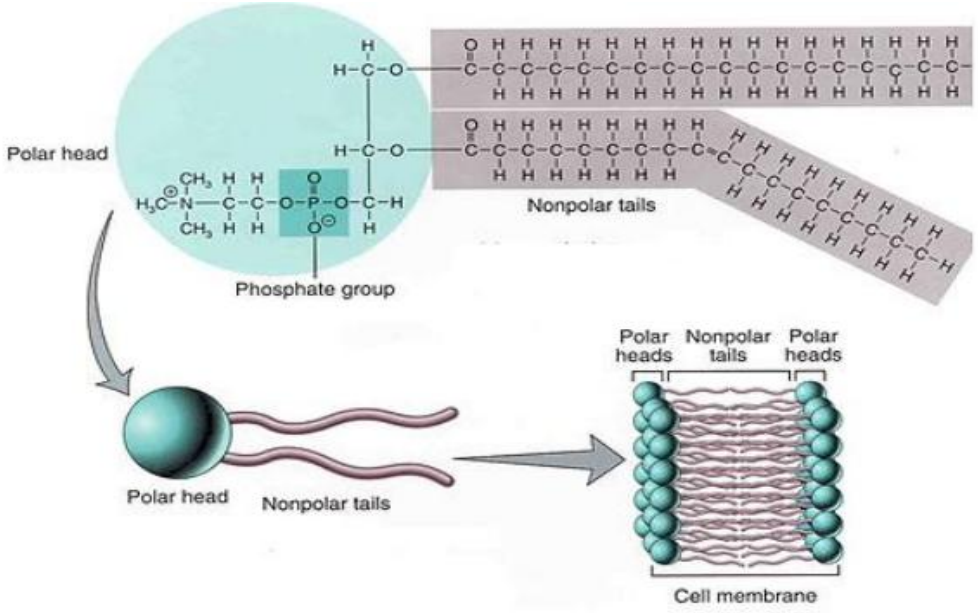


Figure 2.4: Phospholipid structure. Source: Wissmann (2014)

In general the polar headgroup is composed of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylserine (PS) (Cecv, 1993). The length of both the hydrocarbon chain and the types of fatty acid also varies. These unique characteristics

influence phospholipid solubility in water. A study conducted by Handa et al. (1990) indicated that different types of monolayer formed at the o/w interfaces when different types of headgroups were used as the surfactant. It was reported that the emulsion stabilised with PC showed resistance to coalescence, compared to emulsions stabilised with PE and PS as a more compact monolayer was formed at their interfaces. Work by Rosenberg and Ron (1999) suggested that hydrocarbons, with a length between C12 to C24, were suggested to be the most appropriate at reducing the interfacial tension. When the phosphate group is attached with choline, it is called phosphatidylcholine, it may also be known as lecithin. Lecithin is widely used as a natural surfactant in food applications and has been recognised as safe to consume (Bylaite et al., 2001).

Lecithin can be obtained during the degumming process of sunflower oil (Morrison, 1985; Pan et al., 2001) and soya bean oil. However, due to limited sources of non- genetically modified soya bean, sunflower oil is usually chosen as an alternative. Typically, sunflower lecithin contains 43.1% phospholipids, in the form of: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), 33.4% oil and 23.5% in the form of other compounds, including carbohydrate and sterols (Hernández and Quezada, 2008).

2.2.2.2 Galactolipids

Galactolipid is a main component in glycolipid, it can be obtained from extracting lipid components from plants, animal tissue and bacteria. The

main function of this glycolipid is to enhance membrane stability (Chapman et al., 1983). Galactolipids are basically a glycerol moiety link to a galactose group which is a polar headgroup (Benson, 1964) and a hydrocarbon chain which is a non-polar group. The general structures of the galactolipids are shown in Figure 2.5.

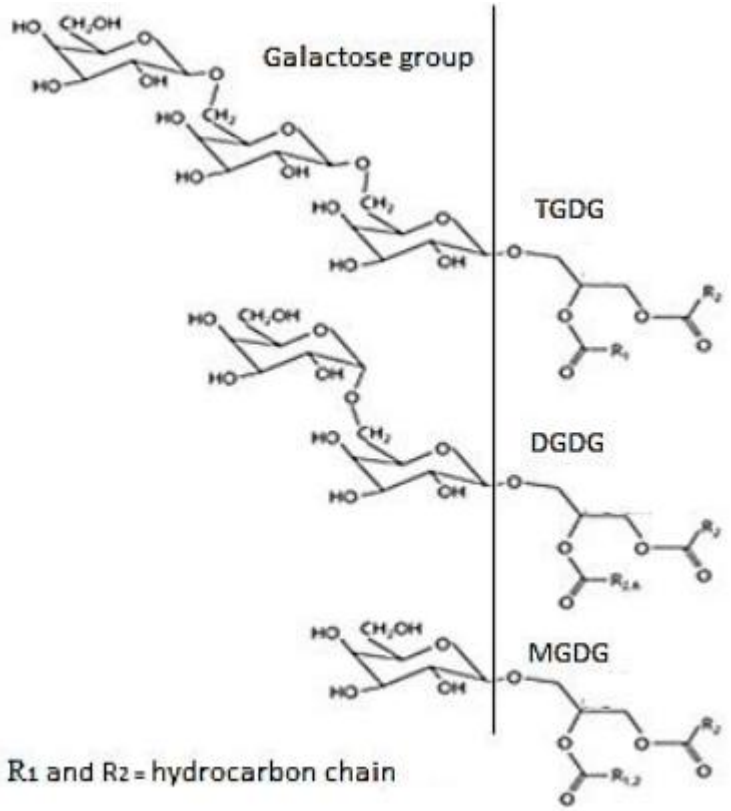


Figure 2.5: Galactolipid structures of MGDG, DGDG and TGDG

The galactose group tends to interact with the water phase while the hydrocarbon chain tends to interact with the oil phase. Two of the most abundant galactolipids in different plant tissues (leaves, unsaturated), compared to seeds (more saturated), membranes are those of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol

(DGDG) (Dorman and Anderson, 2009), these are reportedly rich in spinach, parsley and Chlorella pyrenoidosa. However, the galactolipids occurring in plants differ due to their fatty acid composition which consists of saturated and/or unsaturated fatty acids that are bounded to the glycerol backbone; for example, in spinach, MGDG and DGDG contain polyunsaturated fatty acid (Christie, 1992).

Generally, the fatty acid chain length varies from C16 to C20. MGDG and DGDG are different in their size and shape, due to their headgroup. As MGDG has a smaller galactose headgroup than DGDG, MGDG tends to form a conical shape, whereas DGDG tends to form a cylindrical shape (Aronsson et al., 2008; Kobayashi et al., 2007), as shown in Figure 2.6. These unique biophysical properties of galactolipids reflect the way they differ in terms of their functions, pathways and self-organization properties.

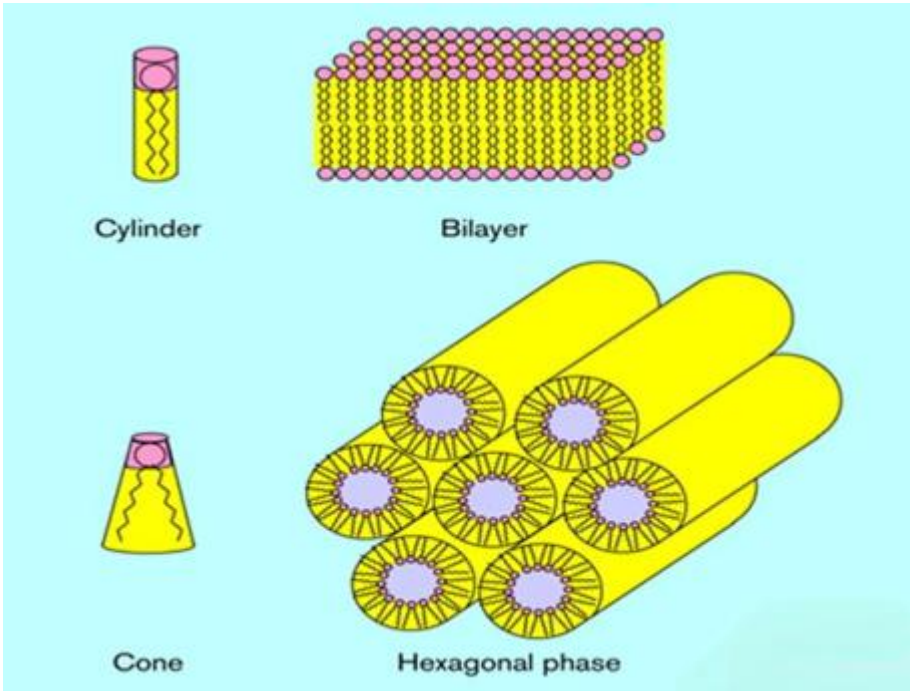


Figure 2.6: The concept of lipid shape and its effects on lipid packing

Source: Current Biology (2000)

In plants, the percentages of the galactolipids of MGDG and DGDG constitute nearly 75% of the total membrane lipids in the leaves (Joyard et al., 1998; Slabas, 1997). Conversely, trigalactosyldiacylglycerol (TGDG) is rare in leafy vegetables, but it can be found abundantly in pumpkin (Ito et al., 1974). The glycolipids composition in various plant sources can be determined through the use of High Performance Liquid Chromatography that is equipped with an Evaporative Light Scattering Detector (HPLC-ELSD), the results of which are displayed in Table 2.1 (Nakea et al., 2000).

Table2.1: The glycolipids composition in various plant sources, as determined by HPLC-ELSD. * DW refers to dry weight ND refers to Not detected.

	MGDG (mg/100 g DW*)	DGDG (mg/100 g DW*)	TGDG (mg/100 g DW*)
Barley	14	60	ND
Corn	341	516	ND
Rice (milled)	0.2	0.7	ND
Rice (bran)	8.4	2.4	ND
Wheat flour	102	225	ND
Soya bean (lecithin)	ND	60	ND
Cacao (raw)	ND	58	ND
Almond (raw)	ND	1.6	ND
Spinach	840	878	ND
Tea	810	736	ND
Carrot	91	112	ND
Pumpkin (seed)	350	420	307

Source: Nakea et al. (2000)

It is worth noting that the amount of glycolipids found may vary depending on the cultivar and growth conditions. It has been considered that TGDG would be more hydrophilic than MGDG and DGDG, as TGDG exhibits a bigger hydrophilic headgroup which contains three galactose residues and is therefore expected to show a stronger lipolysis delay property.

2.2.2.3 Practical uses of surfactant

Interestingly, lipids induce different self-assembling properties in the presence of water; as a result, various structures can be formed, including: micelles, reverse micelles (bilayers), as well as cubic and hexagonal phases (Walde and Ichikawa, 2001). The behaviour of these structures will be dependent on its polarity, the type of acylated fatty acids and saturation of fatty acids present (Ulrich, 2002). Regardless of there formed structure, the general term of "vesicle" will be used throughout this thesis. Depending upon the method of preparation used, the diameter of the vesicle may vary between 20 nm to hundreds of micrometres. Furthermore, as a result of their ability to form different sizes and configurations, vesicles are found in many applications in the food, pharmaceutical and cosmetics industries. The ability to entrap functional components allows these structures to be used as delivery vehicles to increase bioavailability, control release and/or target release (Porter et al., 2008; McClements et al., 2009; Velikov and Pelan, 2008). Phosphatidylcholine (lecithin) is commonly used in the food industry as it is a vesicle- forming amphiphile. When a low lecithin/bile salt molar ratio

is present, lecithin vesicles can be transformed into micelle structures, possibly due to the adsorption of bile salt which impacts on the structure. Consequently, this facilitates the pancreatic lipase to exhibit lipid digestion (Pignol et al., 2000). It is therefore suggested that by modifying the interfacial components it is possible to help minimise lipid digestion within the gastrointestinal tract (GI).

2.3 Galactolipid and satiety studies

Satiety can be defined as a "sensation of fullness", this response is received from the feedback signal triggered in the gastrointestinal tract (Ritter, 2004). Galactolipids, mainly DGDG, can delay the rate of digestion; to illustrate, when a large portion of undigested lipids reach the ileum, the undigested lipids trigger the ileal break mechanism – this controls the way in which satiety is modulated (Chu et al., 2010; Chu et al., 2009: Albertson et al., 2007).

As a result of this mechanism, during lipolysis the bile salts and lipase/colipase absorption onto the substrate surface is restricted. It is believed that restricted access to the substrate is possibly due to the presence of stearic hindrances, such as non-ionic headgroups, which are more surface active than the bile salts (Li et al., 2010). This suggested mechanism is based on the fact that the large headgroups and strong molecular interaction between the galactosyl residues might sterically hinder the bile salts, colipase and lipase from adopting their active

orientation at the interface, as shown in Figure 2.7. The work of Chu et al. (2009) supports this, specifically claiming that galactolipids, particularly DGDG, provide a steric hindrance that slows the duodenum enzyme's ability to absorb the emulsion lipid-water interfaces, and thus impedes the rate at which lipolysis occurs. Tsuzuki et al. (2004) demonstrate that Lysophospholipid inhibited hydrolysis by decreasing both the substrate affinities and the catalytic activity of this lipase; by preventing the lipase from binding to the lipid droplet surface, the emulsifier presumably possesses some inhibitory properties which delay the digestion process.

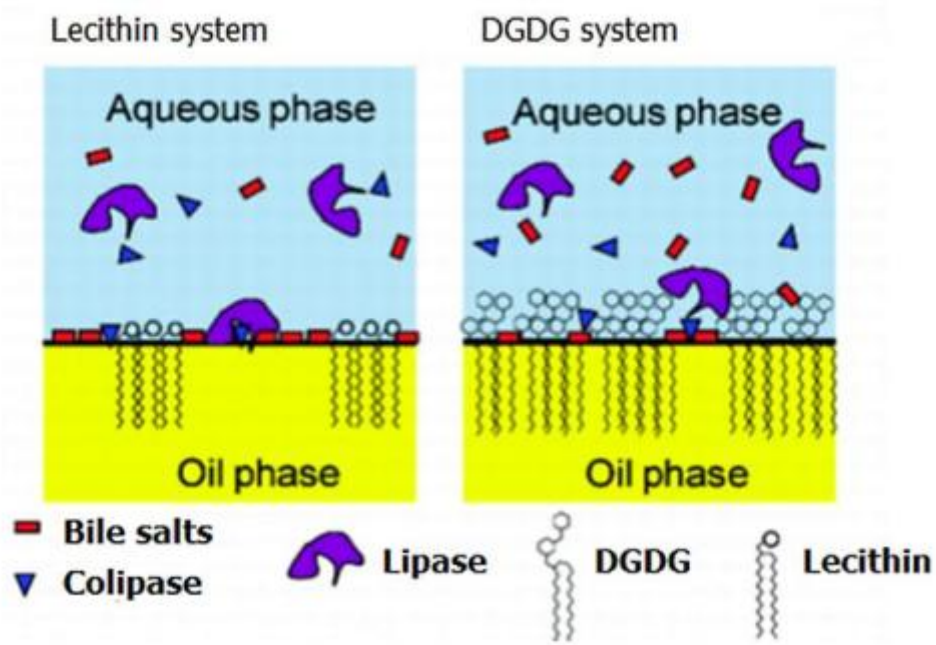


Figure 2.7: O/W interface stabilised with lecithin (left) and DGDG (right) during lipolysis.

Source: Chu et al. (2009)

Furthermore, the molecular structure and the packing density of the components also appear to play an important role in aiding the inhibition

of the lipolysis. Research conducted by Sugar et al. (2001) reported that the adsorption of digestion enzyme onto the substrate surface is strongly dependent on the lipid composition and surface packing.

The fat satiation effect is also affected by lipid's physicochemical structure, specifically the degree of fatty acid saturation and its length. The presence of ALA at both sn-1 and sn-2 positions on the glycerol moiety backbone of DGDG can also help to delay the lipolysis. Research by French et al. (2000) reported that the unsaturated fatty acid (C18:2) can suppress food intake rather than C18:1 and C18:0.

2.4 Controlling lipid digestion

A multitude of studies have focused on the way in which the digestibility of lipids in the human intestinal tract can be controlled. As previously mentioned, the digestion of lipids is an interfacial process; consequently, various studies have focused on modulating the lipid droplets so that they can withstand the digestion enzyme as well as the physical environment encountered during hydrolysis. The crucial factor in modulating the digestion of lipids is concerned with the ability of lipase to be restricted when it comes into contact to the lipid droplets; thereby hindering the hydrolysis process (McClements and Li, 2010). Five different approaches for slowing down the rate of lipid digestion will now be identified.

2.4.1 Interfacial layer

It is predicted that by coating the oil droplet with a layer of absorbed materials or polymers, the surface area would be restricted from coming into contact with the lipase and, thereby, the lipase activity would be slowed somewhat (McClements and Li, 2010). Furthermore, by coating the lipid droplets together with alginate, as an indigestible polysaccharide, the rate of hydrolysis would also be greatly decreased because the polysaccharides linkage occurs during digestion and therefore traps the lipase from interacting with the lipid droplets (Li et al., 2012). Conversely, Chu et al. (2009) indicated that digestion rates can also be delayed by incorporating a surfactant that possesses an inhibitory structure as it hinders the lipase from accessing the emulsified lipids.

2.4.2 Inhibition of by-products

The lipase activity could be inhibited by the accumulation of by-products after hydrolysis, in the form of monoglycerides and free fatty acid molecules (Reis et al., 2009). In fat digestion, the lipase activity can be characterised to be sn-1, 3-regiospecific or non-regiospecific. If hydrolysis creates sn-1, 3-regiospecific, monoglycerides and fatty acid are the by-products, whereas if TG hydrolysis creates a non-regiospecific lipase, fatty acid and glycerol will be the by-products. Thus, if only lipolysis happens with sn-1, 3-regiospecific lipase, the presence of monoglycerides which are interfacially active reported as the by-product can expel lipase from the interfaces (Reis et al., 2008). It is worth noting that monoglycerides are nomally well solublilized by bile salts and this hypothesis is weak and

not well supported by experimental evidence under conditions mimicking the composition of GI tract contents, and this could be well discussed in this research.

2.4.3 Deactivation of lipase

By-products are commonly used within drugs to inhibit the hydrolysis of lipids. The incorporation of the ligand specifically interacts with the lipase active site to prevent any interaction with the lipid droplets (McClements and Li, 2010) – this results in inhibition of the digestion process.

CHAPTER THREE:MATERIALS AND METHODS

3.1. Materials

The interfacial surfactants used in this research were purchased: Purified digalactosyldiacylglycerols (DGDG) in chloroform from Lipid Products (UK); Cocoa particle(contains <1% fat) from Nestle(UK); Whey protein from Bodybuilding Warehouse(UK); Sucrose ester from Sisterna (UK); Sunflower lecithin from Thew Arnott and Co Ltd (Surrey, UK). The following solvents were purchased from Sigma-Aldrich (UK): isopropanol, chloroform and methanol. Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$) and magnesium silicate particles (Florisil-mesh 200) were purchased from Fisher Scientific (UK). All of the chemicals used were of analytical grade. Finally, Organic spinach leaves were purchased from Tesco (Nottingham, UK) and Sunflower oil was purchased from Sainsbury's (Nottingham, UK).

The enzyme used in the research: Porcine gastric Mucin type 3 (glycopeptide), α-amylase from human saliva Type XIII-A, 300-1500 U/mg protein (A-1031), Pepsin from hog stomach (activity=650 units/mg of protein), porcine gastric lipase, porcine bile salt and pancreatin from porcine pancreas(P7545)(activity=100-400 units/mg of protein) were all purchased from Sigma-Aldrich (UK).

3.2. Preparation and preservation of spinach crude Lipid

The spinach crude lipid (SPLIP) was obtained from spinach leaves, because spinach leaves had high yield with less process(Nizaha,2016). The protocol used in the research is shown in figure 3.1.

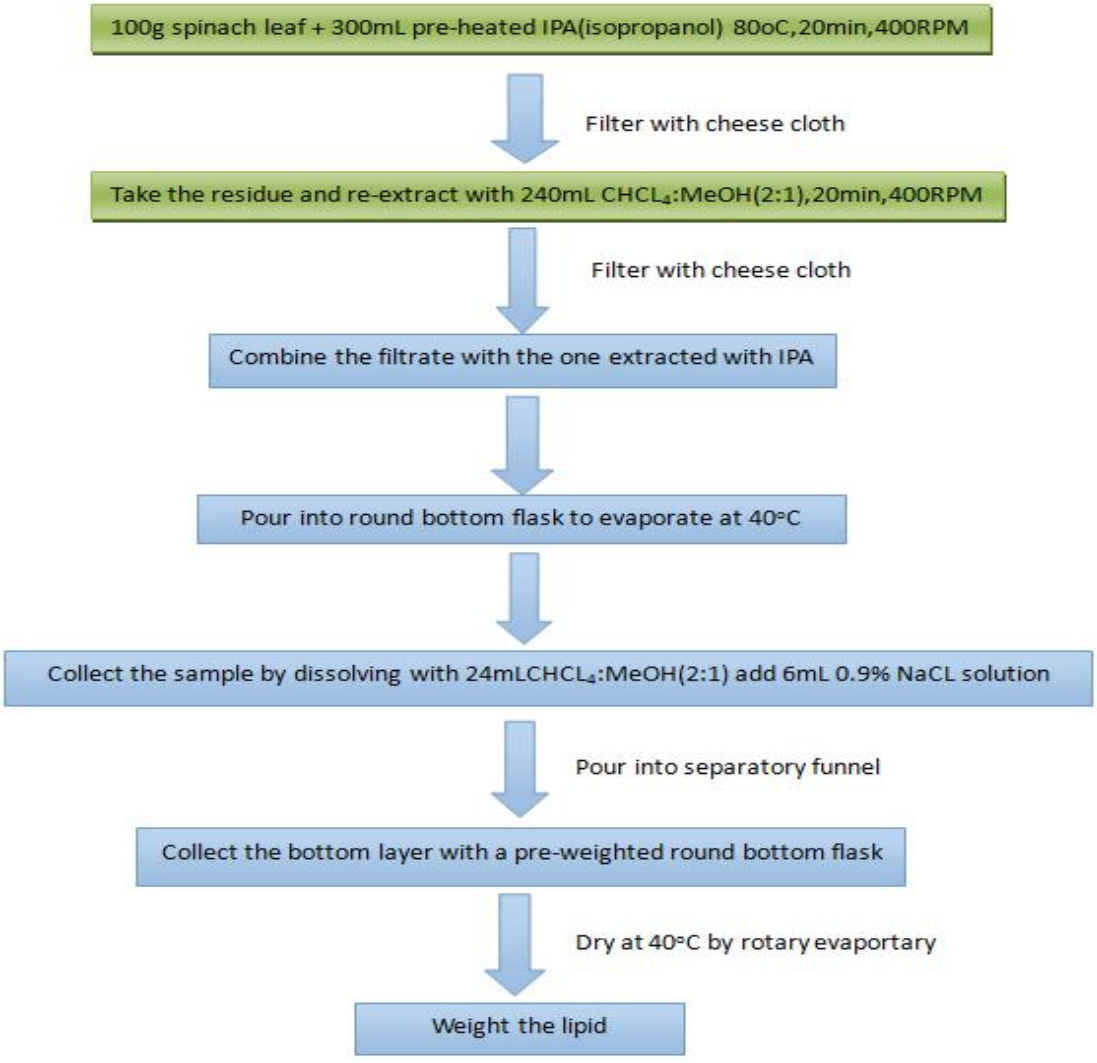


Figure 3.1: Protocol of SPLIP extraction

100g spinach leaves were firstly added to 300mL 80°C pre-heated isopropanol and smashed them by a household blender. The slurry

obtained from previous operation was filtered by using cheese cloth, and the residue was then re-extracted with a 240mL of chloroform and methanol solution (mixed with a 2:1, v/v). The filtrate from isopropanol and chloroform/ methanol were combined together and evaporated at 40 °C in a round bottom flask. After that, the sample was collected from evaporator by dissolving in 24mL mixed chloroform and methanol solution (2:1, v/v). Thereafter, 6mL 0.9% sodium chloride was added to facilitate the partitioning of the lipids into the organic phase (Hincha et al., 1998), the solution was placed into a separatory funnel and the bottom layer collected into a pre-weighted round bottom flask.

The dry SPLIP was collected by chloroform in a pre-weighted bottle, and preserved in -80 °C Freezer.

3.3 SPLIP Analysis

Lipids class analysis was carried out by an external lab (Mylnefield Lipid Analysis at James Hutton Limited, Dundee, UK). Generally, the lipid classes were separated first using thin layer chromatography (TLC) and then the composition of the fatty acids was analysed using gas chromatography-flame ionisation detector (GC-FID). The following descriptions of the procedures are based on information obtained from the external lab.

3.3.1 Thin Layer Chromatography (TLC)

The lipid classes were separated on 1-dimensional glass HPTLC (high performance thin layer chromatography) Silica gel 60 F254 plates (Merck, Germany) 20 cm x 10 cm in size. The polar lipid classes were then separated on 2-dimensional HPTLC Silica gel 60 F254 plates (Merck, Germany) 10 cm x 10 cm in size.

A portion of the sample was taken and the chloroform dried off to obtain the weight of the lipids. A known amount of C17:0 phosphatidylcholine (PC) internal standard was added into the lipid mix to aid analysis with the 1-D TLC plate. The odd carbon internal standard was normally used as it does not overlap with other fatty acids in GC-FID. The mixture of sample and standard was spotted (200 µl) onto a TLC plate along with internal standards and separated in one direction using a 70:30:2 (v/v/v) isohexane : diethyl ether : formic acid solvent mix. The plate was sprayed with primulin and viewed under UV light. Since primulin is non-destructive, the lipid can be removed from the plate for further work to be carried out.

For the polar lipids class separation C17:0 phosphatidylethanolamine (PE) was used as an internal standard and processed using 2-D TLC. A small amount (14 µl for chloroplast and 20 µl for leaves) of sample was spotted near the corner of a TLC plate and separated in two directions using

solvent mixtures. For the first direction, a mixture of chloroform/methanol/water (65:25:2.8, v/v/v) was used followed by a mixture of chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v) for the second direction. Identical plates were run at the same time with commercially bought standards, which would aid with the identification of the spots on the sample plates.

3.3.2 Gas Chromatography- Flame Ionisation detector (GC-FID)

The sample preparation for GC-FID started with removing the polar lipid fraction from the 1-D TLC plate and re-extracting the lipid from the silica. Fatty acid esterification was performed on the lipid before subjecting it to GC-FID for the quantitation of the fatty acids compounds. Lipid fractions removed from the 2-D TLC plate were monogalactosyldiacylglycerol (MGDG), digalactosyldiacyglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), trigalactosyldiacylglycerol (TGDG) and PE before re-extracting from the silica. Each fraction was added to a second internal standard (C21:0) and esterification was carried out as a preparation step for fatty acids analysis using GC-FID.

The esterification procedure was initiated by mixing the lipid fraction with 1 ml of toluene and 2 ml of 1% sulfuric acid in a glass test tube. The mixture was then heated to and held at 50°C for 14 – 16 h. After cooling, it was shaken with 2 ml of isohexane and 5 ml of 5% NaCl solution. The

solvent was then transferred into a new glass tube. The original tube was shaken with another 2 ml of isohexane and the two solvents were combined in the new glass tube. The combined solvents were shaken with 3 ml of 2% $KHCO_3$ solution. The solvents mixture was then transferred into a new tube and 1 ml of toluene was added before blowing off the solvents into dryness with N_2 gas. After that, isohexane and BHT (butylated hydroxytoluene antioxidant) was added to give a lipid concentration of 5 mg/ ml. The fatty acids methyl esters (FAMEs) were then ready to be injected into the GC (Agilent 6890, Agilent, USA). The fatty acids were separated using a capillary column (Cp-wax 52CB, 30 mm x 0.25 mm internal diameter x 0.15 µm, Agilent, UK). Hydrogen was used as the carrier gas at the flow rate of 40 ml/ min. The column temperature initially was held at 170°C for 3 min. The temperature was then increased from 170°C to 220°C at 4°C/ min and maintained at 220°C for 10 min. An amount of 1 µl of sample was injected into a 230°C inlet with a 50:1 split ratio. A flame ionization detector at a temperature of 300°C was used. The data were processed through integrating the area under the curve and the results are reported as normalised area (%) and mg fraction/g lipid by reference to the internal standard.

3.4. Oil-in-water emulsion preparation

3.4.1 Purification of sunflower oil

Bos and Van (2001) reported that the presence of small impurities in oil has an effect on the resulting interfacial rheological parameters. The purification of sunflower oil could remove surface active impurities, and get rid of the impact from other irrelevant surfactants on interfacial measurement.

Magnesium silicate particles was used to purify sunflower oil in this research. 4%(w/w) of magnesium silicate particles was added to sunflower oil, afterwards, stirred sunflower oil for 30 min by a stirrer at 600 rpm at room temperature. After that, the oil was centrifuged at 2700g for 15min to obtain purified sunflower oil.

3.4.2 Vesicles solution

Lecithin is commonly used in the food industry as it is a vesicle-forming amphiphile. Pignol et al.(2000) pointed out that when a low lecithin/bile salt molar ratio is present, the adsorption of bile salt may effect on the structure of lecithin vesicles, the vesicles can be transformed into micelle structures, and this facilitates the pancreatic lipase to exhibit lipid digestion. Consequently, lecithin, purified DGDG and SPLIP were prepared as vesicle solutions to minimise lipid digestion within gastrointestinal tract.

3.4.2.1 Vesicle Solution Preparation

Hydration of dry lipid was used to prepare lipid vesicle solution in this research, because it is simple, easier and wildly used(Lichtenthaler and Park, 1963; Hur et al., 2009). Hydration was processed by adding aqueous medium into a container of dry lipid, while agitating it.

In this research, 95% w/w distilled water was used as aqueous medium, added to a bath sonicator to dissolve the 5% w/w dry lipid, because the final dispersion of vesicle solution would then be subsequently used as the continuous phase of the oil-in-water emulsions. The dispersion then was disrupted by a probe sonicator (Soniprep 150 Plus, at 14%, with 230V.) until the vesicle reached a size ranged from 150 to 240nm.

3.4.2.2 Vesicle Size Measurement

The vesicle size measured by Zetasizer NanoZS (Malvern, UK). 90 μl of vesicle solution was added to 20ml distilled water, and stirred by using a Pasteur pipette. The solution was loaded into a disposable sizing cuvette and measured by the electrophoresis instrument.

The setting parameter were: temperature 25°C; refractive index of dispersant 1.330 and viscosity of dispersant 0.891mPas. Each particle size was reported as the average of three readings that were made per sample.

3.4.3 Emulsion preparation

With respect to characterize the lipolysis rates of four different common types of food emulsifier, 20% w/w oil in water emulsion were prepared to compare the lipolysis rates of whey protein, sucrose ester, lecithin and cocoa particles. The emulsions were prepared by a high shear overhead mixer (Silverson, L5M, Chesham, UK). Operated by the following the concentration and parameters shown in table 3.1, to make similar droplets diameter and surface areas to compare their lipolysis properties after each stage of *in-vitro* digestion:

Table 3.1 Concentration of emulsifiers to prepare emulsions

Emulsifier	Quantity of water(g)	Quantity of oil(g)	Quantity of emulsifier(g)	Speed homogenization(RPM)	Time(min)
lecithin	20	79.2	0.8	3800	2
Whey protein	20	79.2	0.8	4500	2
Sucrose Ester	20	79.2	0.8	3800	2

Cocoa particle	20	75.2	4.8	9000	2

With regard to the emulsion prepared to evaluate the efficacy of spinach crude lipid extracts(SPLIP) to delay in lipolysis, compared with commercial purified DGDG in this research, Oil-in-water emulsion was prepared with 15g sunflower oil and 85g vesicle solution, processed by a high shear overhead mixer(Silverson, L5M, Chesham, UK), at 8000 rpm, 5min. After that the emulsion was pressed by a high pressure homogeniser(Niro Soavi, Italy), running at 300 bar, 30min to complete the emulsification process, this was then increased to 800 bar for 3min to finish the preparation.

The concentration of pure DGDG used was based on the experiment designed by Wilde and Chu (2010), which was 0.05% (w/w). In order to make a closest approximation of phospholipid content with SPLIP, pure DGDG was mixed with a proper amount of sunflower lecithin(LECDG).

The emulsion was stored at 4°C and used in 24h.

3.5. Emulsion Characterisation

3.5.1 Visual observation

Stability of emulsion was observed by placing the emulsion in a tube that is stored in quiescent conditions. When destabilization occurs it was indicated by the separation of the two phases: firstly the top layer is opaque and/or turbid; while, secondly, the bottom layer is transparent (Herrera, 2012). Visual observation was measured by photographs.

3.5.2 Optical Microscopy

Microscope images of samples were obtained by EVOS™ fl Digital Inverted Fluorescence Microscope(Fisher Scientific, UK). In order to observe the microstructures of emulsion, a droplet of the sample was placed on a glass slide and covered with a cover slip, a digital camera was attached to the microscope to capture images of the samples. The scale was showed at the left bottom corner of every image.

3.5.3 Particle Size Distribution

The particle size distributions of emulsion droplets were measured by a low angle laser diffraction particle size analyzer (LS13 320, Beckman Coulter, High Wycombe, UK) fitted with an aqueous dispersion cell (Universal lipid module, LS13 320, Beckman Coulter, High Wycombe, UK).

The samples were shaken gently to make sure the emulsions were homogenous before loading. Samples (1 to 4 mL), were introduced to the universal liquid module, and obscuration was maintained at 10% for all samples by dilution with distilled water. Particles with diameters between 0.3 to 2,000μm were detected. Three independent replicates of each sample was measured and average surface mean diameter ($d_{3.2}$) presented initially using the Fraunhofer diffraction model.

3.6. Human digestion simulation with in-vitro digestion model

3.6.1. Preparation

The artificial saliva preparation: 1.594g of NaCl, 0.202g of KCl and 1g of Porcine gastric Mucin type 3 (glycopeptide) were mixed with distilled water. The pH was adjusted to 7 using 0.1 N NaOH and the volume was made up to 1 L with distilled water (McClement and Li, 2010a)

Ionic juice preparation: 7.02g of NaCl, 0.373g of KCl and 0.6612g of $CaCl_2$ were mixed with 1L distilled water.(Lindahl et al., 1997)

3.6.2 Mouth

4mL of artificial saliva were added into each sample of emulsions (ratio 1 to 4) and the pH value was mantained at 7 by adding 0.1M NaOH solution. After that 0.01g of α-amylase was added into each sample, and the samples were placed in an incubator at 37°C, agitated at 200 rpm for 5min.

3.6.3 Stomach

8.44mL of ionic juice was added to the samples after the oral phase in the section 3.6.2, and pH value adjusted to 2.5 by using 0.1 M HCl solution.

After that, 0.225mg/mL of Pepsin from hog stomach and 0.56mg/mL of porcine Gastric Lipase were added into each sample and stirred by 200 rpm for 2h, at 37°C (Lindahl et al., 1997).

3.6.4 Small Intestine

15.6mL of ionic juice and 8.9mM of Bile salt were added into the sample from the final volume of gastric phase in section 3.6.3 respectively (Zangenberg, 2003), and then used 0.1M NaOH solution to neutralize the pH value to 7. After that added 1.6mg/mL of pancreatin (contained pancreatic lipase closer to 250 μg/mL) which was the concentration

typically found in the human small intestine, and stirred the samples by 400 rpm for 2h, at 37°C(Wickham, 1998).

3.7. pH-Stat Method to characterize lipolysis rates in small intestine phase

In this research, all the pH-stat measurements were focused on small intestine phase, because up to 70% of lipid hydrolysis occurs in small intestine(Wilde and chu, 2010). The release of free fatty acids from emulsified lipid due to pancreatin activity was monitored using a titration method that was modified from Torcello-Gómez et al., 2014.

20mL emulsion sample was placed in a small beaker which was in a water bath at 37 ºC and left to keep the temperature. Then, 1 mL of $CaCl_2$ and NaCl solution (containing 27.7 mg $CaCl_2$ and 219.2 mg NaCl) followed by 3 mL of bile extract solution (containing 125 mg bile extract) were added to the emulsion to mimick the Na^+ and Ca^{2+} environment at small intestine phase and then stirring at 400 rpm on the Metrohm and the system was adjusted back to pH 7 if required. Next, 1 mL of freshly prepared lipase suspension (containing 40 mg pancreatin powder) was added to the above mixture and the titration started. The final concentration of pancreatin was 1.6 mg/mL to simulate that typically found in the human small intestine. The final concentration of bile extract was 9mM, which is close

to the bile salt concentrations reported during the fasted state in the duodenum (3–7 mM). $CaCl_2$ and NaCl had a final concentration of 10 mM and 150 mM, respectively. Finally, the FFAs released were measured by a pH-stat automatic titration unit (Metrohm) by titrating appropriate amounts of NaOH solution (0.1 M) to maintain the pH at 7.0 (Torcello-Gómez et al., 2014).

The Titrino702 machine could automatically display the volume of titrated 0.1M NaOH solution, which was used to neutralize the released FFAs. The consumed volume of NaOH solution was recorded every 2min manually in first 20min, and every 5min in last 100mins.

An equation was used to calculate the percentage of FFAs released:

$$\%FFAs = 100\% * (V_{NaOH} * m_{NaOH} * M_{Lipid} / 2*w_{Lipid})$$

V_{NaOH} is the volume of sodium hydroxide consumed in mL, m_{NaOH} is the molarity of sodium hydroxide used in M, w_{Lipid} is the totally weight of initially oil present in the experiment and M_{Lipid} is the molecular weight of oil (Torcello-Gómez et al., 2011).

3.8. In-vitro digestion interfacial model

The interfacial tension upon initiation of the lipolysis was developed in accordance with the literature provided by Cabrerizo-Vilchez et al. (1999). The *in-vitro* interfacial model aimed to validate the results obtained from the *in-vitro* digestion model as described in introduction part. This was achieved by using the Profile Analysis Tensiometer (PAT1, Sinterface). This technique allowed for an exchange of the internal phase and was therefore suitable for investigating what was happening at the oil/water interfaces of emulsion upon lipolysis. The steps involved in this method included: at the beginning, the first solution (water) droplet was formed through the tip of an external capillary, this was then replaced by the second solution (lipid vesicle) by the internal capillary while extracting the water phase through the external one, as shown in Figure 2.(Cabrerizo-Vilchez et al., 1999).

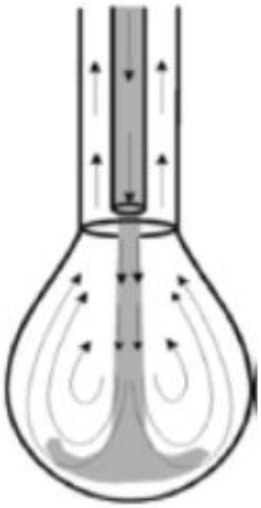


Figure 3.2, Schematic view of the exchange process of double capillary

After the interfacial tension became equilibrium, this procedure was repeated again, the droplet internal phase of the second solution (lipid vesicle) was replaced by the enzyme buffer solution (0.9 mM $NaHCO_3$, pH 7), The concentration of pancreatin, and bile salt were chosen similar to that used in the digestion emulsion model. The change of interfacial tension will display the surfactants activities at interfaces during the lipolysis progress.

3.9. Statistical Analysis

Every example in this research was running in 3 replicates. Statistical analysis was performed by with ANOVA. Assessment of significance was based on a 95% confident limit ($p < 0.05$).

CHAPTER FOUR:RESULTS AND DISCUSSIONS

4.1. Comparation of lipolysis rates of different common types of food emulsifiers

Whey protein, sucrose ester, sunflower lecithin and cocoa particle were selected to represent different types of food emulsifiers: protein, low molecular weight surfactants and particulate emulsifiers.

4.1.1 Characterization of emulsions

The digestion of oil occurs at oil/water interfaces, so the surface areas of emulsion droplets influence the rate and degree of lipolysis, and the smaller droplet sizes, the larger droplet surface areas were. Consequently, before the digestion preparation, the similar droplets size of oil-in-water emulsions should be made. The mean particle diameter was reported as $d_{3,2}$ rather than $d_{4,3}$ since the former was more sensitive to droplet aggregation. (McClement,2005)

The concentration of surfactants used can be found in Table 3.1, all four types of emulsions were stable for at least 2 days, when stored at 4°C.

The mean droplet diameter($d_{3,2}$) of the four types of emulsions were made at 25μm: whey protein was 25.1±1.6μm, sucrose ester was 23.9±0.6μm, lecithin was 24.7±0.8μm and cocoa particle was 25.3±1.3μm. The mean droplet diameter($d_{3,2}$) and droplets size distribution($d_{3,2}$) of prepared emulsions are displayed in figure 4.1 and figure 4.2.

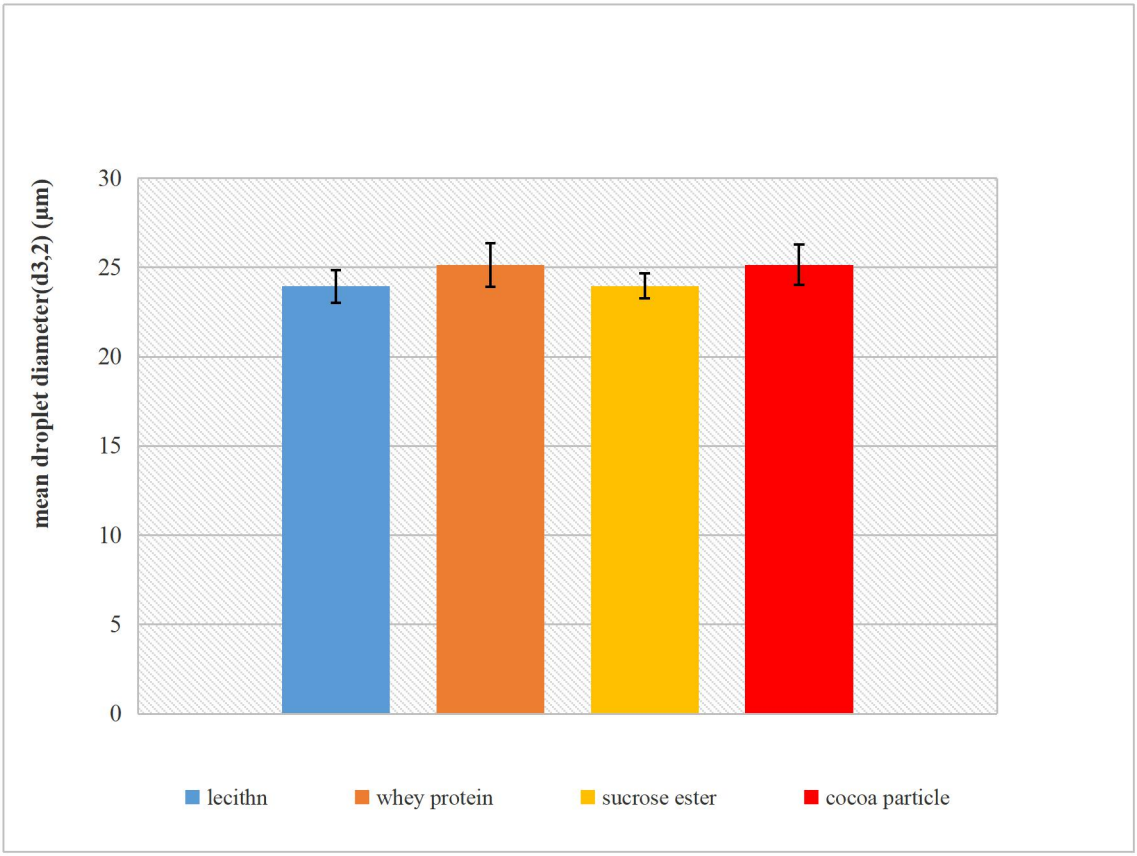


Figure 4.1.: The mean droplet diameter ($d_{3,2}$) of prepared four types of oil-in-water emulsions. Error bars represented standard deviation of the mean. Each sample was analysis in 3 replicates, $P<0.05$.

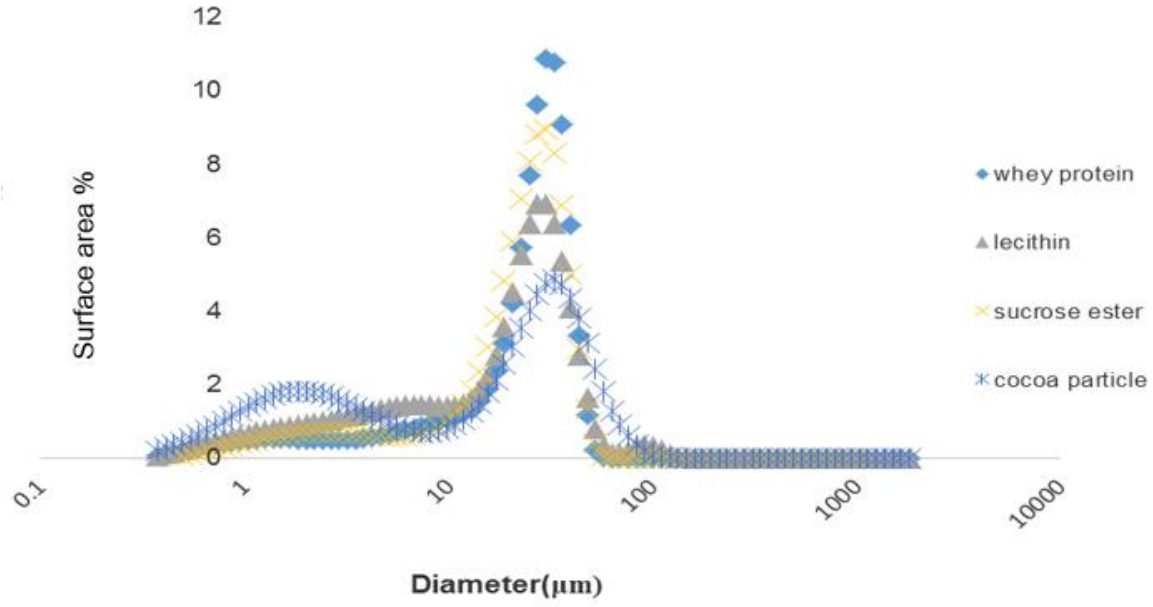


Figure 4.2 The droplet size distribution of prepared four types of oil-in-water emulsions.

The droplet size distribution shown in figure 4.2 displayed that except cocoa particle stabilized emulsion, all the other three types emulsions exhibited a unimodal distribution. Generally, all the emulsions droplet size distributions presented a similar droplet size distribution. The positive skewed distributions (Positively Skewed Distribution is a type of distribution where the mean, median and mode of the distribution are positive rather than negative or zero.) were observed for the four types surfactants stabilized emulsions. Van(2009) explained that the emulsions possibly contained droplets which were relatively larger than the other ones, when a positive skewed distribution observed. As a consequence, the positive skewed distribution refers to an abundance of larger droplets or coarse droplets, as well as the negative skewed

distribution refers to an abundance of small droplets or fine droplets. It is worth noting that, the polydispersity of cocoa particle stabilized emulsion displayed the presence of smaller droplets which a large surface area to volume could impact lipolysis.

4.1.2 Lipolysis rates comparison of emulsions prepared with whey protein, sucrose ester, lecithin and cocoa particle.

As mentioned in materials and methods chapter, the digestion behaviour of oil-in-water emulsions was tested by the *in-vitro* digestion model, simulated human oral phase, stomach phase to small intestine phase. The lipolysis rates of four types of emulsions were evaluated after four stages of in-vitro digestion model: after 5min mouth phase, 1 hour after stomach phase, 2 hours after stomach phase and 2 hours after small intestine phase.

There were two sets of results to determine their properties: the first set was about emulsion breakdown by using optical microscope and droplets size analysis; and the second set was about the amount of free fatty acids(FFAs) released in small intestine phase by means of pH-stat method.

4.1.2.1 Microscopy and droplet size analysis

The analysis for all stages of the model and all emulsifiers has been presented first as a combined analysis to aid comparison: microstructure and mean droplet size.

The optical microscopy pictures helped to distinguish the changes in the microstructure of emulsion droplets digested after each stage. The microstructures of emulsions after each stage of in-vitro digestion model are shown in figure 4.3. These microstructure results are confirmed by the droplet size data presented in Figure 4.4.

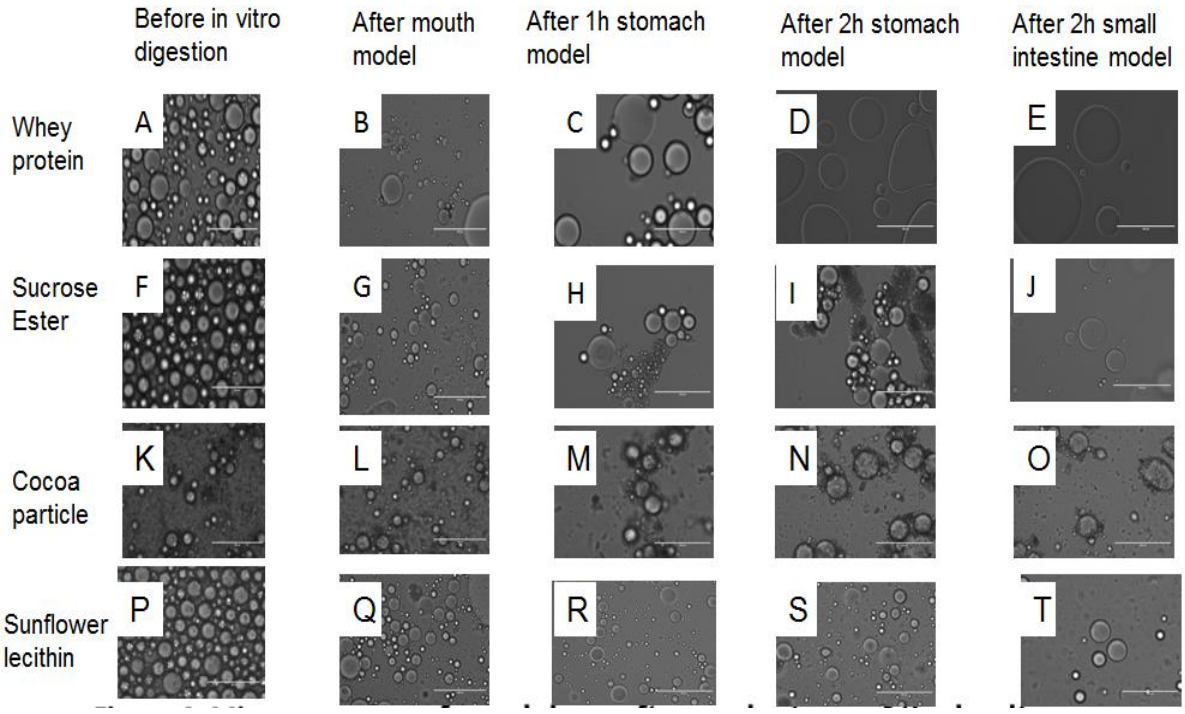


Figure 4.3: Microstructures of emulsions after each stage of the *in vitro* digestion model. Scale bar 100 µm.

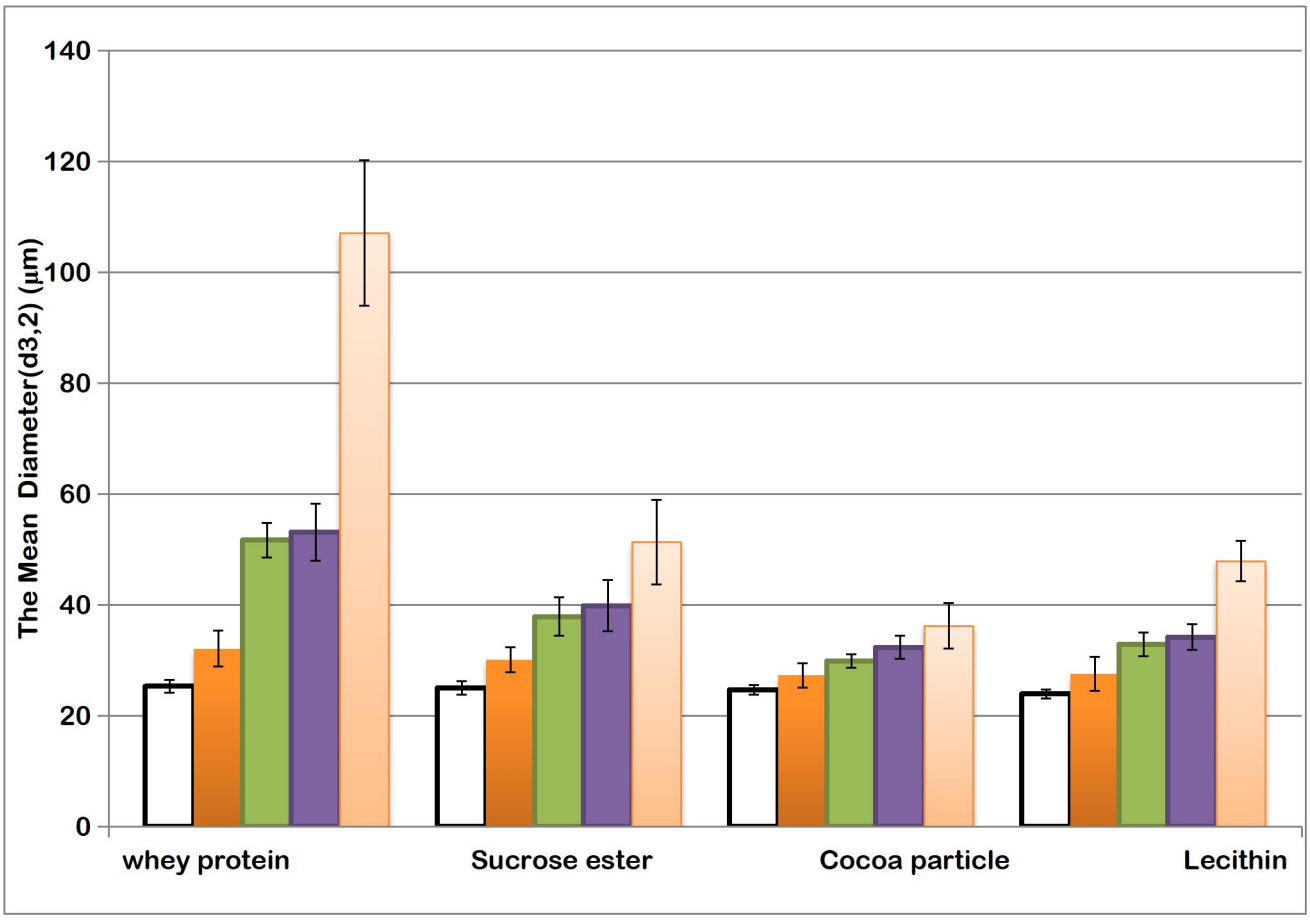


Figure 4.4: The mean droplet diameter ($d_{3,2}$) of emulsions after each stage of the *in vitro* digestion model: before in-vitro digestion (white), mouth (orange), 1h stomach (green), 2hr stomach (purple), and 2h small intestine (light orange). Error bars represented standard deviation of the mean. Each sample represents the average of 3 replicates, $P<0.05$.

Figure 4.3 showed that before digestion work, all four types of emulsions had similar droplets size, which was confirmed Figure 4.1.

Mouth phase

As the Microstructures displayed in Figure 4.3(B, G, L, Q), the mean droplet diameter of these four emulsions had a slightly increase shown in Figure 4.4..

McClements (2005) claimed that flocculation is a kind of aggregation, when two or more droplets are in a close contact, it occurred. Because the porcine gastric mucin concentration used in this model (0.1%) was 5 times than the average mucin concentration in human saliva (0.02%), which was above the critical flocculation concentration observed for porcine gastric mucin (0.4%). The increased droplet size may due to the presence of large salivary mucin aggregates and/or interaction with other salivary proteins (Vingerhoeds et al, 2005).

Stomach phase

Similarly, flocculation was also observed in each emulsion after 1h stomach and was more pronounced after 2h stomach phase digestion, which promoted coalescence. Sucrose ester, sunflower lecithin and cocoa particle emulsified emulsions maintained an emulsion microstructure with the loss of smaller droplets. It is unclear whether the disappeared smaller droplets were digested by gastric lipase or coalesced to become larger droplets. Conversely, whey protein emulsified emulsions could not keep the microstructures especially shown in Figure 4.3(D). It may be the result of the activity of pepsin: most whey proteins could be digested in

2h. And because of that, the emulsions turned unstable. Consequently, the mean droplet diameter of whey protein emulsified emulsion after stomach stage shown in Figure 4.4 was unreliable, because an unstable emulsion's microstructures could be changed by the shearing of the particle size equipment. The mean droplet diameter of all emulsions increased in the stomach model due to droplet coalescence. The smallest increase was seen in the cocoa particle.

Small intestine phase

Small intestine was the main place where lipolysis was expected to occur by the activities of pancreatin and bile salt. The bile salt could replace the emulsifiers at oil/water interfaces and facilitate lipid droplet deformation(McClement and Li, 2010).

After 2h small intestine phase digestion, the microstructures showed in Figure 4.3 (E, J, O, T) that large amount of small droplets were disappeared in all four types of emulsions, and except cocoa particle stabilized emulsions, all the other three presented a significant increase in droplet size, which were also support the data from the mean droplet diameter($p<0.05$) in Figure 4.4. Droplet coalescence creating larger emulsion droplets was evident in all emulsions as the samples progressed through the *in-vitro* digestion model and has also been reported in published studies on *in-vitro* digestion(Mun et al.,2007; Singh and

Ye,2009). It is worth noting that coalescence was caused by the fusogenic properties of free fatty acids, monoacylglycerols and diacylglycerols, during small intestine *in-vitro* process (Pafumi et al, 2002).

All in all, The microstructure and droplet size analysis demonstrated that the presence of cocoa particle had the strongest ability to delay the lipolysis in stomach and small intestine phase, followed by sunflower lecithin and sucrose ester. In contrast, whey protein had the weakest lipolysis delay property of all four types of emulsifiers.

4.1.2.2 Free fatty acids analysis

pH-stat measurement was used to analyse free fatty acids(FFAs) released during lipolysis of emulsions in small intestine phase. FFAs were not determined in the stomach phase, because up to 70% of lipid hydrolysis occurs in small intestine(Wilde and chu, 2010), and only minimal lipolysis activities occurs in stomach phase. FFAs and monoglycerides are actually the by-product during lipolysis process(Reis, 2008), and it is assumed that 1 triacylglycerol molecule produces 2 FFAs and monoacylglycerol (MAG) (McClement and Li, 2010). Furthermore, the rate of lipolysis could be estimated based on the time scales investigated.

Figure 4.5 displays the amount of 0.1M NaOH(mL) consumed to neutralize FFAs during lipolysis progress by time(min) from the four different emulsion systems. During 2 hours of lipolysis progress, the amount of 0.1M NaOH(mL) consumed to neutralise the FFAs released from the emulsion systems were observed in terms of the higher amount to the least amount, as follows: whey protein stabilized emulsion > sucrose ester stabilized emulsion > sunflower lecithin stabilized emulsion > cocoa particle stabilized emulsion. The percentage of FFAs released during the 2 hour lipolysis were respective: Whey protein stabilized emulsion 22.8%, sucrose ester stabilized emulsion 17.52%, sunflower lecithin stabilized emulsion 15.2% and cocoa particle stabilized emulsion 5.69%. It is worth noting that the concentration of purified sunflower oil used in this experiment was much higher than the other studies(20% purified oil w/w was used in this experiment, the other studies used 3-5% purified oil w/w (McClement and Li, 2010)), so the percentage of FFAs released was relative low compared with other studies as well.

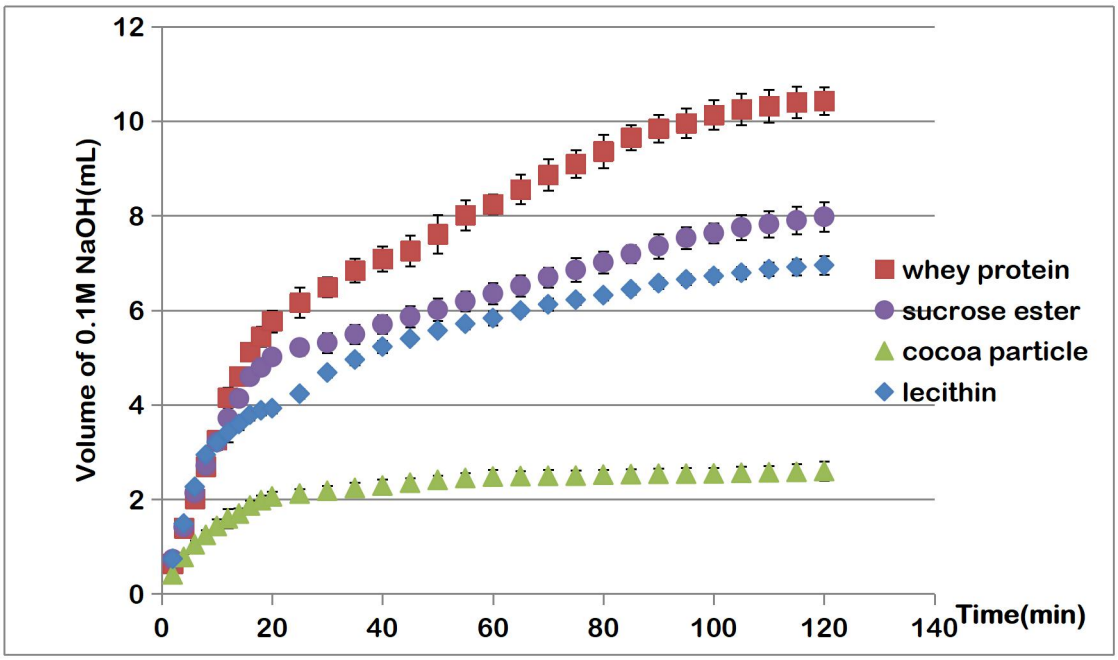


Figure 4.5: Volume of NaOH added to the four different emulsion systems; the pH level was kept at 7, and the experiment was carried out at 37°C. The error bar was present the standard deviation from 3 different samples.

The rate of lipolysis was recorded in a decrease levels for the emulsions stabilized of whey protein, sucrose ester, sunflower lecithin and finally, cocoa particle. At the first 20mins, the consumed volume of NaOH(mL) increased sharply for all four types of emulsions before they reached a fairly constant value. That evidenced FFAs were released rapidly.

However, after 20mins, all four types of emulsions decreased their rate of FFAs released. It is supported by Pafumi et al.(2002) that FFAs and intermediate products presented at the droplet surfaces could result in the inhibition of lipase activity. Due to their surface activity, monoglycerides and fatty acids competed for the surface of lipid droplets displacing lipase

molecules from the oil–water interface at sufficiently high concentrations. Alternatively, they may form a liquid crystalline phase around the lipid droplets, which prevented the lipase from accessing the undigested lipid inside(Patton and Carey, 1979).

The consumed volume of 0.1M NaOH Cocoa particle stabilized emulsion levelled off after 60mins, This could be due to the rate of lipolysis which was relatively slow and there was no more presence of FFAs in the emulsion. It is worth noting that based on the emulsion droplets distribution presented in figure 4.2, cocoa particle stabilized emulsion contained much more small droplets than the other three types of emulsion, which meaned that cocoa particle stabilized emulsion has relative larger surface are. The hypothesis would be that lipolysis would be faster with smaller droplets, but the size did not impact lipolysis in this case.

On the whole, the rate of lipolysis was highly depends on the types of emulsifiers. Cocoa particle stabilized emulsion was recorded with the lowest percentage of FFAs released during lipolysis progress when compared with the other three types of emulsions. Gould(2013) pointed out that, particle stabilized emulsions, in many cases, are more stable than the other surfactants stabilized emulsions. The energy of desorption per particle was very large, and therefore, the particle adsorbed to the interface was considered as irreversibly placed at the interface. When

cocoa particle stabilized an emulsion, the particles were coated at the interface, the large size of particles could contribute a strong steric hindrance to prevent the bile salts and lipase from absorbing the emulsion oil-water interfaces(Gould, 2013).

To conclude, cocoa particle performed the best lipolysis delay property when compared with whey protein, sucrose ester and sunflower lecithin. Sunflower lecithin and sucrose ester were successively weaker than cocoa particle, and whey protein displayed the weakest property of delaying lipid digestion.

4.2 Characterization of spinach crude lipid extract

The method used to obtain spinach crude lipid from spinach leaf was mentioned in chapter 3.2. 2000g spinach leaves were used to extract the SPLIP, and 21.76g of dry SPLIP were obtained in this research.

4.2.1 Composition Analysis of spinach crude lipid extract

Initially, Thin Layer Chromatography analysis(TLC) was used to separate each lipid class from SPLIP, to obtain the qualitative information of phospholipid and DGDG, as these lipids were the core interest of this research. Furthermore, Gas Chromatography analysis(GC) was

investigated to quantify the amount of phospholipid, DGDG, as well as TGDG.

4.2.1.1 Thin Layer Chromatography analysis(TLC)

The results of TLC are shown in Figure 4.6. Sunflower lecithin was measured as reference on the left side and SPLIP was on the right side.

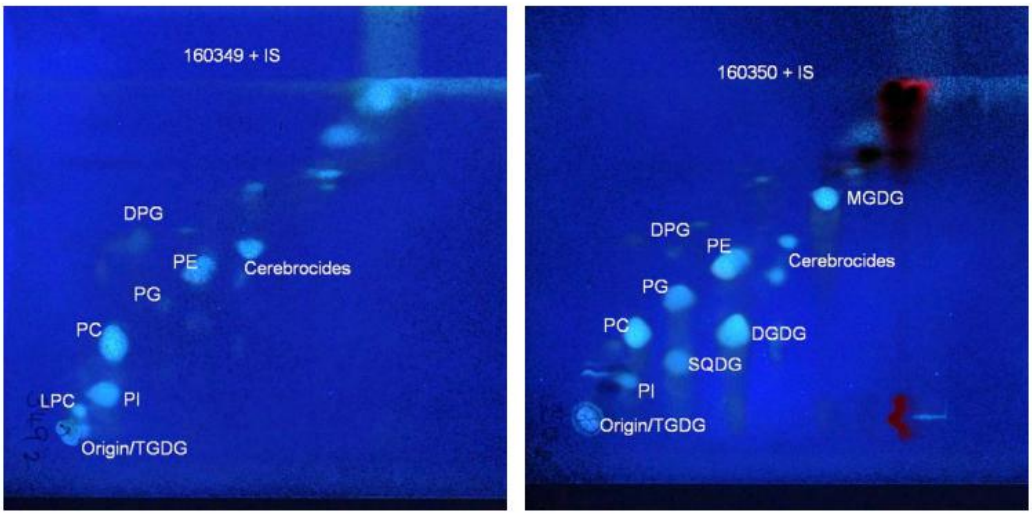


Figure 4.6: 2-D phospholipid species results, Sunflower lecithin (left) and SPLIP (right)

*** TLC Species Initials and Name: LPC – lyso-phosphatidylcholine**

PC – phosphatidylcholine; PI – phosphatidylinositol; PS – phosphatidylserine;

PE – phosphatidylethanolamine; PG – phosphatidylglycerol; PA – phosphatidic acid;

DPG - cardiolipid/di-phosphatidylglycerol; Cereb – cerebrosides;

MGDG – monogalactosyldiacylglycerol; DGDG - digalactosyldiacylglycerol;

TGDG - trigalactosyldiacylglycerol/Origin spot; SQDG-sulphoquinovosyl diacylglycerol.

It can be seen that, for sunflower lecithin, there were many phospholipid displaying in TLC results: phosphatidylcholine(PC), phosphatidylinositol(PI), phosphatidylethanolamine(PE), phosphatidylglycerol(PG), di-phosphatidylglycerol(DPG) and lyso-phosphatidylcholine(LPC). The TLC results of sunflower lecithin were similar to what Holló et al.(1993) stated, the two major compositions of sunflower lecithin are PC and PI, while others such as PE and PG are also present (Holló et al., 1993). However, sunflower lecithin did not contain any TGDG. This could be also validated by GC analysis.

The right picture in Figure 4.6 validated that, SPLIP not only contained a significant amount of MGDG and DGDG, but also have TGDG. As MGDG is less polar than DGDG and TGDG, the interaction with the silica was weak, and therefore would elute longer which resulted in a higher spot being observed. In comparison, DGDG has relative more strong interaction with silica gel, as a consequence, it could dispelled from silica binding place even faster, and this resulted in the identification of DGDG spotting were observed on the lower part of TLC plate. It is worth noting that, TGDG is very polar and the spot did not appear to move from the origin during separation with the solvent mix used. This was supported by Belle et al.(2011) that the polarity of the solvent or a mixture of solvent will influence the way in which it will move or pass via the capillary action on the TLC plate. For example, if highly polar solvents are used, the polar molecule in the solvent will interact with the polar molecules in the sample,

hence there is less interaction with the binding site on the TLC plate, as such no spotting will be observed. TLC lipid identification with reference to standard compounds relies on the fact that a migration occurs and that a migration distance is associated with a specific compound. It is obviously not the case here TGDG remaining at the origin on the TLC plate. so it is not possible to distinguish TGDG from other highly polar compounds remaining at the origin. At this point, SPLIP is worth to do a further investigation to figure out if it contained TGTG or not in the future works.

In addition, SPLIP and sunflower lecithin had the same phospholipids classes except PG. As a result of that, pure DGDG could mixed with sunflower lecithin to make a closest approximation of phospholipid content compared with SPLIP, to do a further investigation to compare their lipolysis rates.

4.2.1.2 Gas Chromatography analysis(GC)

Following the qualitative TLC analysis, the Gas Chromatography(GC) was used to quantify the amount of MGDG, DGDG and TGDG. The quantities of phospholipid components were measured by GC analysis as well. The GC results are displayed in table 4.1.

Table 4.1: The amount of each lipid class of SPLIP and sunflower oil(by dry weight).

LIPID	SPLIP (mg/g)	Sunflower lecithin(mg/g)
Cereb	1.5	1.9
PE	29.7	23.2
PC	65.8	100
PI	13.4	78.4
PS	2.5	6.8
PG	24.8	3.5
DPG	5.0	25.1
MGDG	101.4	0
DGDG	92.1	0
TGDG/Origin	14.4	0
SQDG	26.5	0
Phospholipid totally	141.2	237

As table 4.1 shown that, the SPLIP contained 92.1mg/g DGDG, which is 9.21% weight of SPLIP and 1mg/g of spinach leaf, much more lower than the results from Nakea et al.(2000), who claimed that spinach leaf contained 8.78mg/g. This may be due to the different separating technique, Nakea et al.(2000) used a gradient density method to obtain the pure extract. Therefore, the advance separation may actually impact penetration of a solvent to extract specific components when there are complex structures of the sample.

In addition, the quantification of TGDG obtained from GC analysis was not accuracy, as TGDG is very polar and the spot did not appear to move from

the origin during separation using the solvent mix used. The spot at the origin was removed for analysis, which meaning the spot might contain more than just TGDG.

However, though SPLIP and sunflower lecithin had the same phospholipid classes, the concentrations of each type of phospholipid were different. Especially, SPLIP contained much more PG than sunflower lecithin as 24.8mg/g to 3.5mg/g while sunflower lecithin contained much more PI than SPLIP as 78.4mg/g to 13.4mg/g. Furthermore, the totally phospholipid concentrations of SPLIP and sunflower lecithin were 141.2mg/g and 237mg/g.

The concentration of purified DGDG that Wilde and Chu(2010) used, to obtain a significant lipolysis delay properties at the interface of oil-in-water emulsions with an *in-vitro* digestion model, was 0.05% w/w. In order to make a closest approximation of phospholipid content compared with SPLIP, pure DGDG was mixed with a proper amount of sunflower lecithin to do further investigation. Table 4.2 showed the composition of emulsifiers used in *in-vitro* digestion model and table 4.3 displayed the composition of emulsifiers used in *in-vitro* digestion interfacial model.

Table 4.2: The Composition of emulsifiers: *in-vitro* digestion model

	Concentration used in aqueous phase(mg/100g)		
system	**Total lipid**	**phospholipid**	**DGDG**
LEC	**381**	**90.3**	**0**
LECDG	**439.9**	**90.3**	**58.9**
SPLIP	**639.5**	**90.3**	**58.9**

***LEC for sunflower lecithin, LECDG for pure DGDG + sunflower lecithin**

Table 4.3: The Composition of emulsifiers: *in-vitro* digestion interfacial model

	Concentration used in aqueous phase(mg/100g)		
system	**Total lipid**	**phospholipid**	**DGDG**
LEC	**323.46**	**76.66**	**0**

LECDG	**373.46**	**76.66**	**50**
SPLIP	**542.9**	**76.66**	**50**

4.3 lipolysis rates of spinach crude lipid extract

As mentioned in chapter 3.7 and 3.8, the lipolysis rates of sunflower lecithin(LEC), pure DGDG mixed with sunflower lecithin (LECDG) and SPLIP were compared by using *in-vitro* digestion model and *in-vitro* digestion interfacial model.

The concentration of LEC, LECDG and SPLIP used to prepare emulsions were mentioned in table 4.2 and 4.3. The concentration of DGDG was based on the composition data generated by GC analysis in this study. LEC was used as blank control, which didn't contain any DGDG. LECDG contained pure DGDG which was commercially available, SPLIP also contained DGDG, but it was naturally occurring in spinach crude lipid extract. It is worth noting that SPLIP also contained TGDG (Nakea et al., 2000).

As up to 70% of lipid hydrolysis occurs in small intestine (McClement and Li, 2010), both *in-vitro* digestion model and *in-vitro* digestion interfacial model were focused on small intestine phase. In the *in-vitro* digestion model, two sets of results were determined: emulsion breakdown was followed by using mean droplets size and droplet size distribution analysis; and the second set of result was FFAs released during small intestine phase lipolysis measured by PH-stat method.

4.3.1 Vesicle solutions

As Pignol et al.(2000) pointed out that when a low lecithin/bile salt molar ratio is present, the adsorption of bile salt may effect on the structure of lecithin vesicles, the vesicles can be transformed into micelle structures, and this facilitates the pancreatin to exhibit lipid digestion. Consequently, LEC, LECDG and SPLIP were prepared as vesicle solutions to minimise lipid digestion within gastrointestinal tract.

The concentration of LEC, LECDG and SPLIP used can be found in table 4.2, the vesicle size (nm) of solutions prepared were: LEC 231 ± 3.2 nm, LECDG 227 ± 6 nm and SPLIP 221 ± 7.3 nm.

4.3.2 Characterization of emulsions

The mean droplet diameter ($d_{3,2}$) of the LEC, LECDG and SPLIP stabilized emulsions were made: LEC was 3.5 ± 0.12μm, LECDG was 3.51 ± 0.14μm and SPLIP was 3.34 ± 0.174μm. The mean droplet size diameter ($d_{3,2}$) and droplets size distribution ($d_{3,2}$) of prepared emulsions are displayed in figure 4.7 and figure 4.8. As in this experiment vesicle solutions were used to prepare emulsions with a relative long time processing time(5min compare 2min in section 4.1.1), the droplet size in this section was less than the emulsion droplet size in section 4.1.1.

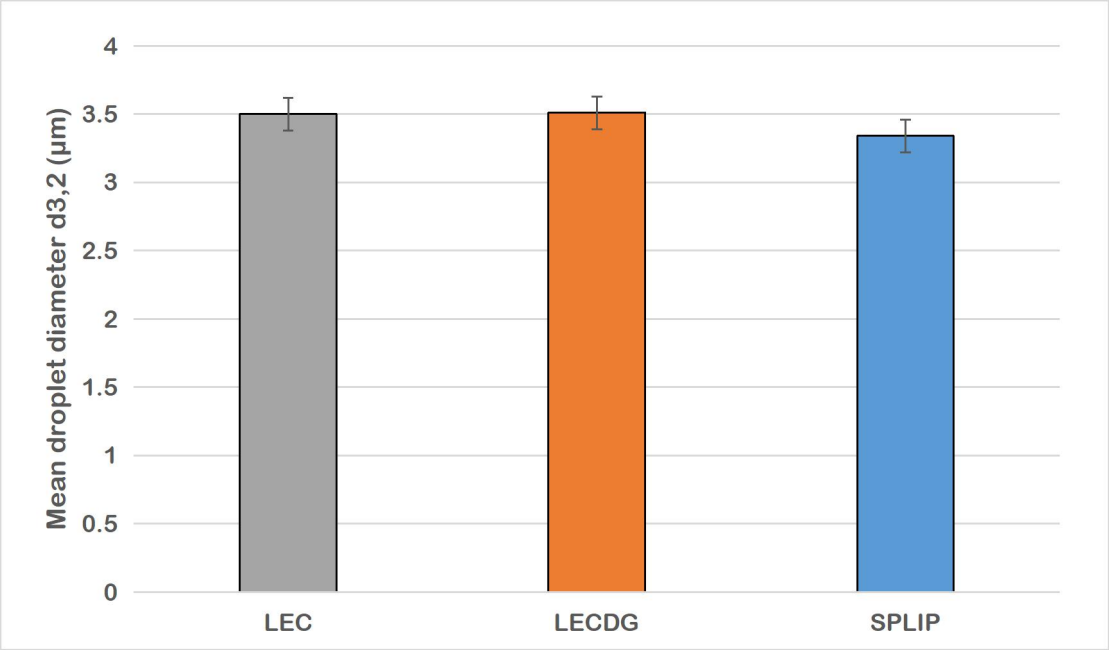


Figure 4.7 Mean droplet diameter ($d_{3,2}$) of LEC, LECDG and SPLIP stabilized oil-in-water emulsion. Error bars represented standard deviation of the mean. Each sample was running with 3 replicates, $P<0.05$.

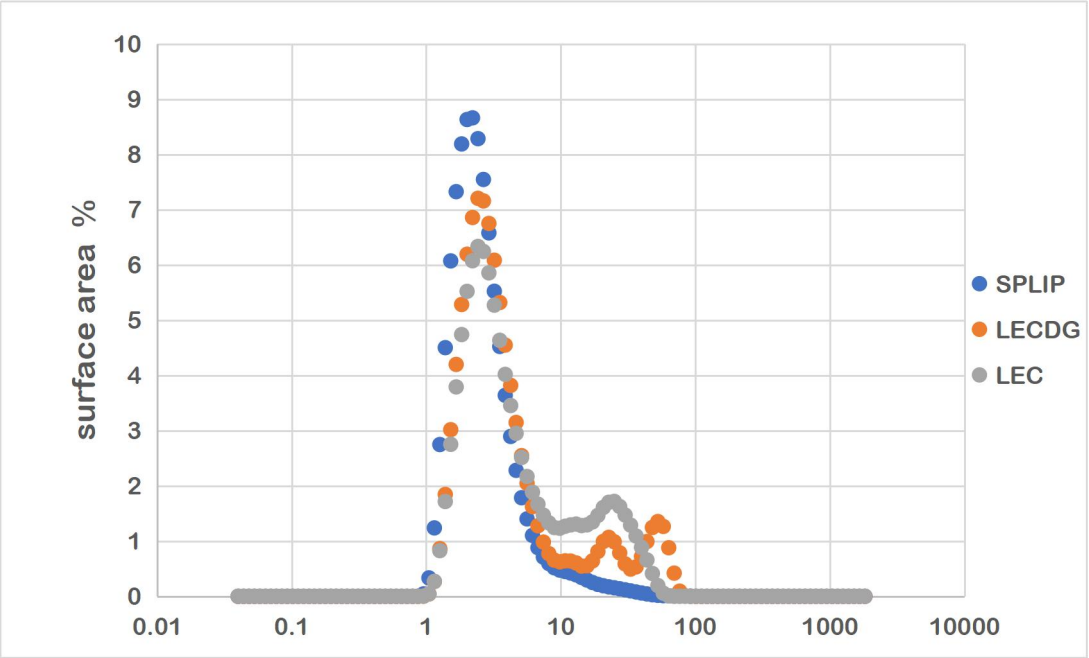


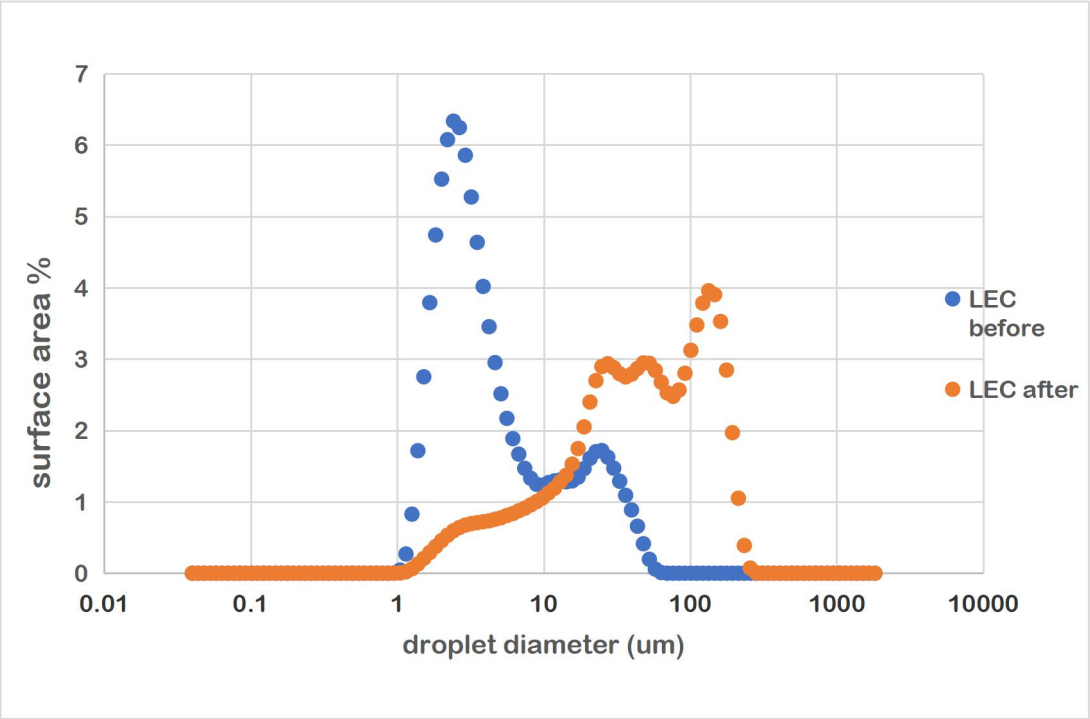
Figure 4.8 The droplet size distribution ($d_{3,2}$) of LEC, LECDG and SPLIP stabilized oil-in-water emulsions.

As the droplet size distribution displayed in figure 4.8, except SPLIP stabilized emulsion, all the other two types of emulsions exhibited a multimodal distribution. Generally, all the emulsions droplet size distributions presented a similar pattern of peak. The positive skewed distributions were observed for all three types of surfactants stabilized emulsions. It is worth noting that the emulsions possibly contained droplets which were relatively larger than the other ones, when a skewed distribution observed (Opedal, 2009). Thus, the positive skewed distribution refers to an abundance of larger droplets or coarse droplets.

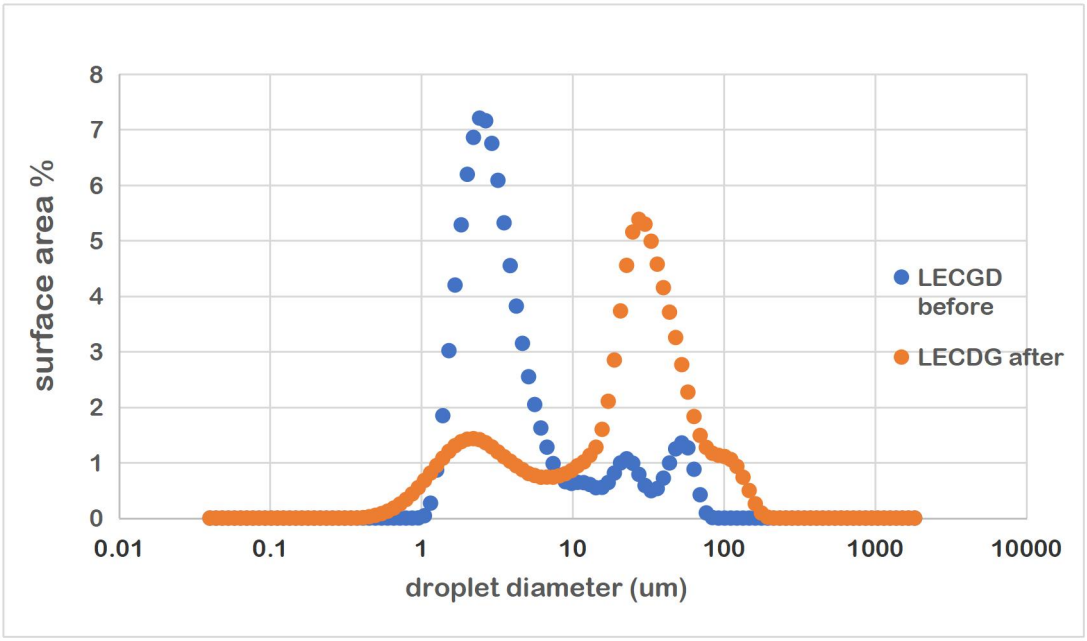
4.3.3 Droplet size distribution and mean diameter analysis

The droplet size distribution of LEC, LECDG and SPLIP stabilized emulsions, before and after in-vitro digestion lipolysis in small intestine phase, are respectively showed in A), B) and C) in Figure 4.9.

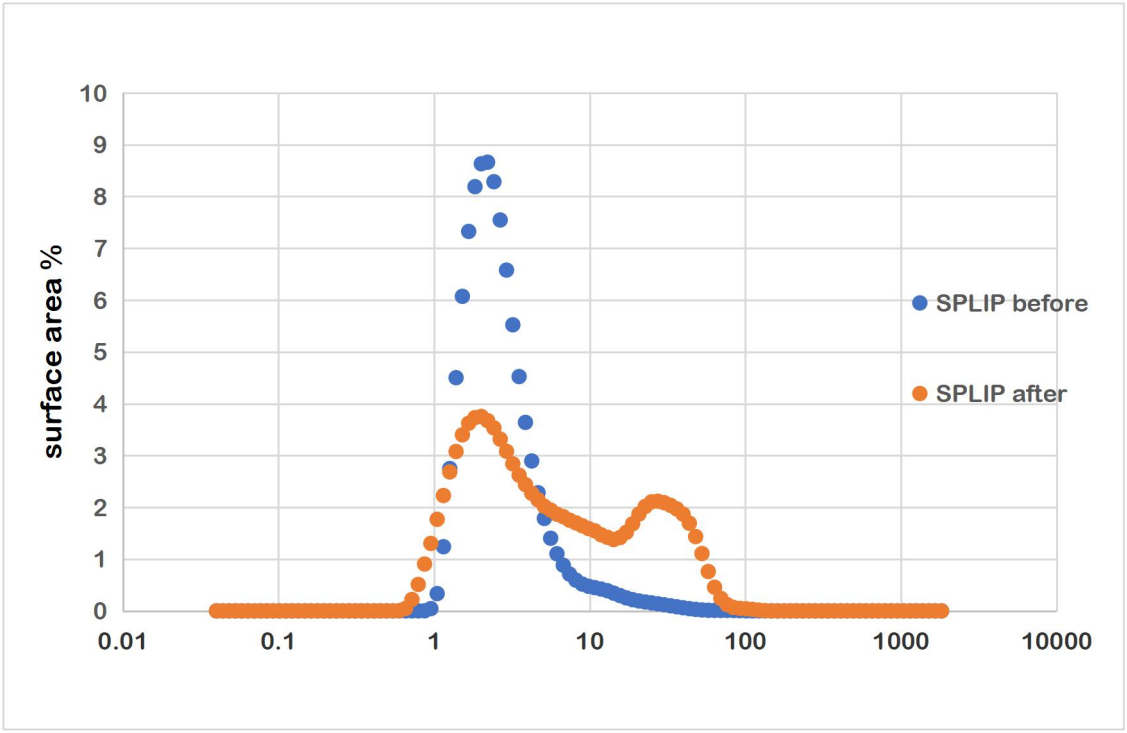
In general, the droplet size increased during lipolysis, these results were validated by the results of mean droplet size diameter displayed in Figure 4.10. In addition, the patterns observed were similar for all three types of emulsions during small intestine phase lipolysis. Especially, SPLIP converted the unimodal distribution to the bimodal distribution during lipolysis, whereas LEC distribution was converted from bimodal distribution to multimodal distribution.



A)



B)



C)

Figure 4.9 Droplet size distribution ($d_{3,2}$) of emulsions before and after small intestine phase lipolysis based on *in-vitro* digestion model: A) LEC, B) LECDG and

C) SPLIP. "before" and "after" meaned before small intestine phase in-vitro digestion and after 2h small intestine phase in-vitro digestion.

It is worth noting that, LEC stabilized emulsion displayed a much more broader distribution peak after small intestine phase *in-vitro* digestion lipolysis, when it compared with the other two emulsion systems. This could be resulted from the abundance of large droplets generated as a consequence of digestion. This illustrated that LEC stabilized emulsion underwent an extensive lipid digestion, which suggests that LEC stabilized emulsion system was less stable against bile salts and lipase during small intestine phase lipolysis, when compared with LECDG and SPLIP. In comparison, the distribution of SPLIP stabilized emulsion showed a smallest changes of its peak during the *in-vitro* digestion lipolysis in small intestine phase, thus suggesting SPLIP had a strongest interfacial property to defend bile salts and lipase during lipolysis compared with the LEC and LECDG.

The mean droplet diameters($d_{3,2}$) of each emulsion system, before and after small intestine phase in-vitro digestion lipolysis, are shown in Figure 4.10. To facilitate further discussion, their numerical values are also shown in Table 4.4. In general, it appears as though the mean diameter of the droplets sizes increased with increasing the digestion time. These findings are similar to those reported by Chanamai and McClements (2000) where the emulsion became less stable when the larger mean diameter sizes of the droplets was observed.

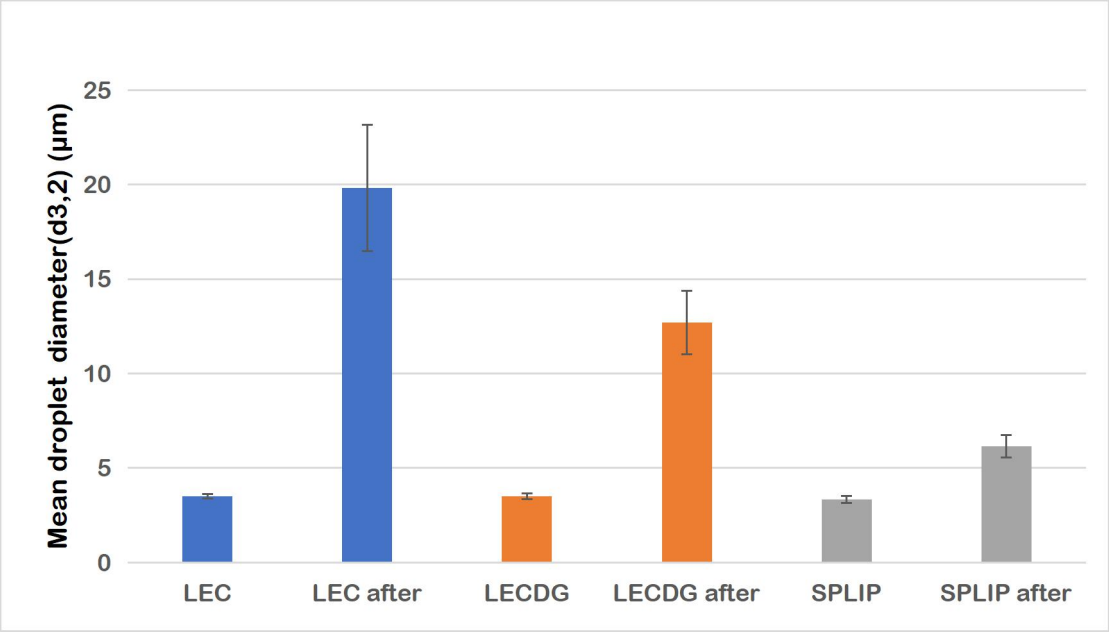


Figure 4.10 The mean droplet diameter($d_{3,2}$) of LEC, LECDG and SPLIP stabilized oil-in-water emulsion before and after small intestine phase lipolysis. Error bars represented standard deviation of the mean. Each sample was running with 3 replicates.

Table 4.4 The mean droplet diameter($d_{3,2}$) of LEC, LECDG and SPLIP stabilized oil-in-water emulsion before and after small intestine phase lipolysis. Error bars represented standard deviation of the mean. Each sample was running with 3 replicates.

Mean droplet diameter (µm)		
Emulsion system	Before digestion	After digestion
LEC	3.5 ± 0.12	19.83 ± 3.34

LECGD	3.51 ± 0.14	12.69 ± 1.68
SPLIP	3.34 ± 0.17	6.14 ± 0.59

The mean droplet diameters($d_{3,2}$) before and after small intestine phase lipolysis are shown in table 4.4. The presence of bile salts and lipase changed the mean size of all emulsion systems significantly($p<0.05$). The increment in diameter of LEC stabilized emulsion observed after small intestine phase digestion, were significantly($p<0.05$) larger than the other two emulsion systems. It stated that LEC stabilized emulsion system was less stable against bile salts and lipase during small intestine phase lipolysis, when compared with LECDG and SPLIP. In comparison, because the presence of DGDG in LECDG and SPLIP, LECDG and SPLIP stabilized emulsion displayed stronger defences against bile salts and lipase, thus a stronger lipolysis delay property than LEC. It confirmed the results from Chu et al.(2010): the presence of DGDG at oil/water emulsion could delay lipolysis progress.

However, although LECDG and SPLIP stabilized emulsion contains 0.05% w/w DGDG, the increments in diameter of their emulsion systems were significantly($p<0.05$) different. SPLIP had a significant less increment than LECDG, which means SPLIP had stronger lipolysis delay property than LECDG. It is important to prove that DGDG in a natural crude extract had a stronger defences against bile salts and lipase during small intestine phase lipolysis, compared with pure DGDG.

4.3.4 Free fatty acids analysis

pH-stat measurement was used to analyse free fatty acids (FFAs) during lipolysis of emulsions in small intestine phase. It is assumed that 1 triacylglycerol molecule produces 2 FFAs and 1 MAG (McClement and Li, 2010). The record of consumed 0.1M NaOH(mL) was calculated to be the percentage of FFAs released by the equation mentioned in chapter 3.7 by Torcello-Gómez et al., 2011.

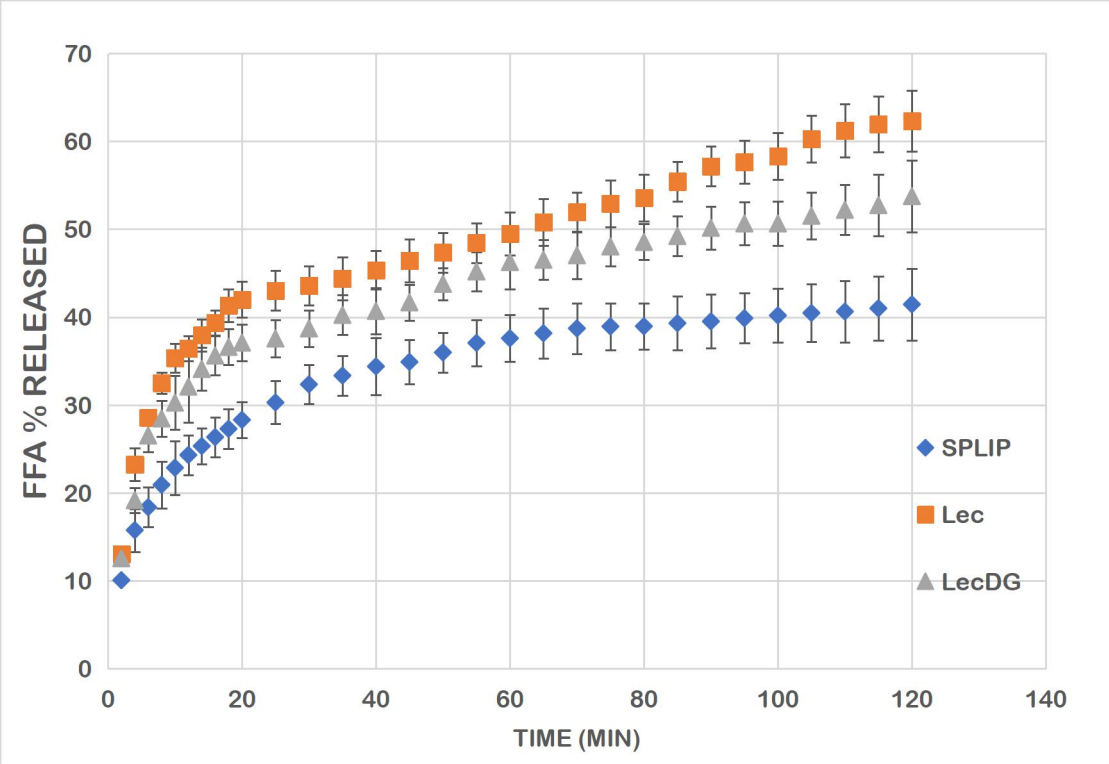


Figure 4.11: Percentage of FFAs released during small intestine phase *in-vitro* digestion. The error bar was present the standard deviation from 3 different samples.

Figure 4.11 shows the percentages of FFAs released during 2h *in-vitro* digestion. As the figure displayed, the percentages of FFAs released from higher amount to the least amount were: LEC stabilized emulsion > LECDG stabilized emulsion > SPLIP stabilized emulsion, which were respectively 62.3%, 53.8% and 41.5%. The rate of FFAs released was displayed in a decreasing level followed respectively by LEC, LECDG and SPLIP.

At the first 20mins, the percentages of FFAs released increased sharply for all three types of emulsions. That evidenced FFAs were released rapidly. However, after 20mins, all three types of emulsions decreased their rate of FFAs released. It is explained by Pafumi et al.(2002) that FFAs and intermediate products presented at the droplet surfaces could result in the inhibition of lipase activity. Due to their surface activity, monoglycerides and FFAs competed for the surface of lipid droplets displacing lipase molecules from the oil–water interface at sufficiently high concentrations. Alternatively, Patton and Carey(1979) pointed out that by-product of lipolysis, such as FFAs and monoglycerides, may form a liquid crystalline phase around the lipid droplets, which prevented the lipase from accessing the undigested lipid inside.

As figure 4.11 shown that, the percentages of FFAs released of both LEC and LECDG stabilized emulsion displayed a gradual increase during 2 hours lipolysis. However, LECDG stabilized emulsion showed a

significant($p<0.05$) lower percentage of FFAs released than LEC, this claimed that the LEC stabilized emulsion underwent a higher degree of lipid digestion compared with LECDG. This stated that the presence of DGDG in oil in water emulsion actually delayed the lipolysis during small intestine digestion, as shown in previous chapters, the presence of DGDG at oil/water interface delayed the lipolysis significantly(Chu et al., 2009).

In comparison, the percentage of FFAs released of SPLIP stabilized emulsion displayed a different curve pattern after 20min when compared with LEC and LECDG stabilized emulsion. This could be a result of the complex properties which are contained SPLIP. In addition, the SPLIP stabilized emulsion had significant($p<0.05$) less percentages of FFAs released at 120mins, when compared with LEC and LECDG, which means SPLIP had stronger lipolysis delay property than LECDG and LEC. It proved that DGDG in a natural crude extract had a stronger defences against bile salts and lipase during small intestine phase lipolysis, compared with pure DGDG. And this results validated the results from droplet size distribution and mean diameter analysis.

With respect to the previous hypothesis, the delay lipolysis property of DGDG is due to steric hindrance from its galactose headgroups; and when a bigger headgroups than DGDG present, such as TGDG, would relatively lower the percentage of FFAs released. On the other hand, Bottier et al.(2007) stated that MGDG, which is also contained in the SPLIP, could

tilt the headgroup of DGDG at the interface. Therefore, it is hypothesised that the presence of MGDG and DGDG may also affect the orientation headgroup of TGDG, enhanced the steric hindrance. Within this experiment, SPLIP stabilized emulsion underwent slower rate of lipid digestion than LEC and LECDG, which means the hypothesis has been confirmed in this experiment.

It is worth noting that the percentage of FFAs released of SPLIP stabilized emulsion levelled off after 80mins, this might be due to the rate of lipolysis was relatively slow and there were very few presence of FFAs in the emulsion.

On the whole, the rate of lipolysis was highly dependent on properties of the interfacial surfactants used. The percentage of FFAs released was significant lower in SPLIP stabilized emulsion, when compared with LEC and LECDG stabilized emulsion, whereas LECDG stabilized emulsion displayed significant lower percentage of FFAs released than LEC stabilized emulsion. To conclude, compared with LEC and LECDG, SPLIP stabilized emulsion was the most stable one against bile salt and lipase during small intestine phase digestion. Furthermore, this finding suggests that SPLIP is effective and even better in hindering the access of lipase molecules, even in presence of bile salts in the system than the pure DGDG, contained in LECDG, stabilised emulsion.

4.3.5 in-vitro digestion interfacial model analysis

The *in-vitro* digestion interfacial model analysis was applied to validate the results obtained from last two subchapters. This model was achieved by using the Profile Analysis Tensiometer(PAT1) with a pendant drop technique, as mentioned in Chapter 3.8, it was to investigate the changes at the interfaces during the small intestine lipolysis when submitted to the sub-phase exchange through interfacial tension. LEC, LECDG and SPLIP vesicle solution and small intestine digestion buffer solution, which contained bile salt and lipase, were prepared at 37°C, pH 7, to simulate small intestine phase digestion.

Before starting the lipolysis using the digestion buffer solution at the oil/water interfaces, the interfacial activity of the surfactant systems needed to be evaluated using a control buffer as a reference. When the interfacial layer was identified, at approximately 8.5 mN/m, the sub-phase exchange was then started by substituting the control buffer with the small intestine digestion buffer solution. The value the interfacial tension, during and after the sub-phase exchange, is shown in Figure 4.12.

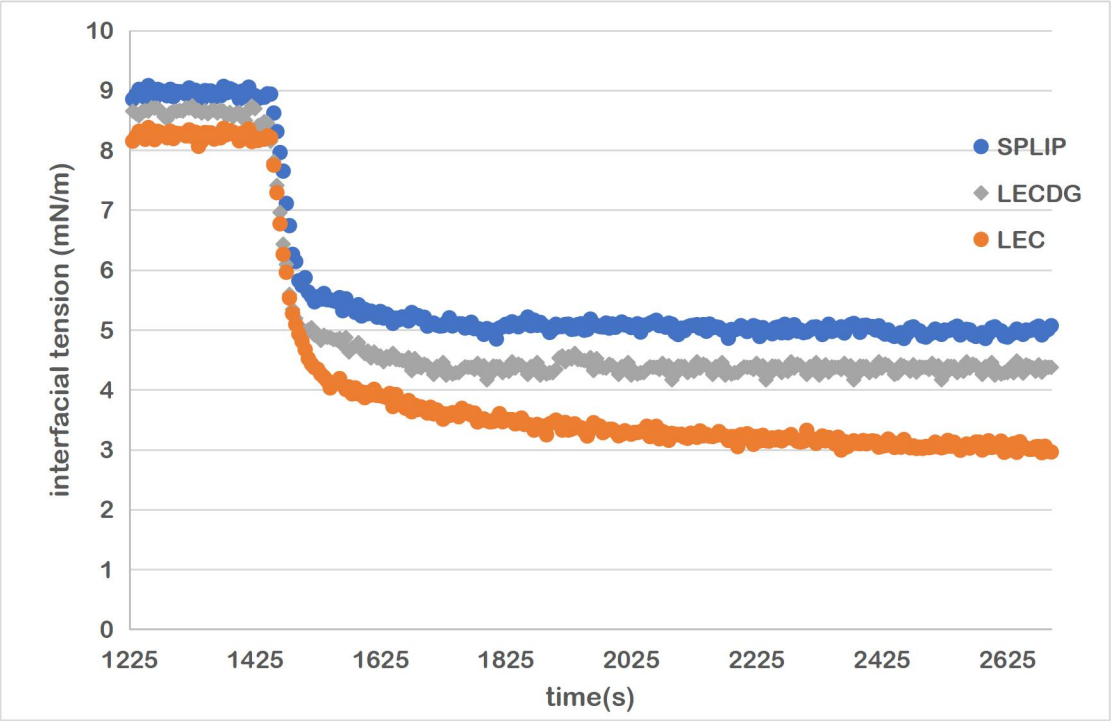


Figure 4.12 Change in interfacial tension during in-vitro digestion, during the sub-phase exchange for the 3 different surfactants stabilized emulsion systems used at 37°C

When the sub-phase exchange was investigated at 1450s, an abrupt decreases in interfacial tension was observed in all the emulsion systems, which continued to gradually decrease over time. These decreases may be the result of hydrolysis which occurred when the digestion buffer solution was introduced. In addition, there was also might be due to a rearrangement of surfactant, which could be instead by the adsorption of bile salt and lipase onto the droplets surface.

The interfacial tension of all emulsion systems reached an equilibrium after 2700 s, this is indicative of saturation of the interface. This might be

a result of the accumulation of fatty acids chains at the interface, which have inhibited the ability of lipase to come in contact with and hydrolyse more lipids (Sarkar et al., 2010). These results are also similar to a study conducted by Maldonado-Valderama et al. (2014). It is worth noting that Chu et al.(2009) pointed out that the abrupt decrease which was due to hydrolysis of Sn 1-3 monoglycerides by lipase, and the continued gradually decrease was a result of the presence of surface active Sn-2 monoglycerides which were not hydrolysed and contributed to populating at the interfaces to displace lipase from the oil/water interfaces.

The interfacial tension value at 2700s were determined for all of the stabilised emulsion systems. the curve pattern decreasing values in interfacial tension from high to low followed: LEC > LECDG > SPLIP. Therefore, when the droplet was covered by LEC, the interfacial tension was the significant($P<0.05$) lowest when compared with LECDG and SPLIP. This is also supported by Chu et al.(2009) that the presence of DGDG at the interface, could have steric hindrance to defend bile salt and lipase. In addition, when the droplet covered by LECDG, the interfacial tension was lower than SPLIP, it could be predicted that the presence of TGDG in SPLIP could provide more steric hindrance due to its larger headgroup. However, the reductions ($p > 0.05$) in the interfacial tension during lipolysis between LECDG and SPLIP stabilised emulsions were not significant ($P>0.05$). This may be a result of the concentration DGDG used were relatively low, and this could have rendered an insignificant data.

To conclude, both LECDG and SPLIP showed relatively higher interfacial tensions when compared to the LEC stabilised emulsion. The presence of both DGDG and TGDG, even though they were only in a relatively small amount, presumably impacts on the interface during the *in-vitro* digestion interfacial model process. Actually, in SPLIP, a more complex interaction occurs between the molecule which possibly influences the organisation of the headgroup in DGDG and in TGDG. Bottier et al.(2007) stated that MGDG, which is also contained in the SPLIP, could tilt the headgroup of DGDG at the interface. Therefore, it is hypothesised that the presence of MGDG and DGDG may also affect the orientation headgroup of TGDG, enhanced the steric hindrance. Although no significant differences of reductions in the interfacial tension between LECDG and SPLIP during the sub-phase exchange, this could still provide the basis for future work.

4.4 Overall Summary

With respect to the comparation of lipolysis delay property of four different types of food emulsifiers, the four different emulsion systems were prepared and tested by *in-vitro* digestion model. The results obtained from microscopy indicated that all emulsion systems were digested by stomach and small intestine enzymes, shown in Figure 4.3. The main physical changes were coalescence and/or flocculation. The changes matched up with the results from the mean droplet diameter($d_{3,2}$). The mean droplet size increased especially after 2hours small intestine

digestion. FFAs was produced during small intestine digestion by pancreatin, with the help of bile salts. The percentage of FFAs released was measured by pH-stat method. Whey protein stabilized emulsion resulted in a massive physical changes as well as the highest percentage of FFAs released(22.8%), which proved that whey protein stabilized emulsion underwent an extensive lipolysis, compared with the other three types of emulsion systems. In comparison, cocoa particle stabilized emulsion displayed fewer physical changes and extremely low percentage of FFAs released(5.69%). It stated that cocoa particle stabilized emulsion had the strongest ability to defend bile salt attack at oil/water interface during lipolysis. All in all, the physical changes and percentages of FFAs released were recorded, from least to most during lipolysis were followed by: cocoa particle stabilized emulsion < sunflower lecithin stabilized emulsion < sucrose ester stabilized emulsion < whey protein stabilized emulsion.

With regard to evaluate the efficacy of SPLIP to delay lipolysis, LEC, LECDG and SPLIP stabilized emulsions were prepared and exposed to *in-vitro* digestion model and *in-vitro* digestion interfacial model. First of all, in order to comment the concentration used, TLC and GC analysis were used to quantify the amount of DGDG and TGDG in SPLIP sample. LEC and LECDG(pure DGDG mixed with approximately amount of LEC) were used as reference. Furthermore, pH-stat method was used to record the percentage of FFAs released during lipolysis. The absence of DGDG in LEC stabilized emulsion recorded the highest percentage(62.3%) of FFAs

released. Which indicated that LEC stabilized emulsion underwent an extensive lipolysis during small intestine digestion when compared with LECDG and SPLIP stabilized emulsion. In comparison, the presence of DGDG in LECDG stabilized emulsion resulted in a relative lower percentage(53.8%) of FFAs released, which proved that the presence of DGDG could protect the droplet from the bile salt attacking. Last but not least, SPLIP stabilized emulsion had significant($p<0.05$) less percentages of FFAs released at 120mins, when compared with LEC and LECDG, which means SPLIP had stronger lipolysis delay property than LECDG and LEC. It proved that DGDG in a natural crude extract had a stronger defences against bile salts and lipase during small intestine phase lipolysis, compared with pure DGDG.

With respect to *in-vitro* digestion interfacial model, both LECDG and SPLIP showed relatively higher interfacial tensions when compared to the LEC stabilised emulsion. The presence of both DGDG and TGDG, even though they were only in a relatively small amount, presumably impacts on the interface during the *in-vitro* digestion interfacial model process. However, there were no significant differences of reductions in the interfacial tension between LECDG and SPLIP during the sub-phase exchange, this could still provide the basis for future work.

CHAPTER FIVE:DISCUSSION

Cocoa particle is a kind of food particulate surfactant. It forms oil in water emulsions by adsorbing to the interface, once it adsorbed at the interface, the energy of desorption per particle was very large, and therefore, the particle adsorbed to the interface was considered as irreversibly placed at the interface. The large size of particles could contribute a strong steric hindrance to prevent the bile salts and lipase from absorbing the emulsion oil-water interfaces(Gould, 2013). In this study, cocoa particles displayed significant stronger lipolysis delay properties than the other three types of emulsifiers, followed by sunflower lecithin and sucrose ester respectively, and finally whey protein.

Lecithin which stand for low molecular weight surfactants presented a better lipolysis delay property than whey protein and sucrose ester, and have a more widely usage than cocoa particle. This result arouse interest in low molecular weight surfactants, and that result in the follow experiment in study SPLIP.

With respect to SPLIP, the suggested headgroup specifically DGDG and presence of TGDG in a whole crude lipid form, was identified as possessing an inhibitory property when compared against the purified DGDG, contained in the LECDG stabilised emulsion. This was observed

from the results conducted in the two sets of experiment: the *in-vitro* digestion model and the *in-vitro* interfacial model. It can be summarised from the *in-vitro* digestion that, the rate of lipolysis was highly depends on the interfacial surfactants used. The percentage of FFAs released was significant lower in SPLIP stabilized emulsion, when compared with LEC and LECDG stabilized emulsion, whereas LECDG stabilized emulsion displayed significant lower percentage of FFAs released than LEC stabilized emulsion. As a consequence, it can be concluded the lipolysis delay property from strong to weak followed by: SPLIP > LECDG > LEC.

Furthermore, when it investigated into *in-vitro* interfacial model, the comparation was shown by changes in the interfacial tension measurements. The interfacial value of the LECDG stabilised emulsion was lower than SPLIP stabilised emulsions. The three galactose moieties (TGDG), present at the interface, could have more steric hindrance than the two galactose moieties (DGDG) as proposed in the literature by Chu et al.(2009). Moreover, Bottier et al.(2007) also stated that MGDG, which is also contained in the SPLIP, could tilt the headgroup of DGDG at the interface. Therefore, it is hypothesised that the presence of MGDG and DGDG may also affect the orientation headgroup of TGDG, enhanced the steric hindrance. However, in this study, it had no significant differences of reductions in the interfacial tension between LECDG and SPLIP during the sub-phase exchange, but this could still provide the basis for future work.

In conclusion, the study presented in this thesis has provided a better understanding of the lipolysis delay properties of four common types of food interfacial surfactants, and moreover to figure out the effectiveness of SPLIP, when tested in an *in-vitro* digestion model and in-vitro interfacial model. The data presented and discussed in this paper will be of particular interest to groups working on surfactants, as well as functional foods aimed at addressing fat regulation.

REFERENCES

Allen, T. (1992) Particle Size Measurement (4th Edition). London: Chapman & Hall.

Albertsson, P. A., Kohnke, R., Emek, S. C., Mei, J., Rehfeld, J. F., Akerlund H. E. & Erlanson-Albertsson, C. (2007) Chloroplast membranes retard fat digestion and induce satiety: Effect of biological membranes on pancreatic lipase/co-lipase. *Biochem. J.*, 401, 727-733

Armand, M., Pasquier, B., Andre, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V., and Lairon, D. (1999) Digestion and absorption of 2 fat 140 emulsions with different droplet sizes in the human digestive tract. Am. J. Clin. *Nutr*., 70, 1096-1106.

Arronsson, H., Schottler, M. A., Kelly, A. A., Sundqvist, C., Dorman, P., Karim, S. & Jarvis, P. (2008) Monogalactosyldiacylglycerol deficiency in Arabidopsis affects pigment composition in prolamellar body and impairs thylakoid membrane energization and photoprotection in leaves. *Plant Physiology*, 148, 580-592.

Bancroft, W. D. (1913) The Theory of Emulsification. *The Journal of Physical Chemistry,* 17, 501-519.

Belle, A. A. & Khale, A. (2011) An overview on thin layer chromatography. *International Journal of Pharmaceutical Sciences and Research*, 2, 256-267.

Benson, A. A. (1964) Plant membrane lipids. *Annu Rev Plant Physio*, 15, 1-16.

Bonnaire, L., Sandra, S., Helgason, T., Decker, E. A., Weiss, J. & McClements, D. J. (2008) Influence of lipid physical state on the in-vitro digestibility of emulsified lipids. *J. Agric Food Chem.*, 56, 3791-3797.

Bos, M.A and van Vliet, T., `Interfacial rheological properties of adsorbed protein layers and surfactants: a review`, Advances in Colloid and Interface Science, 2001, 91 (3), 437-71.

Bottier, C., et al. (2007) Galactosyl headgroup interactions control the molecular packing of wheat lipids in Lahgmuir films and in hydrated liquid-crystalline mesophases. *Biochimica Et Biophysica Acta-Biomembranes*., 1768, 1526-40.

Bremer, L., De Nijs, B., Deriemaeker, L., Finsy, R., Geladé, E. & Joosten, J. (1996) Ostwald ripening of concentrated alkane emulsions: a preliminary report. Part. Part.*Syst. Charact*., 13, 350–353.

Bylaite, E., Nylander, T., Venskutonis, R. & Jönsson, B. (2001) Emulsification of caraway essential oil-in-water by lecithin and [beta]-lactoglobulin: Emulsion stability and properties of the formed oil-aqueous interface. *Colloids and Surfaces B: Biointerfaces*, 20, 327-340.

Cabrerizo-Vılchez, M. A., Wege, H. A., Holgado-Terriza, J. A. & Neumann, A. W. (1999) Axisymmetric drop shape analysis as penetration Langmuir balance. *Review of Scientific Instruments*, 70, 2438.

Cevc, G. (1993) Phospholipids Handbook. New York: Marcel Dekker, 939-56.

Chanamai, R. & McClements, D. J. (2000) Dependence of creaming and rheology of monodisperse oil-in-water emulsions on droplet size and concentration. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 172, 79-86.

Chapman, D. J., De-Felice, J. & Barber, J. (1983) Influence of winter and summer growth conditions on leaf membrane lipids of Pisum Sativum-L. *Planta*., 157, 218-223.

Christie, W. W. (1992) Solid-phase extraction columns in the analysis of lipids. In: W. W. Christie (Editor) Advances in Lipid Methodology-One. *Ayr: Oily Press*, 1-17.

Chu, B. S., Rich, G. T. Ridout, M. J., Faulks, R. M., Wickham, M. S. J. & Wilde, P. J. (2009) Modulating pancreatic lipase activity with galactolipids: Effects of emulsion interfacial composition. *American Chemical Society*, 25, 9352-9360.

Chu, B. S., Gunning, A. P., Rich, G. T., Ridout, M. J., Faulks, R. M., Wickham, M. S. J., Morris, V. J. & Wilde, P. J. (2010) Adsorption of bile salts and pancreatic colipase and lipase onto digalactosyldiacylglycerol and dipalmitoylphosphatidylcholine monolayers. *Langmuir,* 26, 9782-9793.

Dembitsky, V. M. (2004) Astonishing diversity of natural surfactants: Glycosides of fatty acids and alcohols. *Lipids*, 39 (10), 933-53.

Dickinson, E. (1992) Introduction to food colloids. Oxford University Press.

Dickinson, E. (2003) Hyrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, 17, 25-39.

Dorman, P. & Anderson, M. X. (2009) Choroplast Membran Lipid Biosynthesis and Transport. *Plant Cell Monogr*. Dos.

Fernando Leal-Calderon, Veronique Schmitt & J. Bibette. (2007) Book Emulsion Science Basis Principles (2nd Edition).

French, S. J., Conlon, C. A. & Mutuma, S. T. (2000) The effects of intestinal infusion of long-chain fatty acids on food intake in human. *Gastroenterology,* 119 (4), 943-948.

Gould, J., Vieira, j., and Wolf, B., Emulsifier-free emulsions stabilized by food particles, in particular cocoa particles. European Patent application number EP11188128.0, *Nestle PTC York*, 2011.

Handa, T., Saito, H. & Miyajima, K. (1990) Phospholipid monolayers at the triolein-saline interface: Production of microemulsion particles and conversion of monolayers to bilayers. *Biochemistry*, 29 (11), 2884-2890.

Hanif, R., Iqbal, Z., Iqbal, M., Hanif, S. & Rasheed, M. (2006) Use of vegetables as nutritional food: Role in human health. *Journal of Agricultural and Biol Science*, 1, 18- 22.

Harrison, L. J. & Cunningham, F. E. (1985) Factors influencing the quality of mayonnaise: A review. *Journal of Food Quality*, 8, 1-20.

Hernández, E. & Quezada, N. (2008) Uses of phospholipids as functional ingredients. In: F. D. Gunstone (Editor) Phospholipid Technology and Applications. Bridgewater: *The Oily Press*. Chapter 4, 83-94.

Herrera, M. L. (2012) Analytical Techniques for Studying the Physical Properties of Lipid Emulsions. *SpringerBriefs in Food, Health and Nutrition* 3, DOI 10.1007/978-1-4614-3256-2_3.

Holló, J., Perédi, J., Ruzics, A., Jeránek, M. & Erdélyi, A. (1993) Sunflower lecithin and possibilities for utilisation. *Journal of the American Oil Chemists Society*, 70, 997-1001.

Holmberg, K. (2001) Natural Surfactants. *Current Opinion in Colloid & Interface Science*, 6 (2), 148-159.

Hincha, D. K., Oliver, E. A. & Crowe, J. H. (1998) The effects of chloroplast lipids on the stability of liposomes during freezing and drying. *Biochemical et Biophysical Acta*, 1368, 150-160.

Ito, S., Okada, S. & Fujino, Y. (1974) Glyceroglycolipids in pumpkin (in Japanese). *Nippon Nogeikagaku Kaish*i, 48, 431-436.

Jokela, P., Fletcher, P. D. I., Aveyard, R. & Lu, J. R. (1990) The use of computerised microscopic image analysis to determine emulsion droplet size distributions. Journal of Colloid and Interface Science, 134, 417-426.

Joyard, J., Teyssier, E., Miege, C., Berny-Seigneurin, D., Marechal, E., Block, M. A., Dorne, A. J., Rolland, N., Ajlani, G. & Douce, R. (1998) The biochemical machinery of plastid envelope membranes. *Plant Physiol*., 118, 715-723

Kamp, F., D. Zadim, F. Zhang, N. Noy & J. A. Hamilton (1995) Fatty acid flip-flop in phospholipipd bilayers is extremely fast. *Biochemistry.*, 34, 11928-11937.

Kaufman, M. (2002) Principles of Thermodynamics. New York: Marcel Dekker.

Knezevic, Z. D., Siler-Marinkovic, S. S. & Mojovic, L. V. (1998) Kinetics of lipase- catalysed hydrolysis of palm oil in lecithin/isooctane reversed micelles. Appl. Microbiol. *Biotechnol*., 49, 267-271.

Kiokias, S., Reszka, A. A. & Bot, A. (2004) The use of static light scattering and pulsed-field gradient NMR to measure droplet sizes in heat-treated acidified protein-stabilised oil-in-water emulsion gels. *International Dairy Journal*, 14, 287-295.

Kobayashi, K., Kondo, M., Fukuda, H., Nishimura, M. & Ohta, H. (2007) Galactolipids synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis and embryogenesis. Proceedings Natl. Acad. *Sci.* (PNAS), USA, 104, 17216-17221.

Krolova, I., & Sjoblom, J. (2009) Surfactants used in food industry: A review. *Journal of Dispersion Science and Technology*, 30 (9), 1363-1383.

Lairon, D., Play, B. & Jourdheuil-Rahmani, D. (2007) Digestible and indigestible carbohydrates: Interactions with postprandial lipid metabolism. *J. Nutr. Biochem.*, 18, 217-227.

Latimer, P. (1982) Light scattering and absorption as methods of studying cell population parameters. *Annual Review of Biophysics and Bioengineering*, 11, 129-150.

Lambourne, R. & Strivens, T. A. (1993) Paint and Surface Coatings – Theory and Practice (2 nd Edition). Cambridge, UK: Woodhead Publishing Ltd.

Li, Y., Hu, M., Xiao, H., Dua, Y., Decker, E. A. & McClements, D. J. (2010) Controlling the functional performance of emulsion-based delivery systems using multi- component biopolymer coatings. *European Journal of Pharmaceutics and Biopharmaceutics*, 76, 38-47.

Li, Y., J. Kim, Y. Park & D. J. McClements (2012) Modulation of lipid digestibility using structured emulsion-based delivery systems: comparison of in vivo and in vitro measurements. *Food and Function*., 3, 528-536.

Lindahl, et al., Characterization of fluids from the stomach and proximal jejunum in men and women*, Pharm*. Res., 1997, 14(4), 497–502.

Lichtenthaler, H. K. & Park, R. B. (1963) Chemical composition of chloroplasts lamellae from spinach. *Nature,* 198, 1070-72.

Maldonado-Valderrama, J., A. Torcelo-Gomez, T. Castillo-Santaella, J. A. HolgadoTerriza & M. A. Cabrerizo-Vilchez (2014) Subphase exchange experiments with the pendant drop technique. *Adv Colloid Interface Sci*.

Mason, T. G., Krall, A. H., Gang, H., Bibette, J. & Weitz, D. A. (1996) Monodisperse emulsions: properties and uses. In: P. Becher (Editor) Encyclopaedia of Emulsion Technology. New York: Marcel Dekker.

McClements, D. J. (1999) Food Emulsions: Principles, Practice and Techniques. New York: CRC Press.

McClements, D. J. (2004) Food Emulsions: Principles, Practices, and Techniques (2 nd Edition). CRC Series in Contemporary Food Science. New York: CRC Press.

McClements, D. J. (2005) Food Emulsion: Principles, Practise and Techniques (3 rd Edition). New York: CRC Press.

McClements, D. J. & Decker, E. A. (2000) Lipid oxidation in oil-in-water emulsion: Impact of molecular environment on chemical reactions in heterogeneous food system. *Journal of Food Science*, 65 (8), 1270-1282.

McClements, D. J., Decker, E. A. & Park, Y. (2009) Controlling Lipid Bioavailability through Physicochemical and Structural Approaches. *Critical Reviews in Food Science and Nutrition*., 49, 48-67.

McClements, D. J. & Y. Li (2010) Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science*., 159, 213-228.

McClements, D. and Li, Y. (2010a) Review of in-vitro digestion models for rapid screening of emulsion-based systems. *Food & Function*, 1 (1), 32-59.

McClements, D. J. & Li, Y. (2010b) Structured emulsion-based delivery systems: Controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science*., 159, 213-228.

Morrison, W. H. I. (1985) Sunflower Lecithin. In: B. F. Szuhaj and G. R. List (Editors) Lecithins. *American Oil Chemists' Society Press*, 97-103.

Mun, S., Decker, E. A. & McClements, D. J. (2007) Influence of emulsifier type on invitro digestibility of lipid droplets by pancreatic lipase. *Food Research International*, 40, 770-781.

Nakea, T., Kometani, T., Nishimura, T., Takii, H. & Okada, S. (2000) Effects of Glyceroglycolipids Prepared from Variuos Natural Materials on Polymorphisc Transformation of Cocoa Butter. *Food Sci*. Technol. Res., 6, 320-323.

Opedal, N. van der T., Sørland, G. & Sjöblom, J. (2009) Methods for droplet size distribution determination of water-in-oil emulsions using low-field NMR. The OpenAccess Journal for the Basic Principles of Diffusion Theory, *Experiment and Application*, 7, 1-29.

Packer, K. J. & Rees, C. (1972) Pulsed NMR studies of restricted diffusion. I. droplet size distribution in emulsions. *Journal of Colloid and Interface Science*, 40 (2), 206-218.

Pan, L. G., Noli, A., Campana, A., Barrera, M., Toma´s, M. C. & Aȓȯn, M. C. (2001) Influence on the processing conditions on degumming process in sunflower seed oil. J. Am. *Oil Chem*. Soc., 78, 553-554.

Pignol, D., Ayvazian, L., Kerfelec, B., Timmins, P., Crenon, I., Hermoso, J., Fontecilla-Camps, J. & Chapus, C. (2000) Critical Role of Micelles in Pancreatic Lipase Activation Revealed by Small Angle Neutron Scattering. *Biol. Chem*., 275.

Porter, C. J. H., Pouton, C. W., Cuine, J. F. & Charman, W. N. (2008) Enhancing intestinal drug solubilisation using lipid based delivery systems. Adv. *Drug Deliv*. Rev., 60, 673-691.

Reis, P., Holmberg, K., Watzke, H., Leser, M. E. & Miller, R. (2009) Lipases at interfaces: A review. Adv. *Colloid Interface Sci*., 147, 237-250.

Reis, P., Raab, T., Chuat, J. Y., Leser, M. E., Miller, R., Watzke, H. & Holmberg, K. (2008) Influence of surfactants on lipase fat digestion in a model gastro-intestinal system. *Food Biophysics*, 3, 370-381.

Ritter, R. C. (2004) Increased food intake and CCK receptor antagonists: Beyond abdominal vagal afferents. Am. J. Physiol. Regul. Integr. Comp. *Physiol*., 286, 6, R991-3.

Rosenberg, E. & Ron, E. Z. (1999) High- and low-molecular-mass microbial surfactants. *Applied Microbiology and Biotechnology*, 52, 154-62.

Ruiz, M. C., Lermanda, P. & Padilla, R. (2002) Drop size distribution in a batch mixer under breakage conditions. *Hydrometallurgy,* 63, 65-74.

Sajjadi, S., Zerfa, M. & Brooks, B. W. (2002) Dynamic behaviour of drops in oil/water/oil dispersions. *Chem. Eng*. *Sci.*, 57, 663-675.

Singh, H., Ye, A. & Horne, D. (2009) Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Progress in Lipid Researc*h, 48, 92-100.

Slabas, T. (1997) Galactolipid biosynthesis genes and endosymbiosis. *Trends Plant Sci*., 2 (5), 161-162.

Stang, M., Schuchmann, H. & Schubert, H. (2001) Emulsification in high pressure homogenisers. *Engineering in Life Science*, 1, 151-157.

Sugar, I. P., Mizuno, N. K., Momsen, M. M. & Brockman, H. L. (2001) Lipid lateral organisation in fluid interfaces controls the rate of colipase association. *Biophysical Journal*, 81, 3387-3397.

Sarkar, A., Horne, D. S., & Singh, H. (2010) Pancreatin-induced coalescence of oil-inwater emulsions in an in vitro duodenal model. *International Dairy Journal*., 20, 589597.

Tadros, F. T. (2009) Emulsion Science and Technology: A General Introduction.

Torcello-Gómez, A., Jódar-Reyes, A. B., Maldonado-Valderrama, J., & MartínRodríguez, A. (2014) Effect of emulsifier type against the action of bile salts at oil– water interfaces. *Food Research International*., 48, 140-147.

Tsuzuki, W., Ue, A., Nagao, A., Endo, M. & Abe, M. (2004) Inhibitory effect of lysophosphatidylcholine on pancreatic lipase-mediated hydrolysis in lipid emulsion. *Biochim. Biophys*. Acta., 1684, 1-7.

Ulrich, A. (2002) Biophysical aspects of using liposomes as delivery vehicles. Bioscience Reports., 22, 129-150.

Velikov, K. P. & Pelan, E. (2008) Colloidal delivery systems for micronutrients and nutraceuticals. *Soft Matter*, 4, 1964-1980.

Vibhute, B. P., Bhide, D. R., Karadbhajne, V. Y., Kulkarni, A. S. & Khotpal, R. R. (2013) Fatty acid profile of pumpkin and bael seed lipids of Central India Region. Research and Reviews: *Journal of Botanical Sciences* (RRJBS), 2 (2) 1-3.

Walde, P. & Ichikawa, S. (2001) Enzymes inside lipid vesicles: preparation, reactivity and applications. *Biomolecular Engineering*, 18, 143-177.

Wickham M., M. Garrood, J. Leney, P. D. G. Wilson and A. Fillery- Travis, J. *Lipid Res*., 1998, (39), 623–632.

Wilde, P. J. & Chu, B. S. (2010) Interfacial and colloidal aspects of lipid digestion. *Advances in Colloid and Interface Science*., 165, 14-22.

Wintermans, J. F. G. M. (1960) Concentrations of phosphatides and glycolipids in leaves and chloroplasts. *Biochimica et Biophysica Acta*., 44, 49-54.

Zangenberg N. H., A. Mullertz, H. G. Kristensen and L. Hovgaard, Eur. J. *Pharm. Sci*., 2001, 14, 237–244.