DARK CHOCOLATE: UNDERSTANDING THE IMPACT OF LIMONENE ON THE CRYSTALLIZATION PROPERTIES AND APPLICATION OF GREEN LEAF LIPID EXTRACT AS A FLOW ENHANCER

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ABSTRACT

Dark chocolate as the main subject matter in this study was investigated for physical changes when formulated with two different lipids based ingredients impacting on the chocolate flow properties. The first ingredient was limonene, known to reduce viscosity when substituting a small fraction of the cocoa butter and to affect cocoa butter crystal morphology, but without knowledge of the impact on bloom formation. The second lipid ingredient was a polar lipid extract from green leaf tissue hypothesised to show functionality as a flow aid, potentially enabling the replacement of currently applied commercial surfactants in chocolate. The driver for this part of the PhD study was to uncover alternatives for the commercial surfactant polyglycerol polyricinoleate (PGPR) which has a negative consumer image. This study was also carried out to devise an opportunity for functionalisation of green tissue waste, although in this first investigation, fresh spinach was used as the raw tissue material. As DGDG was claimed to be the polar lipid compound that caused the low viscosity reduction of dark chocolate by oat lipids, DGDG as well as MGDG are also available abundantly in spinach. The limonene work was using the techniques of whiteness index (WI) to detect bloom, X-ray diffraction (XRD) for crystal identification and differential scanning calorimetry (DSC) for the melting behaviour. Limonene significantly influenced the cocoa butter crystal transformation rate in chocolate which further promoted the development of clearly visible bloom. Nevertheless, limonene may still commercially can be applied in chocolate filling or white chocolate bars where bloom formation is not visible. Polar green leaf tissue lipids were extracted from both spinach leaf and spinach chloroplast due to their difference in composition. Based on compositional analysis of the lipids and their fatty acids by thin layer chromatography (TLC) and gas chromatography (GC), respectively, it was confirmed that leaf contained higher proportion of phospholipids compared to chloroplast, which was highly concentrated with glycolipids. The surface active nature of both lipid

extracts was confirmed by interfacial tension measurements at the oil/water interface. Leaf and chloroplast lipid showed a comparable surface activity and demonstrated to be more surface active than lecithin and PGPR. Before applying as flow aid into a complex chocolate formulation, sugar/oil suspensions with added surfactant were rheologically analysed. The performance of the two green tissue extracts was compared to the commercially applied lecithin and PGPR in a concentration range of 0.1 % to 0.7 % based on total suspension containing between 40 and 50 % sugar by weight. Both spinach leaf and chloroplast lipids showed a comparable result in influencing the rheological properties of chocolate model. Therefore, due to the higher lipid recovery from leaf compared to chloroplast as the basic of parent leaf, application in chocolate to verify the results obtained for the model system was executed only for leaf lipid extract. Based on the model suspension results addition at 0.3 and 0.6% on chocolate by weight was considered. Spinach leaf lipid decreased the viscosity of chocolate to value lower than lecithin and PGPR added at the both levels. Yield value measured at 5 s^{-1} was also reduced; at both levels of addition providing an advantage over lecithin which was shown and is known to increase yield stress at higher level of addition. On the other hand, the yield (5 s^{-1}) lowering capacity was less pronounced than PGPR whereas it was found to be effective based on the model chocolate system. The present results show promise for green leaf tissue lipid to be applied as surfactant in chocolate or fat based food suspension although further research is required to develop the full potential of this natural surfactant system.

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LIST OF SYMBOLS AND ABBREVIATIONS

η : Viscosity

Φ_m : Maximum packing fraction

τ_{SFC} : Induction period

$\dot{\gamma}$: Shear rate

ΔH: Enthalpy

ΔS: Entropy

a.u.: arbitrary unit

AI: Acetone insoluble

ANOVA: Analysis of variance

BHT: butylated hydroxytoluene

CBS: Cocoa butter substitutes

CER: Cerebrocides

CH.SPLIP: Spinach chloroplast lipids

-COOH:Carboxylic acid

DAG: Diacylglycerol

DGDG: Digalactosyldiacylglyceride

DPG: Diphosphatidylglycerol

DSC: Differential Scanning Calorimetry

FAMEs: Fatty acids methyl esters

FDA: Food and drug administration

FFA: Free fatty acids

FS: Free sterol

G: Gibbs free energy

GC: Gas chromatography

GL: Glycolipids

HPTLC: High performance thin layer chromatopgraphy

ICA: International confectionery association

IOCCC: International office of cocoa, chocolate and sugar confectionery

LEC: Lecithin

LPC: Lysophosphatidylcholine

MAG: Monoacylglycerol

MCT: Medium chain triglyceride
MGDG: Monogalactosyldiacylglyceride
-OH:Hydroxy group
PA: Phosphatidylglycerol
PC: Phosphatidylcholine
PG: Phosphatidylglycerol
PhL: Phospholipids
PI: Phosphatidylinositol
PL: Polar lipids
PR: Polyricinoleic
PS: Phosphatidylserine
PSI: Photosystem I
PSII: Photosystem II
SE: Sterol ester
SFC: Solid fat content
SG: Steryl glycoside
SPH: Sphingomyelin
SPLIP: Spinach leaf lipids
SQDG: Sulfoquinovosyldiglyceride
T: Temperature
TAGs or TG: Triacylglycerols or triacylglycerides
TGDG: Trigalactosyldiacylglyceride
TLC: Thin layer chromatography
T_m: Melting temperature
W: heat flows or watt
WI: Whiteness index
WT: well-tempered
XRD: X-ray powder diffraction
YN: Ammonium phosphatides
τ : Shear stress

1 GENERAL INTRODUCTION: THESIS OUTLINE AND RESEARCH OBJECTIVES

1.1 THESIS OUTLINE

This PhD study focused on lipid phase modification in chocolate. Molten chocolate, simply referred to as chocolate in the following text, represents a food suspension where sugar and cocoa particles are dispersed in the liquid cocoa butter phase. Chocolate is formulated close to its maximum packing fraction, Φ_m, where reducing the amount of cocoa butter as a strategy to formulate reduced fat chocolate becomes unfeasible as the viscosity becomes so high that flow is inhibited. Therefore, in this study, the modification looked at viscosity behaviour and two main objectives were investigated. The first objective related to the substitution of an amount of cocoa butter with another lipid, which has already been shown to impact on viscosity, and in this PhD the impact on the crystallization behaviour and bloom of the chocolate was investigated. Bloom is a chocolate defect which is characterized by the loss of initial gloss, a rougher surface texture with a whitish appearance on the surface of the chocolate. The second objective was to study the potential of lipids from a photosynthetic plant to be applied as a natural surfactant in chocolate. The impact on the flow properties of chocolate in comparison to commercially applied emulsifiers was investigated.

There is no chapter on general methods in this thesis due to the different research approaches taken to obtain the results reported in the results section of each chapter. This thesis is organised into five main chapters, each of which features an introduction, the materials and methods used to obtain the results reported and discussed in the chapter. Each main chapter finishes with conclusions drawn from the results obtained. The final thesis chapter features an overall conclusion and future work on the two main objectives of this PhD study.

The study relating to cocoa butter substitution was a continuation of previous work by Beckett (1999) where limonene substitution at 5% was reported to reduce the viscosity of low fat chocolate. Do et al. (2008) had developed a reduced fat chocolate using 3% of limonene, which resulted in a chocolate with lower viscosity and melting temperature. The low melting point was concluded to be due to a lower type of crystal formed in presence of the limonene (Do et al., 2008). However, Ray et al. (2012) found that the limonene did not decrease the crystallinity (i.e. change the crystal form to a lower degree) of cocoa butter as was stated by Do et al. (2008). Instead, Ray et al. (2012) suggested that the limonene did not crystallize with the cocoa butter triglycerides but would accelerate the phase transformation of cocoa butter crystal to a larger crystal (Form VI) (Ray et al., 2012). Cocoa butter has six forms of crystals, starts with the most unstable crystal, Form I, followed by Form II, III, IV, V and

ending with VI as the most stable crystal. Nevertheless, the impact of limonene on the bloom formation of chocolate has not yet been studied and was included as the first piece of research in this PhD study.

The remainder of the research in this PhD was related to the polar membrane lipids of spinach and their utilization in chocolate. The properties of spinach lipids was reported in previous studies, especially in delaying fat digestion, by interacting at the oil/water interface of a lipid droplet. The process was also found to promote satiety hormones (Rayner et al., 2011a). The high surface activity of polar membrane lipids was claimed to be due to the presence of monogalactosyl diacylglyceride (MGDG) and digalactosyl diacylglyceride (DGDG). The presence of MGDG and DGDG in oat lipids was also reported to show an ability in reducing the low shear rate viscosity of chocolate to a value lower than lecithin but comparable to polyglycerol polyricinoleate (PGPR) (Evans et al., 1991). There was also a pull from industry to use natural surfactants and replace synthetic ones like PGPR in chocolate. PGPR has a unique characteristic where it can reduce the yield stress of chocolate without significantly affecting the viscosity of the chocolate. However, the reason to search for a replacement is due to it originating from castor oil and the long chemical process of producing PGPR, as well as increasing demand from consumers for natural products even though PGPR has been proved to have no detrimental effect on human health

of a two years study (Wilson et al., 1998). Therefore, there is a benefit in investigating the potential of spinach lipids as a natural surfactant in chocolate, as conducted in this thesis.

The remainder of Chapter 1 explains on the knowledge from literatures which includes the introduction on chocolate ingredients especially cocoa butter, the process of chocolate making, the common used emulsifiers and also some introduction on the green leaves tissue and polar membrane lipids.

1.2 RESEARCH OBJECTIVES

This thesis consists of two main objectives, relating to the overall properties of chocolate. The first objective was a continuation of previous studies into the effects of the incorporation of limonene into chocolate. This work was focused on the impact of limonene on the crystallization and the bloom of chocolate.

The second objective was to investigate the impact of spinach lipids on the rheological properties of chocolate model systems, in comparison to the functional behaviour of lecithin and PGPR as the most commonly used surfactants in chocolate. In order to relate the functional behaviour of leaves lipids and chloroplast lipids from

spinach to the rheological properties of the chocolate model systems, the composition of polar lipids and fatty acids were analysed quantitatively. Verification of the spinach lipids function was then carried out on real chocolate.

1.3 CHOCOLATE INGREDIENTS

Chocolate generally consists of three main ingredients; sugar, cocoa solids and cocoa butter (Lonchampt and Hartel, 2004). Emulsifier is also added in small quantities to improve the flow properties of molten chocolate and which consequently affects the sensory and overall quality of chocolate.

Sugar is crystalline sucrose either refined from sugar cane or sugar beet. It is a hydrophilic compound. Sugar crystals with 100% purity of saccharose have a density of 1.5805 g/ml (Johansson and Bergenstahl, 1992a). Sugar used in chocolate consists of crystals which generally have a size of 0.6 – 1.0 mm. Sugar also easily absorbs moisture and thus must be kept in conditions with a surrounding humidity between 20 - 60%. At that humidity range, sugar maintains a moisture between 0.01% and 0.02% (Beckett, 2008).

The term “cocoa solids”, refers to the non-fat part of cocoa beans either in powder form or in liquor. Dark chocolate has a high concentration (65-75%, v/v) of suspended solid particles comprising of sugar crystals and cocoa solids, dispersed in a continuous matrix of cocoa butter (Afoakwa et al., 2009b). Cocoa liquor is the roasted, hulled, ground substance obtained from fermented and dried cocoa beans. Most of the cocoa liquor that is used to make cocoa powder is alkalized (though liquor that is used to make chocolate is rarely treated this way) in order to make the powder less likely to agglomerate or sink to the bottom when it is added to milk or a water-based drink (Beckett, 2008). Cocoa powder is normally used to produce cake with chocolate flavour or chocolate drinks.

Cocoa butter, the central fat in chocolate, is produced by pressing cocoa liquor. Pure pressed cocoa butter has a flavour, which will become part of the whole chocolate. For white chocolate, this flavour is unpleasant thus a deodorized cocoa butter will normally be used. The deodorization process is done by steam distilling the cocoa butter under vacuum (Beckett, 2008).

Emulsifiers that are commonly used in chocolate are lecithin and PGPR. In chocolate, the sugar particles are hydrophilic, which means the sugar attracts water but repels fat. Liquid chocolate flows because the sugar and other solid particles are able to move past one another

as they are coated individually by the liquid fat. The emulsifier adsorbs at the solid particles and then helps dispersion in the lipid phase (Beckett, 2008). Lecithin is very effective at reducing the viscosity of chocolate while PGPR is often added as well to reduce the yield value of the chocolate.

There are also additional ingredients used in making chocolate such as milk powder and milk fat. The population of some countries prefer milk chocolate more than dark chocolate as it has a creamier taste and texture (Beckett, 2008). Cow milk has a major component of water. The addition of 3% or 4% of water into liquid chocolate will produce a very thick paste. Approximately, for every 0.3% of water left within the chocolate at the end of conching, the manufacturer must add an extra 1% of fat. Cocoa butter is by far the most expensive component in chocolate, thus it is very important to ensure the chocolate is free of water. Therefore, milk is usually used in powder form, produced either by spray or roller drying. Both types of milk powder can be purchased in full cream or skimmed versions (Wohlmuth, 2009). Milk fat is the second largest component in dehydrated milk and it is very important in giving milk chocolate its distinctive texture and flavour. Milk fat also can change the snap of chocolate and can prevent the formation of bloom (Beckett, 2008).

1.4 CHOCOLATE MANUFACTURING PROCESS

1.4.1 Overview of the process

Chocolate processing generally has seven main steps, starting from mixing of all ingredients until removing chocolate from its mould. The general process of chocolate manufacturing can be seen in Figure 1.1.

The first three steps to produce molten chocolate are mixing, refining and conching (Afoakwa et al., 2007). Chocolates contain cocoa liquor, sugar, cocoa butter, milk fat and milk powder are firstly mixed together to obtain a premix. The mix of sugar, milk solids and cocoa liquor with an overall fat content of 8 – 24%, is then refined using a roll-refiner to obtain particle sizes of <30 μm. To melt the mass into a uniform chocolate paste, the conching process is carried out at >50°C (Beckett, 2008). During this stage, additional fat and emulsifier are added to further coat the particles to ensure the chocolate has the correct flow properties for the next processing stages. This step is followed by tempering, moulding, cooling and de-moulding of the solidified chocolate. Each step is discussed in more detail in the following sections.

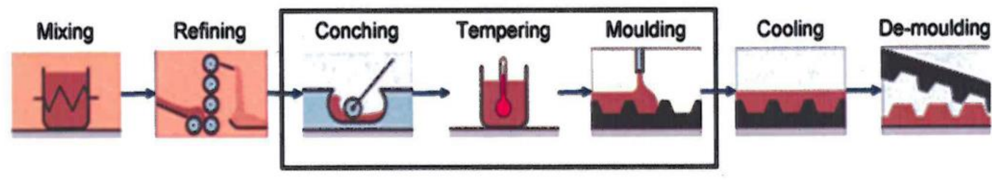

Figure 1.1 General steps in chocolate processing, provided by Nestle PTC York (Do, 2008)

1.4.2 Chocolate refining

Chocolate refining is very important to ensure that the particle size of the dispersed (solid) phase is sufficiently small, so that the chocolate does not feel gritty when eaten. However, eating quality is not determined by particle size alone but also the fat content which determines the overall texture of the chocolate (Wille and Lutton, 1966). There are two ways to refine chocolate, either by "fine (or separate) ingredients" or by "combined milling" (Beckett, 2008). In the fine ingredient process, the solid particles (non-fat components) are milled separately and then added to the cocoa liquor, cocoa butter and other liquid ingredients in the conche. This process is carried out using a classifier mill, which has a series of milling stages that take place before the finely ground particles leave the mill.

In many chocolate factories, a combined milling is normally preferable which is where all ingredients of the chocolate premix are mixed together, generally using a five-roll refiner (Beckett, 2008). A schematic diagram of a five-roll refiner can be seen in Figure 1.2. The five roll refiner consists of five horizontal cylinders, four of which are placed one above the other. The first cylinder, which is the feed cylinder is placed at the bottom, but is put on the side so that a trough is formed between the first and the second cylinder (Beckett, 2008). The other four cylinders provide four crushing gaps and therefore produce a very finely ground chocolate paste.

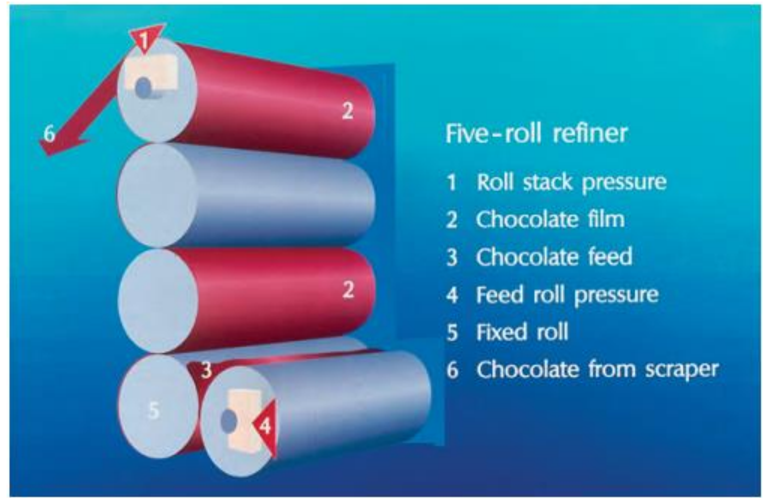

Figure 1.2 Schematic diagram of a five-roller refiner (Beckett, 2008)

The cylinders are hollow so that water can be used to cool and heat the cylinders. Each successive roller is faster than the previous one so that it can attract the chocolate film from the previous roller, and eventually bring the chocolate film up the refiner. The normal operating condition for a five-roll refiner is shown in Table 1.1. This illustrates that, if two surfaces are travelling at very different speeds and are very close together, there is a very high shearing action which will pull the particles apart, and so, will cause the chocolate film to rise up the refiner. The process by which the chocolate film is attracted from a roller to another roller can be seen in Figure 1.3. The thickness of the film depends on the gap between the particular roller and the one below. The gap sizes are between often 44.5 µm – 13.3 µm, from the bottom to the top of the refiner.

Table 1.1 Example of operating conditions for a five-roll refiner (Ziegler and Hogg, 2009)

Roll	RPM	Temperature (°C)
R1	<58	35-40
R2	58	35-40
R3	155	42-48
R4	268	50-60
R5	380	35-40

* Roll number is from bottom to the top of the refiner

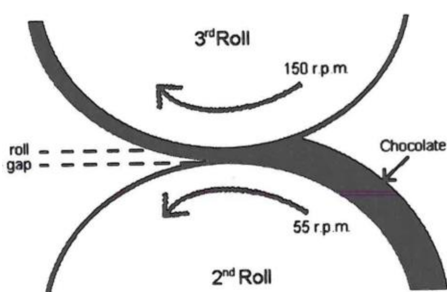

Figure 1.3 Close-up of the gap between the second and third rolls of a five-roll refiner (Beckett, 2008)

1.4.3 Conching

Conching is a process that contributes to development of viscosity and final texture and flavour (Afoakwa et al., 2007). The unpleasant flavour that normally develops during fermentation and roasting of cocoa beans is removed during conching (Beckett, 2008). Besides that, the main objective of conching is to turn chocolate from a flaky, powdery dry mass into a free flowing liquid that can be used to make the final products. This process involves coating the surface of the solid particles with fat so that they can slide past one another (Beckett, 2008).

The conching process generally consists of three stages in order to obtain a well processed chocolate (Beckett, 2008):

1. Dry conching

   Chocolate at this stage is still powdery and has some excess moisture. The chocolate is heated and mixed to remove the excess water which is detrimental to the flow properties of the final product. The moisture content is decreased to approximately 0.7% (Ley, 1994). As the moisture is removed, it takes with it unfavourable flavour volatiles such as volatile fatty acids and acetic acid generated by fermentation and roasting. Figure 1.4 shows the longitudinal conche which had been used by Rudi Lindt in 1878.

2. Pasty phase

   As the temperature rises, more of the cocoa butter melts and a thick paste is formed which allows the shearing action to break up agglomerates, release the cocoa butter contained within them and coat the non-fat solid particles with fat. It is important to ensure that the particles' surfaces are coated as much as possible, so that the chocolate flows well during the later processing stage and in the mouth.

3. Liquid conching

   Final additions of fat and emulsifier are carried out in this step in order to ensure that the chocolate has the correct flow properties for the next processing stage.

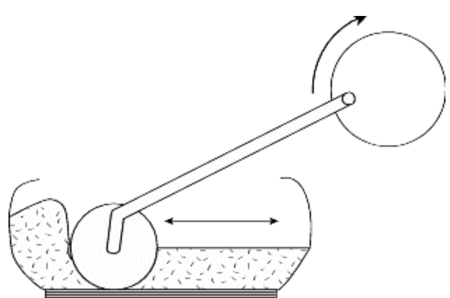

Figure 1.4 Longitudinal conche (Beckett, 2009b)

1.4.4 Tempering

Tempering is the process of the pre-crystallization of chocolate in order to form the stable Form V crystal (further detail on the crystals in chocolate can be found in section 1.6.1.). Crystals formed during tempering will induce the crystallization of the rest of the fat to be in Form V. Small scale chocolate manufacturers can just add a small amount of set chocolate into a molten chocolate at 30°C to achieve tempering.

Tempering depends on the temperature and the rate of mixing and shearing. An example of a tempering sequence for milk chocolate is shown in Figure 1.5. For dark chocolate, a temperature of 2 to 3°C higher than those indicated would be used. This is because milk fat can delay the formation of the crystal lattice due to an eutectic effect which results in a lower melting temperature, softening of texture and thus lowering the tempering temperature needed to obtain seed crystals (Afoakwa et al., 2007). However, the exact temperature to be used depends on the recipe of the final product, the tempering equipment and the purpose for which the chocolate will be used. A well-tempered chocolate will have a good snap, colour, gloss, contraction from the mould and a longer shelf life than a poorly tempered chocolate (Afoakwa et al., 2007).

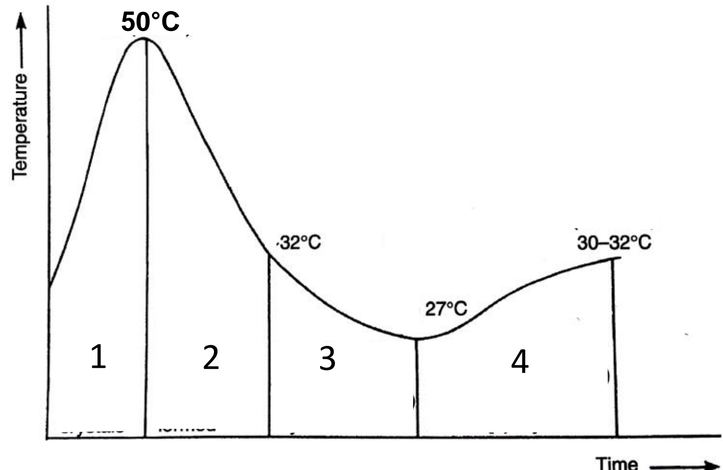

Figure 1.5 Milk chocolate tempering sequence (Talbot, 2009). Stages 1 to 4 are explained in the main body text.

The most commonly used method of tempering involves the following steps as described by Talbot (2009):

1. Complete melting

   Chocolates are melted at about 50°C (112°F) to ensure complete melting of the fat and erase previous crystal memory (Svanberg et al., 2013).

2. Cooling to the point of crystallization

   The melted chocolate is cooled to a holding temperature where Form II and Form III crystals are created.

3. Crystallization

   The process of crystallization is carried out by mixing and shearing the chocolate on a marble slab and blending it with a

flexible spatula until it begins to thicken; which normally occurs at 27°C. At this point, both stable (Form V) and unstable polymorphic forms (Form IV) are formed.

4. Melting out of unstable crystals

   The thick ‘mush’ is heated (30 - 32°C) to melt out the unstable crystals (Talbot, 2009), leaving predominantly Form V crystals.

1.5 COCOA BUTTER AS MAIN INGREDIENT IN CHOCOLATE

1.5.1 Cocoa butter

Cocoa butter is the main fat used in chocolate. However, other ingredients in the recipe of the finished product may be a source of fat in chocolate, such as milk and nuts. Other vegetable fats used as cocoa butter substitutes (CBS) which are usually from coconut or palm kernel oils (Wainwright, 2000), can provide a new flavour or to enhance the physico-chemical properties of chocolate (Lonchampt and Hartel, 2004). Cocoa butter is a fat that is made up of several different triacylglycerols (TAGs) which solidify at a different temperatures and at different rates (Beckett, 2008). Overall the fat content of chocolate is 25 – 35% by weight (Beckett, 2008).

1.5.2 Cocoa butter structure

Cocoa butter is mainly made up of monounsaturated TAGs (Lonchampt and Hartel, 2004). The three main fatty acids in cocoa butter are oleic acid (O) (C18:1), stearic acid (S) (C18:0) and palmitic (P) (C16:0) which account for over 95% of all fatty acids found in cocoa butter. Cocoa butter consists of a mixture of about 40 – 50 different TAGs, with POS at 35%, SOS at 23% and POP at 15% (Schenk and Peschar, 2004). Figure 1.6 shows the structure of the POS molecule with palmitic (P) in position 1, oleic acid (O) in position 2 and stearic (S) in position 3. Stearic and palmitic are saturated fatty acids where the hydrocarbon chain does not contain any double bonds. Oleic acid is the only one of the three main fatty acids found in cocoa butter that has a double bond. Figure 1.6 shows the TAG molecule with a symmetrical structure in terms of saturated and unsaturated fatty acids; they are often referred to as sOs triglyceride, where “s” refers to any saturated fatty acid. About 80% of cocoa butter is in this form where oleic acid situated at position 2 (Beckett, 2008). About 1 to 2% of TAGs in cocoa butter are made up of all

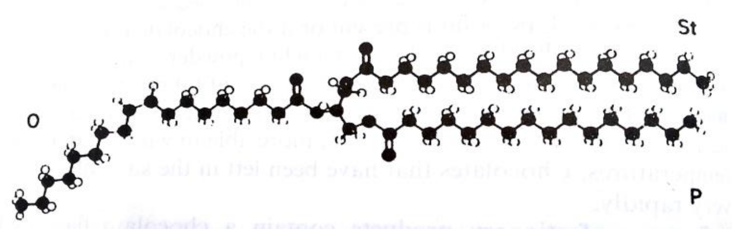

Figure 1.6 Structure of triglycerides (Beckett, 2008)

saturated fatty acids (sss triglyceride, where the saturated fatty acid is mainly palmitic and stearic), melting at a much higher temperature than common sOs. For example, sOs has melting temperature of 39.2°C while sss has a melting temperature of 73.3°C (List et al., 2004). The other 5 to 20% of TAGs are mostly two oleic acid molecules and mainly liquid at room temperature (Beckett, 2008). Therefore, when all of the TAGs are combined together in cocoa butter, some of the fat will be in liquid form at room temperature. Some chocolate may have milk fat and other vegetable oils. Chocolate containing milk fat usually has a softer texture due to higher proportion of TAGs with two oleic acid molecules. As the temperature rises, the fat will melt according to the proportions of the different types of fat present.

The TAG composition of cocoa butter originating from cocoa trees in different countries can be seen in Table 1.2. Different environmental conditions for cocoa growth have an effect on the hardness of cocoa butter, its proportion of fat and variety of cocoa bean. Lower environmental temperatures generally give cocoa butter a softer texture or a lower melting point (Fowler, 2009). According to Beckett (2008), on average, the nearer the place of cocoa growth to the equator, the harder the fat of the cocoa will be. The TAG composition of cocoa butter in terms of saturated and unsaturated fat is actually the main reason for this phenomenon, where the hardness of cocoa butter is particularly affected by the sOs/sOO ratio.

Table 1.2 Triacylglycerol composition of cocoa butter from different countries (Chaiseri and Dimick, 1995)

	Malaysia	Ivory Coast	Ghana	Ecuador	Dominican Republic	Brazil
	[% wt]					
PLO	0.4	0.7	1.0	0.5	0.7	0.9
PLP	1.1	1.7	1.8	1.6	1.8	1.7
OOO	0.1	0.4	0.8	0.7	0.6	0.7
POO	11.0	1.8	2.0	2.7	3.8	5.8
PLS	2.6	3.7	3.6	3.1	4.2	3.9
POP	12.6	15.0	14.5	14.1	14.6	13.9
SOO	1.8	2.3	2.8	3.3	4.4	6.7
SLS	1.6	1.7	2.0	1.6	1.8	2.1
POS	46.9	46.3	42.8	45.4	42.8	40.2
PPS	0.7	0.7	0.8	0.8	0.7	0.6
SOS	29.8	24.0	26.3	24.8	22.8	21.7
PSS	0.4	0.5	0.6	0.4	0.5	0.5
SOA	0.9	0.8	1.0	0.8	1.0	0.9
SSS	0.2	0.4	0.2	0.3	0.4	0.6

* P- palmitic; L- linoleic; O- oleic; S- stearic; A- arachidonic

In general, cocoa butter consists of three forms of triglycerides that are important in fat crystallization. They are sss (all saturated), sOs (saturated-oleic-saturated) and ssO (saturated-oleic-oleic). When the cocoa butter is melted and then cooled again, the three types of TAG once again behave differently. Figure 1.7 shows a graph representing the time taken for the three TAGs to crystallize after

being melted. It illustrates that, upon cooling, sss will crystallize first followed by sOs crystals. After sss triglycerides have crystallized, the liquid chocolate becomes thicker due to the decreased liquid fat level. Triglycerides of sOs are the most abundant triglyceride in cocoa butter, thus it is most significant in determining the texture of cocoa butter.

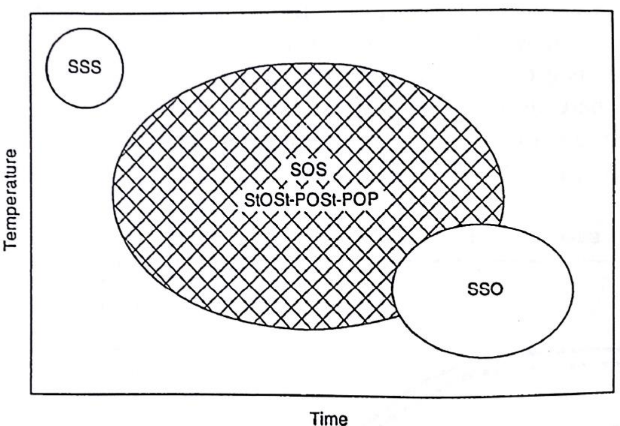

Figure 1.7 Crystallization rate at different temperature of different triglycerides groups (Talbot, 2009)

1.6 CRYSTALLIZATION AND POLYMORPHISM

1.6.1 Crystallization

Controlling the crystallization of cocoa butter is critical in making good chocolate. Poor crystallization in chocolate results in the formation of bloom where the external surface of the chocolate becomes greyish

white. Crystallization also affects the organoleptic and physical characteristics of the final products (snap, mould contraction and glossy appearance) (Loisel et al., 1998). In chocolate, cocoa butter can exist in six different crystalline forms. This is referred to as polymorphism, where each of the crystal form has a different thermodynamic stability and melting point (Timms, 1984, Wille and Lutton, 1966).

The process of crystallization starts with nucleation and is followed by crystal growth. Nucleation involves the formation of aggregates of molecules that have exceeded a critical size and are therefore stable. Once a crystal nucleus has formed, it begins to grow by incorporating other molecules from the adjacent liquid layer, which is continuously filled by the supersaturated liquid that is around the crystal (Boistelle and Astier, 1988).

The characterization of the crystallization kinetics can be done according to the induction period (τ_{SFC}) or nucleation period (time to the beginning of crystal formation) and maximum solid fat content, SFC_{max} (Ribeiro et al., 2015). Induction time reflects the time required for a stable nucleus of critical size to be formed in the liquid phase (Himawan et al., 2006). In other words, τ_{SFC} is defined as the time required to obtain a crystal nucleus per volume (Ribeiro et al., 2015).

The polymorphic crystallization is primarily determined by the rate of nucleation which is governed by thermodynamic and kinetic factors (Sato, 2001). According to the rule of Ostwald, metastable nucleates form first before the formation of the most stable form, when nucleation is induced under large kinetic factors such as supercooling or supersaturation (Sato, 2001). When the kinetic factors are minimized and some external factors such as pressure, temperature fluctuation, ultrasonic stimulation and seeding are applied, the "rule" is broken and the more stable forms are nucleated at reduced kinetic factors. At this time, direct crystallization of β'- or β-forms from melts tends to occur (Himawan et al., 2006). β' and β are types of polymorph of crystals which are further explained in the next section on polymorphism.

1.6.2 Polymorphism of fat crystals in cocoa butter

Polymorphism is the ability of a molecule to take more than one crystal form depending on its arrangement within the crystal framework. A TAG usually possesses three polymorphs which are α, β' and β (Sato, 2001). The three main TAGs found in cocoa butter determine its polymorphic nature which exhibits as six polymorphs from Form I to Form VI, according to the nomenclature given by Wille and Lutton (1966). The polymorphic forms of cocoa butter are also referred to by Greek letters (γ, α, β'_2, β'_1, β_2, β_1) (Vaeck, 1960), in

increasing order of melting points. However, for commercial chocolate production, only the Forms IV to VI are important (Svanberg et al., 2011). Form V is the preferred polymorph, Form IV is found in untempered chocolate, and Form VI is found in bloomed samples (Timms, 2002)

The relative stability of polymorphs and the driving force for transformations between them at constant temperature and pressure are determined by their respective Gibbs free energies (G) (Himawan et al., 2006). The polymorph which has the lowest Gibbs free energy is the most stable. Gibbs free energy-temperature diagrams are used to map the thermodynamic stability of the polymorphs as shown in Figure 1.8. Gibbs free energy is defined as a function of enthalpy (H), entropy (S) and temperature (T) as in Equation 1.1;

$$G = H - TS \qquad \text{(Equation 1.1)}$$

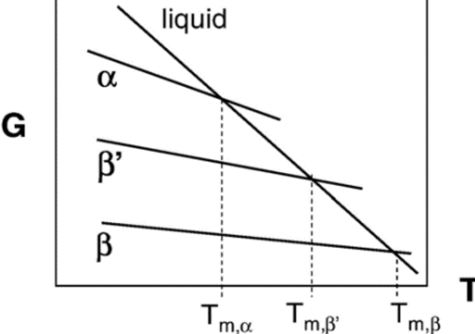

Figure 1.8 The relation between Gibbs free energy and temperature for the three main polymorphs (Himawan et al., 2006)

Form I (γ) is very unstable and melts at about 17°C (McGauley and Marangoni, 2002). Due to its instability, it can easily transform into the Form II (α). This form then will slowly turn into Form III and IV (β') if stored at higher temperature (0°C to 20°C) (McGauley and Marangoni, 2002). According to Beckett (2008), cooling a liquid chocolate of 30°C in temperature in an airflow of 13°C will produce chocolate with a majority of Form IV crystal. The Form IV crystal is relatively soft; thus the chocolate will not have any snap. After a few days of storage, this crystal will turn into Form V. The different polymorphic forms have a different range of melting temperature as shown in Figure 1.9.

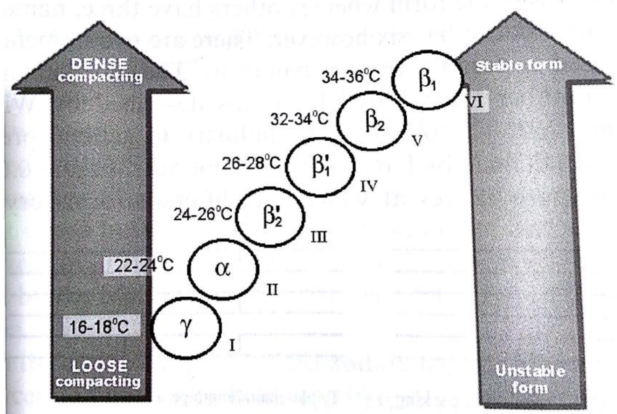

Figure 1.9 Temperature ranges for the stable formation of the six different crystalline forms of cocoa butter (Beckett, 2008)

In chocolate processing, cocoa butter is tempered to ensure all crystals formed are in the state of Form V. Form V crystal structure of cocoa butter will make the chocolate look glossy, have a good snap and will be relatively resistant to bloom. This form also makes the chocolate contract well when it sets in a mould.

The Form VI crystal structure is the most stable form. However, these crystals make chocolate become harder in texture and influences the sensory properties by making it more difficult to melt in mouth due to the higher melting point. This form of crystal can only be achieved from a solid state condition (from Form IV or Form V) or by seeding with a Form VI crystals (Beckett, 2008).

TAG molecules are three legged molecules that can pack in a "chair" configuration where the acyl chain in the 2 position is alongside the chain on either the 1 or 3 positions (Himawan et al., 2006). Alternatively, a "tuning fork" configuration can be adopted where the acyl chain in the 2 position is alone and the chains in the 1 and 3 positions pack alongside each other (Himawan et al., 2006). Different polymorphic forms or packing configurations of TAGs in cocoa butter are characterized by differences in the distances between the glyceride chains and in the 'angle of tilt' relative to the plane of the end methyl group of the chain, see Figure 1.10. The α configuration

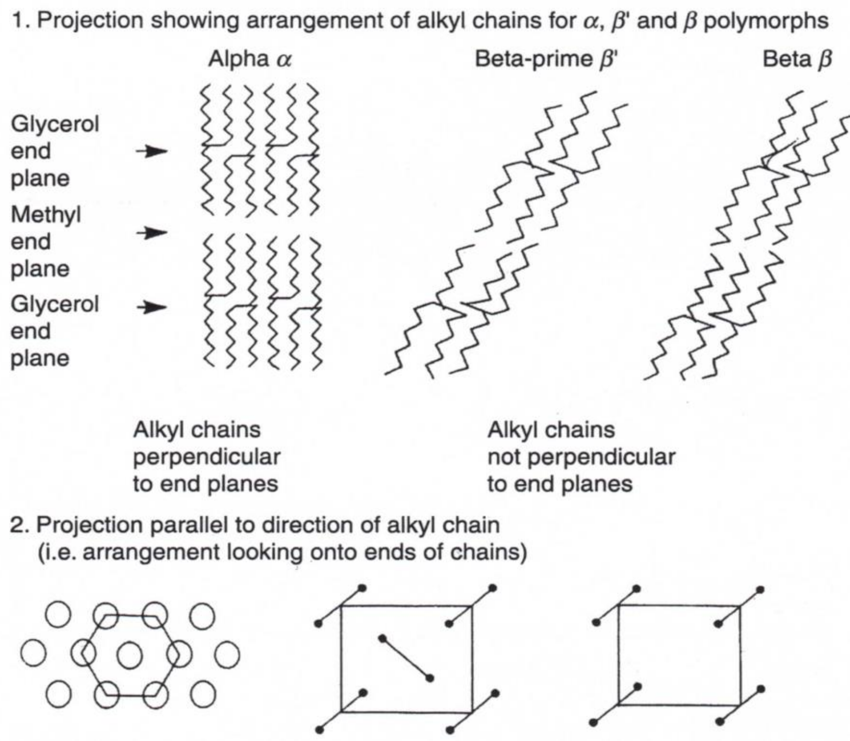

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Figure 1.10 Crystal packing of triglycerides. (1) Projection showing arrangement of alkyl chains for ɑ, β and β′ polymorhs. (2) Projection parallel to direction of alkyl chain (arrangement looking onto ends of chains, from left to right, α (hexagonal), β (orthorhombic) and β′ (triclinic)) (Talbot, 2009).

has alkyl (fatty acid) chains which are both straight and parallel to each other, but which are also perpendicular to the end planes of the molecules. By looking at the end of the chains, they would appear to have a cross-section of hexagonal symmetry (Talbot, 2009). The β′ and β polymorphs show considerably more order in their crystal packing. Both display an angle of tilt relative to the end plane of the molecules. The β′ form has slightly greater angle (i.e. closer to perpendicular) than the β form. The packing of the chains in the β′

form is in an orthorhombic sub-cell in which adjacent zigzag fatty acid planes are mutually perpendicular when viewed end on. On the other hand, the chains pack of the β form are in a triclinic sub-cell with all the zigzag fatty acid chains being parallel to each other (Talbot, 2009).

These structures' alkyl chain arrangement can be identified by powder X-ray diffraction patterns (Sato, 2001, Loisel et al., 1998) where long spacings give information on the repeat distance between crystal planes (chain length packing) and short spacings give information on subcell structure (interchain distances) (Himawan et al., 2006). The chain length packing of double (2L) and triple (3L) are shown in Figure 1.11.

The determination of fat whether to crystallize in a double or triple chain form is dictated by its triacylglycerides composition and, particularly, by the positional distribution of fatty acids. If the

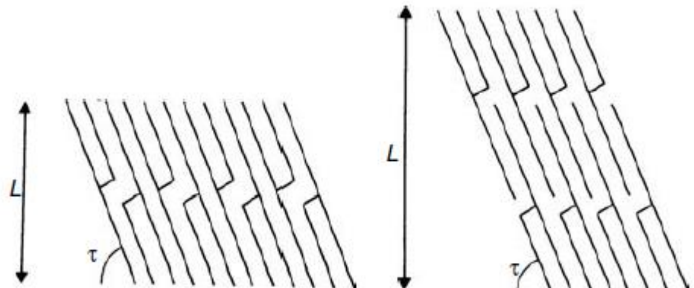

Figure 1.11 Double- and triple-chain packing configuration (Talbot, 2009)

triacylglycerides are crystallizing in a double chain form, the sn-2 position of oleic acid of one molecule would adjacent to the saturated acids in the 1- and 3-positions of the next molecule. On the other hand, if the triacylglycerides are crystallizing in a triple chain form, the sn-2 position of oleic acid of one molecule would lie adjacent to the sn-2 position of oleic acid of other molecules. Previous study have shown that the Form III and IV cocoa butter have a double chain packing of TAGs while the Form V and VI have a triple chain packing of TAGs (Talbot, 2009).

1.6.3 Fat bloom

Fat bloom in chocolate is normally related to colour changing and loss of gloss, and a greyish or whitish appearance on the surface (Lonchampt and Hartel, 2004). It may due to the migration of less stable fat crystals to the surface of chocolate during the transition to more stable crystal polymorphs. It can occur as a result of exposing chocolate to temperatures above the melting temperature of some of the fat crystals as may happen in the distribution chain or the consumer’s home. The melted fats will diffuse to the surface of the chocolate and recrystallize to form a higher polymorphic form of crystalline cocoa butter.

Fat bloom can be largely prevented through correct tempering during the chocolate manufacture process to ensure the cocoa butter crystallises in Form V. However, even a well-tempered chocolate can develop bloom as a result of slow transition from Form V to Form VI after long storage (Kinta and Hatta, 2012). Figure 1.12 shows bloom formation on the surface of chocolate.

The term “bloom” also relates to sugar bloom (Kinta and Hatta, 2012). Sugar bloom is caused by changes in the morphology of the sugar crystals. Sugar crystallization is usually mediated by water, for example if the chocolate is placed in high humidity or being in contact with water. The water dissolves sugar on the surface of the chocolate. As the water dries, the dissolved sugar crystallizes and precipitates onto the surface of the chocolate. The small crystal formation of sugar will give a dusty appearance on the surface of chocolate.

Both types of blooms are attributable to changes in crystal morphology of chocolate. However, fat crystals are more sensitive to environmental conditions and are thus less stable than sugar crystals (Kinta and Hatta, 2012). Therefore, fat bloom is more frequently attributed to be the cause of a case of bloom on chocolate than sugar recrystallization.

Figure 1.12 Chocolate bloom (Widlak and Hartel, 2012)

1.7 TECHNIQUES USED TO STUDY POLYMORPHISM

The structural properties of fats are normally studied by X-ray diffraction (XRD). In the study of fat polymorphism, XRD is normally accompanied by the analysis of the thermal behaviour of fats using differential scanning calorimetry (DSC).

1.7.1 X-Ray powder diffraction

X-Ray powder diffraction is a frequently applied technique to study the phase transition phenomena of TAG polymorphic forms. XRD can be used to characterize and identify the extent of polycrystalline phases in mixed amorphous–crystalline systems by measuring the average spacings between layers or rows of atoms. XRD can also be used to understand the crystal structure of an unknown material. The

application of XRD in chocolate is very important when studying the crystallization behaviour of fat crystals (Loisel et al., 1998, Wille and Lutton, 1966, Cebula and Ziegleder, 1993).

A crystal lattice consists of individual units of atoms or molecules that arrange themselves into a regular, repeating three-dimensional structure. In such a crystal lattice, the distance between individual units is well defined and constant. The continuous planes in a crystal may act to deflect X-rays that impinge upon them at a particular angle. The angle of incidence of the X-ray is equal to the angle of diffraction, as illustrated in Figure 1.13.

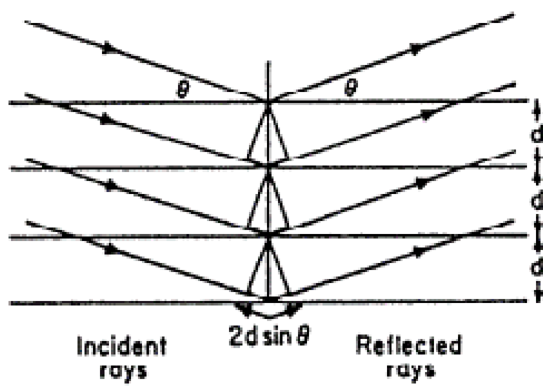

Figure 1.13 Diffraction of X-rays in a crystal lattice (deMan, 1992)

The relationship between the wavelength of the X-rays, the spacing d between crystal planes and the angle of diffraction is given by Bragg's law (Equation 1.2);

$$n\lambda = 2d \sin \theta \qquad \text{(Equation 1.2)}$$

where n is an integer, λ is the wavelength of the X-rays, d is the interplanar spacing and θ is the diffraction angle. The d-spacing also define the subcell within the crystal lattice (Small, 1966), which can be hexagonal (α), orthorhombic (β') or triclinic (β). X-ray diffraction is the most suitable technique to be used as the wavelengths generated are in the general order of magnitude of the spacings found in crystals. A study of crystals using this technique needs X-rays that are composed of only one wavelength (monochromatic).

Analysis of fat crystals using X-ray diffraction involves two types of spacings to be recognized. Long spacings are observed in the 2θ range of 1 – 15° while short spacings are in the range of 16 - 30°. The long spacings (small angle) correspond to the planes formed by the methyl end groups of the triacylglycerides, the chain length and the angle of tilt of the component fatty acids. The short spacings (wide angle) correspond to the cross-sectional packing of the hydrocarbon chains and are independent of chain length (deMan, 1992). Short spacings are used for the characterization of the polymorphic crystals

form present in the sample. The example of short spacing line from the XRD is shown in Figure 1.14.

XRD identifies the α form as one strong short spacing line near 0.42 nm, β′ form as two strong short spacing lines at 0.37 - 0.40 nm and at 0.42 – 0.43 nm while the β form as a strong spacing line at 0.46 nm (Himawan et al., 2006). Larsson (1966) introduced the criteria for classification of the different crystal forms similar to the one stated by the AOCS method, but with the slight difference that the β polymorphic form should display three peaks at positions d= 4.6 Å, d=3.8 Å and d = 3.7 Å.

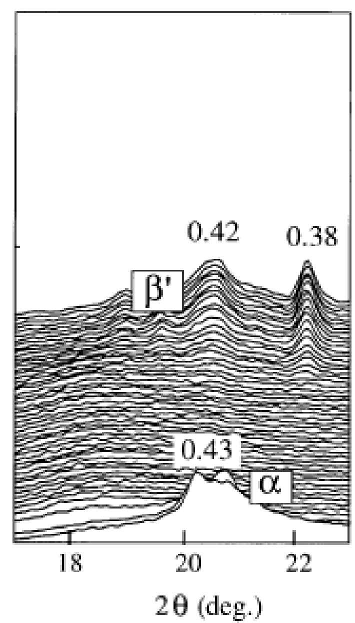

Figure 1.14 XRD pattern showing the short spacing line of cocoa butter crystals (Sato, 2001)

1.7.2 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermal analysis technique used to measure changes in heat flow associated with material phase transitions. DSC measurements provide both qualitative and quantitative data on endothermic and exothermic processes. DSC is widely used to investigate the melting and crystallization behaviour of confectionery fats and cocoa butter (Aronhime et al., 1988, Spigno et al., 2001).

The two common types of DSC are heat-flux DSC and power compensated DSC. The heat-flux DSC system is composed of a single furnace containing the sensor upon which both sample and reference are placed and heated up. The power compensated DSC consists of two separated furnaces which heat up the sample and reference individually. The DSC systems are pictured in Figure 1.15; both

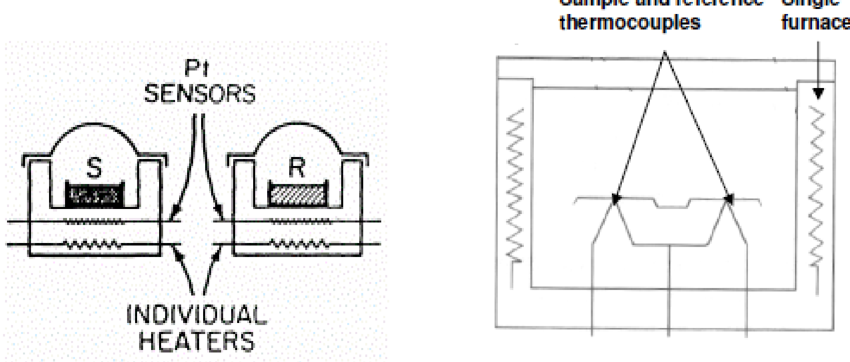

Figure 1.15 Schematic diagram of the principle of a DSC system. The left one is the power compensated DSC while the right one is the heat-flux DSC (Baichoo, 2007)

systems use similar principles in measuring the heat flow as a function of temperature or time. “S” refers to “sample” while “R” refers to “reference”. An empty pan is normally used as reference. The amount of sample added is normally around 10 to 15 mg. The sample and reference are heated by separate electrical heaters programmed to heat or cool the sample and reference exactly at the same rate. The change in sample temperature during endothermic or exothermic processes will cause a modification to the power supplied to the sample heater in order to maintain the sample at the same temperature as the reference. The difference in power supplied to the sample and reference heaters is accurately monitored and represents the energy change or enthalpy change (ΔH) in the sample (Sivasankar, 2008).

Thermograms are created which show the curve related to the phase changes of fat samples as the temperature is increased or decreased at a controlled rate. Typical thermograms display the difference in heat flow on the Y axis, while the temperature or time is shown on the X axis, see Figure 1.16 for an example. The parameters that are commonly analysed are the temperature of the summit of the peak of crystallization, T_C; the summit of the peak of melting, T_M; the onset temperatures for crystallization, T_{OC} and for melting, T_{OM}; and the enthalpy of crystallization, ΔH_C and fusion or melting ΔH_M. The heat flow for the crystallization is from right to left, where the fat is cooled

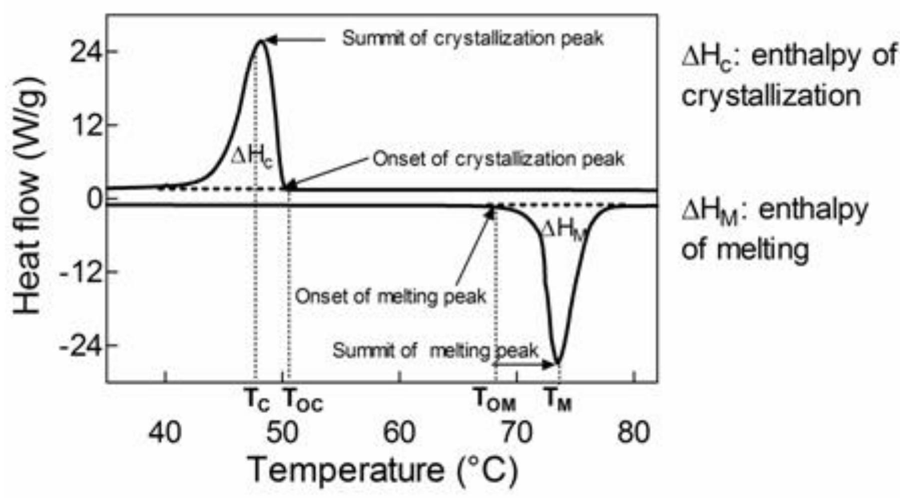

Figure 1.16 The schematic diagram of a thermogram with the common parameters measured from DSC (Marangoni and Peyronel, 2014)

from higher temperature to lower temperature and heat is released during the crystallization process. On the other hand, the heat flow for melting is from left to right where the fat is heated from lower temperature to higher temperature and heat is absorbed during the process. The summit of the peak, either the crystallization peak or melting peak, displays the highest heat flow where, at that temperature, at least half of the lipid species have gone through a phase transition. The onset temperature is defined as the temperature at which the first crystallites melt or form, which is observed as a deviation from the baseline. The area under curve corresponds to the enthalpy of the phase transition, ΔH.

1.8 LIMONENE

As part of this PhD study, the first objective was to investigate the impact of limonene substitution on the crystallization behaviour of cocoa butter and chocolate. Limonene is a terpene (Figure 1.17) that exists in D- and L- forms of isomers. It is naturally present in its D-form in various essential oils and is frequently found in food products, generally as a flavour compound. Foods containing limonene have an orange flavour and products where limonene is used include chocolate, ice cream, desserts and chewing gum. Food grade limonene is 96% to 97% pure and it is extracted from the rinds of citrus fruits (Florida, 2000). Limonene is a volatile oil and its boiling point is around 175.5°C to 176°C. Its vapour pressure is 133 Pa at 14°C and 665 Pa at 40.4°C (Program, 1990).

CH_3

H_3C CH_2

Figure 1.17 D-form of limonene

1.9 RHEOLOGICAL PROPERTIES OF CHOCOLATE

Rheology is the study of flow behaviour matter, primarily in a liquid state or as "soft solids". A good understanding of the rheological properties of chocolate is important in the manufacturing process in order to obtain a chocolate with a well-defined texture and maintain optimal sensory properties of chocolate (Servais et al., 2003). The flow properties of chocolate are non-Newtonian because the viscosity decreases with an increase of shear rate. Therefore, it is important to measure viscosity in terms of the shear rate to which it is exposed. (Beckett, 2009a).

In this thesis, steady shear rheology is considered and so it is important to understand the theory behind steady shear rheology. Viscosity is defined as shear stress over the shear rate. Figure 1.18 shows a diagram which illustrates shear. Shear stress is the shear force, *F* (*N*) per the shear area, *A* (m^2) while shear rate is defined as velocity difference, *v* (*m/s*) per distance, *h* (*m*). Shear stress is represented by the symbol of τ (tau) while shear rate is represented by the symbol $\dot{\gamma}$ (gamma dot).

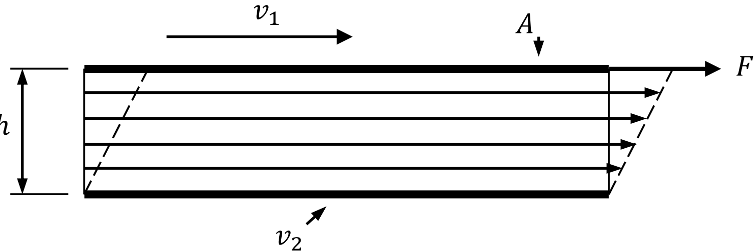

Figure 1.18 Diagram of shear to illustrate the velocity distribution of a flowing fluid (Nelson and Beckett, 1999)

The SI unit of the shear stress and shear rate are Pascal (Pa) and 1/s, respectively. When the upper plate is moved by the (shear) force F and the lower plate is stationary, there will be a gradient in the velocity (v_1-v_2) of fluid over the distance of h. Therefore, the viscosity (η) relates the shear stress (F/A) and shear rate (dv/dh) through Equation 1.3;

$$\eta = \tau/\dot{\gamma} \qquad \text{(Equation 1.3)}$$

The units of viscosity are Pascal seconds (Pa.s) or Poise (0.1 Pa.s). At any particular shear rate, the value of viscosity η, is known as the apparent viscosity. The relationship between shear stress and shear rate with the viscosity can be seen in Figure 1.19 for a Newtonian fluid. However, chocolate flows in a different way as a consequence of the interaction of the particles and this affects the flow properties. At low shear rates, the interaction between particles is strong, thus restricting the movement of the particles in the liquid which consequently increases the apparent viscosity. As the shear rate increases, the chocolate flows faster because the particles all move together with the flow rather than resisting it, so the chocolate has a lower apparent viscosity and behaves like a thinner liquid. The plot of shear stress and apparent viscosity of chocolate against the shear rate for a non-Newtonian fluid like chocolate is shown in Figure 1.20.

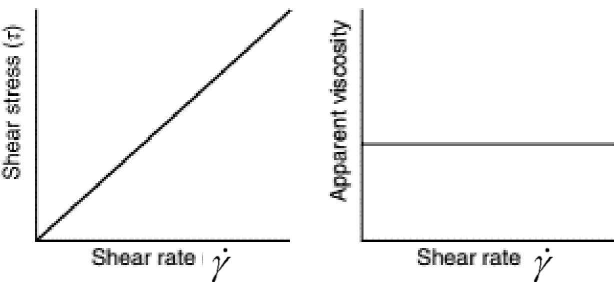

Figure 1.19 Plot of shear stress and apparent viscosity as a function of shear rate for a Newtonian fluid (Beckett, 2009a)

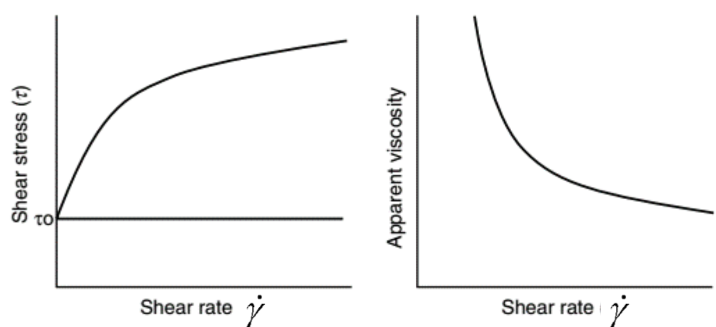

Figure 1.20 Plot of shear stress and apparent viscosity as a function of shear rate for a non-Newtonian fluid (Beckett, 2009a)

As mentioned before, chocolate is shear thinning as increasing the shear rate will reduce the viscosity. The slope of the viscosity curve decreases with increasing shear rate until a Newtonian plateau is reached. At low shear rate, the interactions among particles are high and the chocolate will not flow until a minimum shear stress is applied to start the flow. This is known as yield value or yield stress (Beckett, 2009a) and indicates the transition from elastic to viscous

deformation (Goncalves and Lannes, 2010). Yield stress is important for moulding and coating while shear viscosity is related to pumping characteristics, coating and sensory character of chocolate mass (Goncalves and Lannes, 2010). Therefore, the rheological properties of chocolate are always defined by yield stress and shear viscosity (Fernandes et al., 2013). Chocolate with an incorrect viscosity will produce chocolate with poor quality. If too low, the coating of the chocolate will be too thin during enrobing, while if too high it will produce chocolate with 'feet' as shown in Figure 1.21. Bubbles are also difficult to eliminate from a chocolate moulded tablet with incorrect viscosity. Furthermore, the viscosity of chocolate affects sensory acceptance of the chocolate. Chocolate with high viscosity will have a harder texture and give a pasty taste in the mouth (Beckett, 2008).

Figure 1.21 Chocolate with 'foot' due to incorrect viscosity (Beckett, 2008)

1.9.1 Factors affecting the flow behaviour of chocolate

The flow behaviour of chocolate is influenced by the processes and the formulations of chocolate manufacture. These include refining and conching, and the amount of fat, the amount and type of emulsifiers added and the particle size distribution of the solids, especially sugar (Schantz and Rohm, 2005).

During processing, the refining process will affect the particle size distribution of the solids (sugar and cocoa particles) in the formulation. Increasing particle size was reported to decrease the Casson plastic viscosity, Casson yield value, apparent viscosity and yield stress of dark chocolate (Afoakwa et al., 2008). The Casson model is explained in detail in Section 1.9.2. A given mass of large particles have smaller surface area compared to the same mass of small particles. Increasing the surface area will increase the amount of fat required to coat the particles or the contact point of particles to the cocoa butter (Middendorf et al., 2015, Mongia and Ziegler, 2000), thus increasing the yield stress and plastic viscosity.

Generally, the solid particles in the chocolate premix are refined to particle size below 30 μm using five-roll refiners (Beckett, 2008). The final particle size not only affects rheological, but also sensory properties. Particle size distribution is important in controlling the consistency of solid-liquid mixtures to aid pumping and mixing of

molten chocolate (Mongia and Ziegler, 2000). According to Beckett (2008), the largest particles are important for mouthfeel with respect to grittiness, but the smaller ones are more important with respect to chocolate flow properties. The texture of milk chocolate is reported to be improved by a bimodal distribution of particles with a small proportion up to 65 μm but most lower at <35 μm, although these values are influenced by product type and composition (Ziegler and Hogg, 2009). Many chocolate products have bimodal and trimodal particle size distribution (Afoakwa et al., 2007).

The conching process is the endpoint of chocolate manufacturing where chocolate mass is turned into a flowable liquid. Conching develops flavour and the final chocolate viscosity is adjusted by adding some additional cocoa butter and emulsifier. At the early stage, moisture is reduced and undesirable flavour-active volatiles are removed. Through the process of shearing and longitudinal mixing, fat in the chocolate mass agglomerates will be released and will promote interaction between the particles and the continuous phase (Afoakwa et al., 2007). The longer the chocolate is conched, the thinner the chocolate becomes. However, using a conching time which is too long will give a less significant effect in lowering the viscosity of chocolate and consequently adversely affect the cost of processing (Beckett, 2009a).

The addition of fat and emulsifier into chocolate is the most significant factor on the quality of chocolate; the rheological properties of liquid chocolate, release from mould, snap, gloss, prevention of bloom, melting properties and flavour release (Timms, 1980). Fat and emulsifier are normally added at the final stage of conching to give a significant effect on the final viscosity of the chocolate.

Cocoa butter is the only liquid ingredient in chocolate available to make the solid particles flow. As the fat content increases, the distance between the solid particles increases which causes the viscosity to be decreased (Beckett, 2009a). The effect of increasing fat is proportionately much higher on the shear viscosity than the yield value (Afoakwa et al., 2007). This is because addition of extra fat will increase the amount of free fat (Beckett, 2008) which aids the flow and decreases the shear viscosity dramatically (Afoakwa et al., 2007). The yield value is more pronounced because of the forces between the solid particles, which is closely related to the absolute distance between particles, hence is less affected by fat additions (Afoakwa et al., 2007).

Addition of surfactant also affects the flow properties of the tempered chocolate (Beckett, 2009b). Lecithin is commonly used to lower the viscosity of chocolate as it can reduce the viscosity ten times more effectively than the same amount of cocoa butter (Beckett, 2009b).

Addition of lecithin at 0.1 – 0.3% dramatically reduces the yield value and plastic viscosity of chocolate. However, at more than 0.5% lecithin increases the yield value of chocolate (Beckett, 2008, Chevalley, 1994). Lecithin has a hydrophilic head that attaches to sugar particles and its lipophilic tails point out into the fat phase of chocolate. The interaction of lecithin at the interfaces of sugar/oil helps sugar particles keep a distance from each other and helps them to move easily past one another (Beckett, 2008). This reduces the contact point of sucrose surfaces with cocoa butter which results in reduction of the amount of immobilized fat (Middendorf et al., 2015). Other surfactants like PGPR are normally used to reduce the yield stress of chocolate and has only a small impact on the viscosity (Schantz and Rohm, 2005).

1.9.2 Measurement of rheological properties of chocolate

Rheological measurement on chocolate are often carried out using rotational viscometers with concentric cylinders (bob and cup geometry) (Rao, 2014, Goncalves and Lannes, 2010). It is also possible to measure viscosity using a cone and plate geometry, however the results tend to be less reproducible than those obtained with the concentric cylinder systems (Aeschlimann and Beckett, 2000). This is because the concentric cylinder geometries are better

in controlling temperature during measurement due to larger contact of sample with the wall of the cup (Mezger, 2014).

Concentric cylinder geometries consist of a cylinder (bob) placed concentrically (coaxially) inside a cup containing a selected volume of the test fluid. In a controlled rate rheometer, the speed of the bob is controlled where the bob is rotated and its rotational speed varied either from low speed to high speed or from high speed to low speed. In controlled-stress instruments, the variable controlled is the stress (torque) and the measured quantity is the rotational speed. The resulting shear stress is measured and the viscosity data over a wide range of shearing conditions are obtained. However, most of the confectionery industry prefer to evaluate chocolate at a given rate and measure the force required to do so (Beckett, 2009a).

The recommended concentric cylinder for measuring chocolate viscosity by The International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC) is depicted in Figure 1.22. The cone shape cylinder and the Couette geometry are designed to minimize the end effect (shear) at the bottom of the cylinder. The Couette geometry provides an interface of air to minimize any significant drag on the ends of the rotating cylinder (Rao, 2014). It is also important that the chocolate moves uniformly in the gap between the bob and cup. If

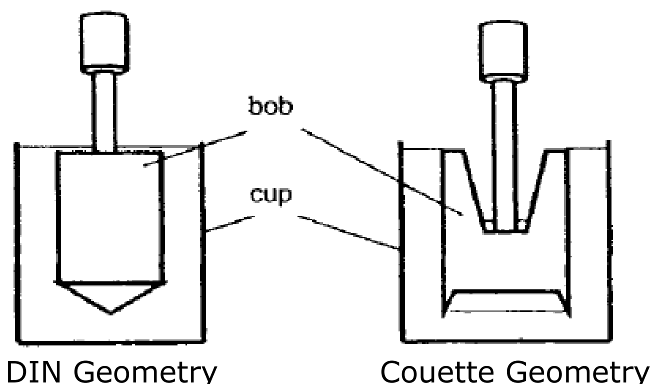

Figure 1.22 Bob and cup geometry recommended for chocolate quality control (IOCCC, 2000).

the gap is too wide, only a layer of chocolate near the moving bob will be affected. Therefore, IOCCC (2000) has recommended that the ratio of the bob diameter to the cup should be greater than 0.65.

In 2000, the International Confectionery Association (ICA) recommended measurement of stress and viscosity at shear rates between 2 s^{-1} and 50 s^{-1} using up and down curves in shear rate, preceded by a pre-shear at 5 s^{-1} for 5 min (Afoakwa et al., 2009a). The Casson model (Equation 1.4) is commonly used to characterize the rheological properties of chocolate and other food dispersions even though it was originally developed for characterizing printing inks (Rao, 2014).

$$\tau^{1/2} = \tau_C^{1/2} + (\eta_C.\dot{\gamma})^{1/2} \quad \text{(Equation 1.4)}$$

where τ is the shear stress, τ_C is the Casson yield stress, η_C is the Casson plastic viscosity and $\dot{\gamma}$ is the shear rate.

According to this model, the square root of the shear stress is plotted as a function of the square root of the shear rate which produces a straight line (Servais et al., 2003). The square of the slope is the Casson plastic viscosity while the square of the intercept is the Casson yield stress. The Casson model is a structural-based model which may be used together with experimental data to estimate the value of parameters in order to characterize the rheological behaviour of a food sample (Rao, 2014).

The Casson model however is known to not provide reproducibility at low shear rates and, as a result, the rheological data produced do not fit the Casson equation well (Aeschlimann and Beckett, 2000). At low shear rate, the Casson yield value often does not correspond to the actual value, especially when the material testing has thixotropy behaviour where this affects the reproducibility of the Casson model (Servais et al., 2003). Thixotropy refers to the continuous decrease of apparent viscosity with time under shear and the subsequent recovery of viscosity when the flow is discontinued (Mewis and Wagner, 2009). It is normally occur in low fat chocolates (Afoakwa, 2016). The Casson model has also been reported to be only suitable

for chocolate with a low amount of particles (Bouzas and Brown, 1995). Therefore in 2000, ICA recommended the use of interpolation data (calculate within the data range) for chocolate viscosity, between 2 s^{-1} and 50 s^{-1} as mentioned in the previous paragraph.

Servais et al. (2003) suggested reporting the yield stress measured at 5 s^{-1} and viscosity as apparent viscosity at 40 s^{-1}. The regression coefficient shows that the apparent yield stress at 5 s^{-1} using the flow curve was highly correlated with the true yield stress which was measured using a vane geometry. Vane geometry has long been used to measure the true yield stress as it acts uniformly on the side and ends of an equivalent fluid cylinder formed when the vane is rotated (Dzuy and Boger, 1983). However, a lower value of regression coefficient was obtained between the Casson yield stress and the true yield stress. This shows that the apparent yield stress is closer to the true yield stress than the Casson yield value with the viscosity is as repeatable as the Casson plastic viscosity and more reproducible. The yield stress produced by vane geometries (which consists of four thin blades arranged at equal angles around a small cylindrical shaft) occurs along the localized cylinder surface circumscribed by the vane. This means that the material yields within itself, so that all the problems and errors associated with slip flow on smooth surfaces are absent (Liddel and Boger, 1996). It is also suggested that thixotropy values are obtained as the difference between the viscosity at 40 s^{-1} from the ramps up and down (Servais et al., 2003).

1.10 SURFACTANTS IN CHOCOLATE

Surfactants in chocolate are very important in modifying the rheological properties of chocolate. The interaction of the surfactant with the interface between the sugar and the cocoa butter allows the high amount of sugar to flow in cocoa butter which consequently affects the rheology and tempering behaviour of chocolate (Minifie, 1989).

There are many surfactants used in the food industry but lecithin is the most common one used in chocolate. Ammonium salts of phosphatides (YN) and polyglycerol polyricinoleate (PGPR) are also used in chocolate (McClements, 2015). YN, known as synthetic lecithin, is obtained from partially hardened rapeseed oil after glycerolysis, phosphorylation and neutralization (Beckett, 2009a). Meanwhile PGPR, a product of polyglycerol-polyricinolic acid esterification, is used to reduce the yield value of chocolate as introduced in Section 1.1, in the fourth paragraph.

1.10.1 Lecithins

Lecithins are the most widely used emulsifier in chocolate as a flow aid agent since the 1930s (Beckett, 2008). Lecithin was first discovered in 1845 – 1847 by Théodore Nicolas Gobley, who isolated lecithin from egg yolk (Garti and Aserin, 2012). Lecithin is not a uniform, standard material, but a natural mixture of a series of surface/interface active components that contribute to overall emulsifying performance (Bueschelberger et al., 2014). It consists of a blend of various phospholipids and adsorbs on the hydrophilic surface of sugar particles in chocolate (Schantz et al., 2003). The first individual substance isolated from lecithin was phosphatidylcholine choline (Bueschelberger et al., 2014).

Lecithin made from soybean is the type most often used in chocolate. Commercial soybean lecithin is a complex mixture containing about 65 - 75% (w/w) of phospholipids (Scholfield, 1981). The phospholipid fraction is mainly composed of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS) and some other components (Garti and Aserin, 2012). Phospholipids are generally derived from *sn*-glycero-3-phosphate and therefore have the basic structure shown in Figure 1.23. The fatty acids in the components of lecithin may differ in chain length and saturation depending on their origin. The fatty acid attached to R2 is usually less saturated than the one attached to R1 (Bueschelberger et al., 2014).

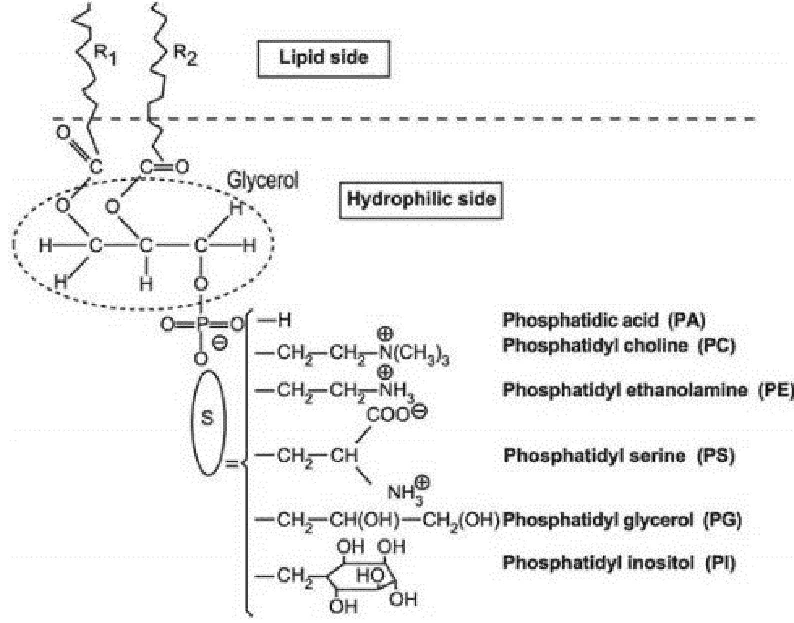

Figure 1.23 Chemical structure of lecithin (Garti and Aserin, 2012)

Phospholipids occur in all living organisms as a functional unit and can be obtained from a wide variety of raw materials (Bueschelberger et al., 2014). Plant raw materials have a low phospholipid content compared to animal raw materials. Table 1.3 shows the phospholipid compositions of some plant and animal raw materials.

Table 1.3 Average composition of plant and animal oil-free phospholipid extracts (Bueschelberger et al., 2014)

Phospholipid	% of total phospholipids					
	Soya	Rapeseed	Sunflower	Corn	Egg yolk	Milk
Phosphatidylcholine	24	25	25	30	74	27
Phosphatidylethanolamine	22	22	11	3	19	36
Phosphatidylinositol	15	15	19	16	1	
Phosphatidic acid	7		3	9		
Sphingomyelin					2	29
Lysophospholipids	3	5		5	3	
Other phospholipids	5	19			1	8

Lecithin is waste product of the oil refining process and is obtained from the degumming step. The water degumming process is carried out by mixing 1 – 3% of water with the oil at 50 – 70°C for about an hour. The phospholipids are transferred into the water phase as the addition of water to the oil hydrates the polar lipids in the oil and forms a gum with a higher specific density than the oil. After the hydration process, the gums are separated from the oil by centrifugation at 50 – 70°C. The amount of lecithin gum obtained is between 30 – 39% oil based on dry weight. Gums normally contain a maximum of 50% water, a minimum of 33% acetone insolubles (AI) and a maximum of 17% oil (van Nieuwenhuyzen, 1976). The lecithin gum then is dried to a moisture content of <1% to achieve a long time shelf life. Drying the gum for a longer time will produce severe

darkening due to Maillard reaction of the adherent sugars and the Amadori reaction between sugars and PE (van Nieuwenhuyzen, 1976).

The crude lecithins produced are not yet ready for food application. They undergo further processing such as microbiological standardization, enzymatic or chemical modification, de-oiling to remove oil and fractionation to obtain certain emulsifiying properties of lecithin (Bueschelberger et al., 2014).

Lecithin as Flow Aid Ingredient in Chocolate

Lecithins have long been used in chocolate to modify the viscosity, normally at 0.1 – 0.5% of the total chocolate ingredients (Beckett, 2008). The phospholipids align their hydrophilic head onto the surface of sugar crystals with their lipophilic tail oriented into the oil phase, which make the sugar crystals act as if they are‘lipophilic’ at their outside. This kind of molecular layer acts as a lubricant and therefore reduces the internal friction and viscosity (Bueschelberger et al., 2014).

The mechanism of how lecithin interacts with sugar particles can be seen in Figure 1.24. The strong adsorption of lecithin to the sucrose particles has been shown by Ziegler et al. (2003) and Johansson and Bergenstahl (1992a), and explains why lecithin is such a good emulsifier in reducing the viscosity of suspensions. However, when lecithin is added at higher levels (above 0.5%), the excess lecithin will attach at the outer layer of another lecithin through their lipophilic tails and form micelles or a bilayer around the sugar (Beckett, 2008). This causes a reverse effect to the flow behaviour of suspensions and increases their yield stress. Due to this, PGPR is added in a small amount (0.2 – 0.3% of the total chocolate ingredients) to a synergistic effect which reduces the viscosity and yield stress of chocolate to a relatively low value.

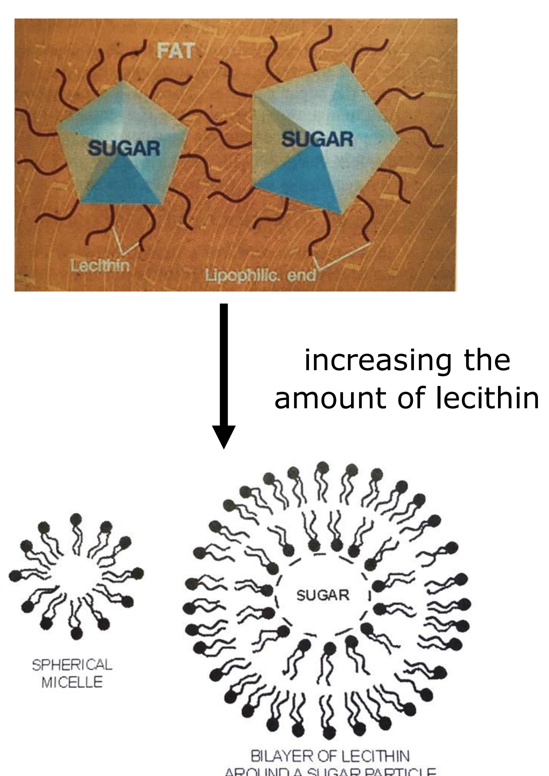

Figure 1.24 Adsorption of lecithin on sucrose particles in oil (Beckett, 2008)

1.10.2 Polyglycerol polyricinoleate

Polyglycerol polyricinoleate (PGPR), also known as Admul-WOL, was originally developed for use in the baking industry. PGPR is a product of esterification of condensed castor oil fatty acids with polyglycerol. PGPR is a non-ionic emulsifier with a high lipophilic character, and is thus more likely to be soluble in a fat matrix (Christiansen, 2014). PGPR is generally recognized as safe by FDA (FDA, 2006). PGPR has unique surface active properties where it can lower or remove the yield value of chocolate without a significant effect on viscosity (Schantz and Rohm, 2005, Rector, 2000a).

Synthesis of PGPR starts with the synthesis of polyglycerol and the synthesis of polyricinoleate done separately. Ricinoleic acid (12-hydroxy-9-cis-octadecenoic acid) is a hydroxy- and mono-unsaturated fatty acid (Figure 1.25) that naturally occurs in the mature castor plant (*Ricinus communis* L.) of the family Europhobiaceae (Bastida-Rodríguez, 2013). About 90% of the fatty acids in castor oil are ricinoleic acid, while the remaining 10% are oleic acid, linoleic acid, stearic acid and small amounts of other fatty acids (Christiansen, 2014).

PGPR is synthesized through direct esterification between polyglycerol and polyricinoleate. Polyglycerol and polyricinoleate are mixed together with added catalyst and heated under vacuum until reaction

temperature (180 – 220°C) is reached. The heating is maintained at the temperature until a certain level of acid value (Christiansen, 2014) is reached. The esterification reaction is depicted in Figure 1.26. PGPR is a highly viscous liquid of yellow to light brown colour, with a neutral taste and smell, and is soluble in vegetable oil. The melting point is below 0°C and the boiling point is unknown, due to molecular structure rearrangement, breakdown or decomposition before reaching the boiling temperature (Christiansen, 2014). PGPR is surface active due to the lipophilic character of polyricinoleate and the hydrophilic character of polyglycerol. The HLB value was reported to be in the range of 1 – 5 (Griffin, 1949), thus it has the property to stabilise water-in-oil emulsions.

Figure 1.25 Ricinoleic acid

Figure 1.26 Synthesis of PGPR (Christiansen, 2014)

The use of PGPR in food products requires a declaration on the product labelwhich is normally shown as E-476. Consumers are becoming increasingly concerned about additives in their food which have “E numbers” or have with a chemical sounding name. JECFA, in 1974, and EC/SCF, in 1979, set the maximum daily intake for PGPR to be 7.5 mg/kg body weight/day, however in 1992, the UK FAC estimated that the maximum per capita mean daily intake of PGPR was only 2.64 mg/kg body weight/day (Wilson et al., 1998).

PGPR as Flow Aid Ingredient in Chocolate

Until recently, little was known about how PGPR gives the macroscopic effect of reducing the yield stress of chocolate without affecting significantly the viscosity (Middendorf et al., 2015). Most manufactures claimed that PGPR forms a monolayer around the sugar particles (Dieffenbacher and Martin, 1987). The adsorption of PGPR on sugar particles was demonstrated to be not as strong as that of lecithin so it was concluded that PGPR absorbs on the surface of sucrose particles in a loosely packed monolayer (Ziegler et al., 2003). The polar moieties of PGPR (hydroxyl and carbonyl groups) were suggested to not form a polar “head” as in the case of lecithin, but are scattered throughout the complex and branched structure of the PGPR molecule. Therefore, this causes PGPR to adsorb “flat” and form a film on the surface of the sucrose particles with the lipophilic tail pointing into the oil phase (Ziegler et al., 2003). The mechanism of

how PGPR adsorbs at the interface of sugar/oil was discussed in more detail with the result obtained in this study in Chapter 4.

1.10.3 Ammonium phosphatides (YN)

Another emulsifier was developed by Cadbury Brothers Ltd. to avoid the off-taste in milk chocolate caused by lecithin (Pedersen, 2012). Ammonium phosphatides (YN) was developed from hydrogenated rape seed oil as an alternative to soya lecithin (Norn, 2014) and its structure can be seen in Figure 1.27. YN acts in the same way of as lecithin but exhibits a more constant composition and efficiency in affecting the flow properties of chocolate (Schantz and Rohm, 2005). Due to a high amount of trans fatty acids (TFA) in YN, its use is limited to 0.4%. When exceeding 0.5 – 0.6%, YN could create serious tempering problems in chocolate due to β′ crystallization provoked by trans fatty acids (Pedersen, 2012). However, increasing the concentration of YN does not lead to an increased yield value as is shown with lecithin. Increasing the amount to more than 0.5% continues to reduce the plastic viscosity while keeping the yield value at the same level (Pedersen, 2012).

CH_2-O-R_1

CH-O-R_2

O

CH_2-O-P-O-NH_4

OH

Figure 1.27 Chemical structure of ammonium phosphatide (Pedersen, 2012).

1.11 GREEN LEAVES TISSUES FOR DEVELOPMENT OF A NOVEL FLOW AID

Green leafy vegetables are important for human diet as a source of vitamins, fibre and phytonutrients. These vegetables are also rich in β-carotene, ascorbic acid and minerals. There are a wide variety of leafy vegetables but the most common are lettuce, spinach, and kale. A lot of the research focussing on the consumption of green leafy vegetables have claimed that they are high in antioxidant activity (Oboh and Akindahunsi, 2004, Oboh, 2005). The increase in awareness among consumers on the importance of eating green leafy vegetables as a way of preventing diseases has led to the increased production of fruits and vegetables. As a side effect, this has contributed to the increase of food waste around the world as these vegetables are perishable. One of the strategies employed to reduce

waste problems is to use fruit and vegetable waste as livestock feed (Wadhwa and Bakshi, 2013). Spinach is included in this category of waste but it is a plant often used in research related to dark green leafy vegetable waste due to its all year round availability.

Spinach (*Spinacia oleracea*) is an edible plant in the family of Amaranthaceae native to central and western Asia. Spinach is cultivated in many countries around the world but in the United States the most abundant producer is California and Arizona (Simko et al., 2014). It grows up to 30 cm tall, is cultivated throughout the year and it is not an expensive vegetable to grow (Lee et al., 2002). Spinach is eaten raw or cooked. It is considered to be very nutritious due to the high content of minerals, vitamins and pigments it contains (Bhattacharjee et al., 1998). Spinach also has a high amount of carotenoids (yellow, orange or red), which are masked by green chlorophyll (Yamauchi et al., 1986). Carotenoids have been proven to be more stable to oxidation during thermal processing as compared to chlorophylls (Shiota et al., 2010).

1.11.1 Structure of spinach leaves

Leaves are the major sites of photosynthesis. Leaves are connected to the plant by petiole. The structure of leaves can be seen in Figure 1.28. The top and bottom surfaces of the leaves are formed from a layer of epidermal tissue which is covered by a waxy cuticle that keeps the leaves from drying out and resist pathogen attacks. Unfortunately, it also prevents gas exchange because the cuticle is not gas permeable. However, the lower epidermis contains openings called stomata that allow gases to diffuse into and out of the leaves. Each stoma has two guard cells that regulate its opening and

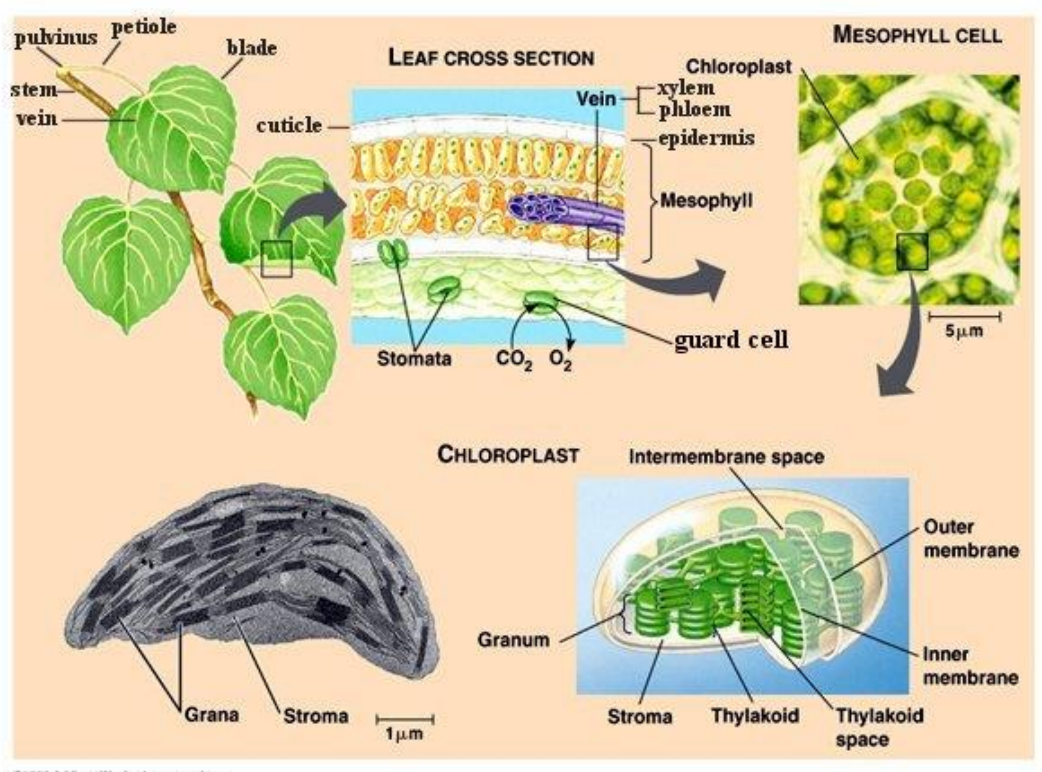

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Figure 1.28 Leaves structure (Universe-review, 2016)

closing(Universe-review, 2016). Carbon dioxide enters the leaves, and oxygen exits. The leaves also need water to survive and this is absorbed by the roots and is delivered to the leaves in veins.

The chloroplast is located mainly in the mesophyll, the interior tissue of the leaves. A typical mesophyll cell has about 30 – 40 chloroplasts. Chloroplasts in land plant like spinach are generally lens-shaped, 5 – 8 μm in diameter and 1 – 3 μm thick (Wise and Hoober, 2007). Chloroplasts have a double-membrane envelope consisting of an outer envelope and an inner envelope. In addition to the outer and inner envelope membranes, chloroplasts have an extensively developed internal membrane system, the thylakoid membrane, where photochemical and electron transport reactions take place (Kobayashi, 2016). In the thylakoid membrane, thousands of proteins, pigments and photosynthetic cofactors are embedded in the bilayer formed by glycerolipids (Kobayashi, 2016).

The thylakoids form a continuous 3-D membrane network that surrounds a single chamber, the thylakoid lumen (thylakoid space). Thylakoids control the transport of metabolites, lipids and proteins into and out of chloroplasts. Thylakoids normally form stacks of disks which are called grana. Mature chloroplasts may contain 40 – 60 grana stacks with diameters of 0.3 to 0.6 μm. Stroma is the aqueous matrix and stroma thylakoids are the non-stacked thylakoids. The

stroma thylakoids are in direct contact with the stroma (Staehelin, 2003). The thylakoid lipids provide a lipid bilayer matrix for photosynthetic protein-cofactor complexes, support electron transportation and avoid free generation of a proton motive force via photosynthetic activities (Kobayashi, 2016). The amount of polar lipids in spinach thylakoids is very similar to the polar lipid composition of the inner membrane of the chloroplast envelope: 78% galactolipids, 15.5% phospholipids and 6.5% sulfolipids (Block et al., 1983).

1.11.2 Lipids in spinach leaves

Lipids are very important in all living cells for biochemical and development systems. Membrane lipids play an active role in organization, expansion and maturation of the plant cell (Benson, 1964). Lipid molecules in cell membranes are amphiphilic, which means they have a hydrophilic head and a hydrophobic tail (Alberts et al., 2002). Most lipids are organised in the form of a bilayer, surrounding cells in order to separate the interior of the cell from the environment (Dörmann, 2013). Phospholipids are the major membrane lipids in bacteria and animals, however, in photosynthetic plants and cyanobacteria, galactolipids are highly abundant (Dörmann, 2013). The galactolipids in plants are different from the galactolipids in animals as the sugars are linked to diacylglycerol

(glycoglycerolipids) while in animals the sugars are bound to a ceramide moiety (glycosphingolipids) (Dörmann, 2013).

Spinach, a photosynthetic plant, is also claimed to be high in galactolipids (Douce, 1974, Chen, 2016). The polar lipids are concentrated in the chloroplast and the main lipids reported are monogalactosyl diacylglyceride (MGDG), digalactosyl diacylglyceride (DGDG), and sulfoquinovosyl diacylglyceride (SQDG). Besides the galactolipids, the main phospholipids found are phophatidylglycerol (PG) and phosphatidylcholine (PC). PG is found in thylakoid membranes while PC is found in the chloroplast envelope (Mazliak, 1977b). Galactolipids are neutral lipids while SQDG and PG each carry one negative charge (anionic) in their head groups (Dörmann and Benning, 2002). Chloroplasts are surrounded by two membranes, of which the inner envelope has a glycerolipid composition which is very similar to that of the thylakoid. The lipid composition of the outer membrane of spinach chloroplasts has been found to be 47% phospholipids, 46% galactolipids and 6% sulfolipids, while the inner membrane has been found to contain 15% phospholipids, 79% galactolipids and 5% sulfolipids (Block et al., 1983). MGDG is more abundant in the inner envelope and thylakoid while DGDG is more abundant in the outer envelope. In addition, PC is relatively high in the outer envelope of the chloroplast but low in the inner envelope and thylakoid (Dörmann, 2013). On comparison, whole leaves was

reported to contain more phospholipids than the chloroplast (Wintermans, 1960).

The structure of glycerolipids in spinach is shown in Figure 1.29. Since the head group of MGDG consists of only one galactose moiety compared to two galactose moieties in the case of DGDG, the size of the head group is comparatively small. Based on the shape concept of lipid phase behaviour a lipid molecule with a small head group and bulky diacylglycerol moiety will exist in a conical shape and not form bilayers in pure lipid-water mixtures (Webb and Green, 1991). Instead, pure MGDG normally forms inverted hexagonal-II or cubic phases in water (Nishihara et al., 1980, Quinn and Williams, 1983). DGDG on the other hand has a larger sized head group and was reported to form cylindrical shapes in pure lipid-water mixtures (Chu et al., 2009). Therefore, DGDG can form bilayers, and are similar in shape to many phospholipids. The shapes of lipid phase behaviour are illustrated in Figure 1.30.

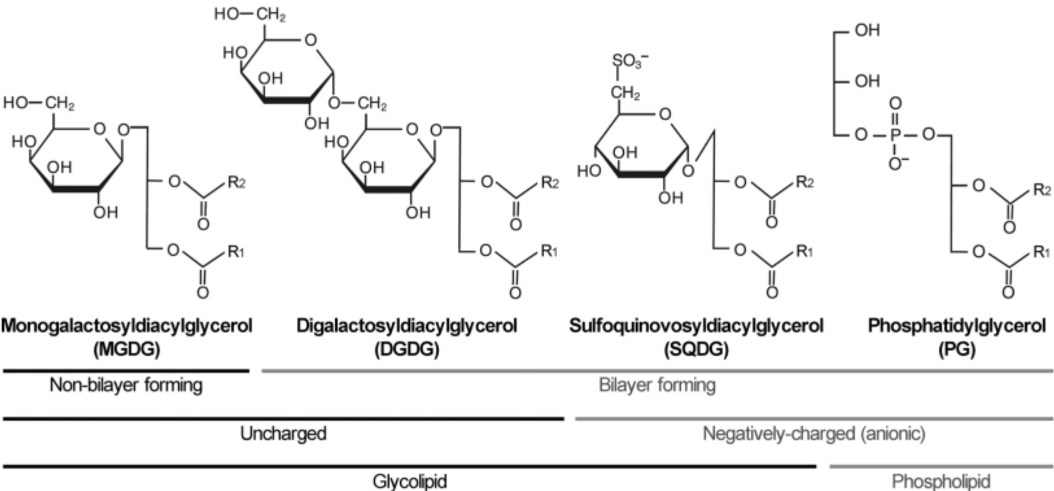

Figure 1.29 Structure and characteristic of glycerolipids in the chloroplast. R_1 and R_2 denote hydrocarbon chains of fatty acids (Kobayashi, 2016)

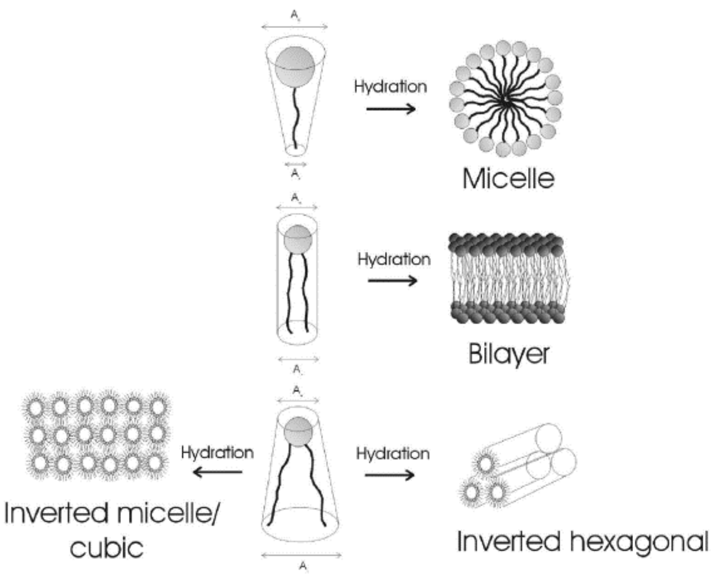

Figure 1.30 Molecular geometry of lipids. ‘A’ is the cross sectional area of hydrophilic head and hydrophobic tails (Hafez and Cullis, 2001)

However, increasing the chain length of fatty acids and the unsaturation number also can lead to the formation of an inverted hexagonal phase (HII) (Jouhet, 2013). They also influence the ability of polar lipid molecules to pack against one another, thereby affecting the fluidity of the membrane (Alberts et al., 2002).

Longer tailed lipids normally have more area to interact with one another thus increasing the strength of the interaction (by Van der Waals forces) between lipid molecules which consequently decreases the lipid mobility. Therefore, at a given temperature, a short-tailed lipid will be more fluid than an otherwise identical long-tailed lipid. However, an insertion of a single *cis* double bond into a fatty acid chain can cause many degrees of reduction in transition temperature from liquid-to-gel phase transition (Phillips et al., 1972). An unsaturated double bond can produce a kink in the alkane chain leading to the disruption of regular packing (Niemelä et al., 2006). Linolenic acids (18:3) are found in in high numbers in MGDG and DGDG. The trienoic acids usually constitute more than 80-90% of the acyl groups found in MGDG and DGDG. Lipids high in linolenic acid exhibit greater solubility and lower melting points than other lipids (Benson, 1964). The membrane in which they lie, therefore, probably possess a liquid lipid phase rather than the quasi-crystalline array anticipated in a simple compressed monolayer of saturated surfactant (Benson, 1964). Therefore, the high amount of trienoic acid found in plants is very important in maintaining membrane fluidity at low

temperature (Andersson and Dörmann, 2009, Gounaris et al., 1983a). Besides, fatty acids with three double bonds (for example α-linolenic acid) are the substrate for lipoxygenase reaction that introduces a hydroperoxy group into the acyl chain (Farmer and Ryan, 1992). The lipoxygenase pathway leads to the synthesis of several important compounds involved in plant defence (Dörmann and Benning, 2002).

SQDG and PG on the other hand contain a comparatively larger proportion of more saturated fatty acids. These two acidic lipids are present in relatively low proportions and have been reported to account for about 15 mol% for SQDG and 5 mol% for PG (Gounaris et al., 1983b). In the plant Arabidopsis, SQDG is used for functional and conformational maintenance of the PSII (photosystem II) complex (Sato, 2004). SQDG is replaceable under nutrient-sufficient conditions in maintaining the amount of total anionic lipids in the photosynthetic membranes when PG content is decreased (Yu et al., 2002). SQDG and PG play specific roles in maintaining the structural and functional integrity of the PSII complex, while PG also contributes to the biogenesis of the Chl-protein complexes such as the PSI complex (Sato, 2004). Their presence is important for the structure of the lipid ensemble in aqueous media (Quinn, 2012).

During photosynthesis, photosystem II uses light energy to oxidize two oxygen molecules. During the process, four electrons are removed from water molecules and are transferred to PSI through cytochrome *bf* (Miles, 2003), as illustrated in Figure 1.31. PSI optimally absorbs photons of a wavelength of 700 nm while PS II absorbs photons of a wavelength of 680 nm.

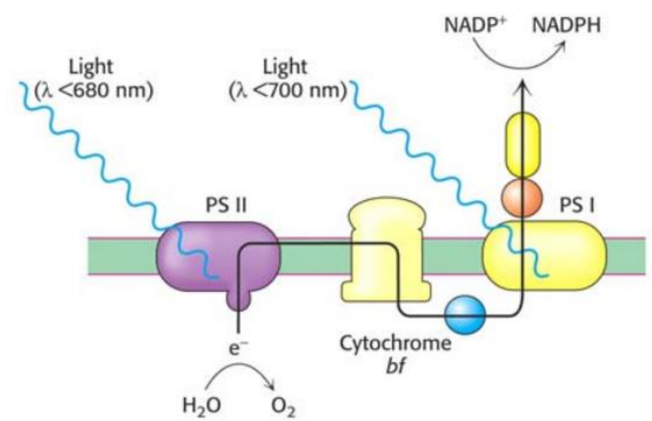

Figure 1.31 Photosystem I and II (Miles, 2003)

1.11.3 Application of glycolipids as a flow aid

Glycolipids are naturally surface active lipids found in plants. As mentioned before, glycolipids are abundant in spinach dominated by MGDG and DGDG. Published research showed that MGDG and DGDG extracted from spinach during digestion is used in the stomach because of their ability as an emulsifying agent in order to delay lipid

digestion (Chu et al., 2009, Rayner et al., 2011a, Rayner et al., 2010). MGDG and DGDG extracted from other plants like wheat have been reported to have a better potential as a bread improver compared to a commercial surfactant like lecithin (Selmair and Koehler, 2008). In addition, the potential seen in oat lipids as a flow enhancer in reducing the viscosity of chocolate was also claimed to be related to the action of DGDG in the mixture (Evans et al., 1991).

Therefore, part of this PhD research was to focus on the potential of total spinach lipids to act as a flow enhancer in chocolate using their natural as surface active lipids. However, the roles of PG, SQDG and other phospholipids were also included in the discussion in order to more fully understand the mechanisms affecting the flow properties of chocolate.

2 IMPACT OF LIMONENE ON THE BLOOM OF COCOA BUTTER AND CHOCOLATE

The addition of limonene to cocoa butter has shown a positive result in lowering the viscosity of chocolate. However, in order to implement a chocolate formulation with the incorporation of limonene, it is important to investigate the quality aspects such as bloom. Therefore, the impact of limonene on the crystallization and melting behaviour were studied and reported in this PhD.

2.1 INTRODUCTION

Chocolate is a highly filled composite material formulated from sugar, cocoa liquor and cocoa butter with added surfactant. Milk fat and cocoa butter equivalent such as shea butter may be chosen to replace some of the cocoa butter to lower the cost. The surfactants lecithin, polyglycerol polyricinoleate (PGPR) and ammonium phosphatide, or a combination thereof, is also often used. The addition of the surfactant enhances the flowability of molten chocolate.

If cocoa butter is reduced the chocolate will reach its maximum packing fraction. At this point, the chocolate will have a high viscosity which has a negative bearing on processing properties and eating quality is affected; a harder texture and slow to melt in the mouth

has been reported (Beckett, 2001). One patented approach to overcome these negative impacts has been to replace up to 5% of cocoa butter with limonene (Beckett, 1999). Limonene is a terpene which can be found in citrus oils. It exists in either the D- or L- form; the D-form is the most widely used form in commercial essential oils. Limonene imparts an orange flavour to the food products into which it is added.

The viscosity reducing effect of limonene replacement of cocoa butter at a level of up to 3% has previously been reported (Do et al., 2008). Limonene was claimed to lower the viscosity of fat reduced chocolate significantly but produces a chocolate with a soft texture. The resultant behaviour was claimed to be due to lower amounts of crystal being formed. Indeed, limonene did accelerate the phase transformation of cocoa butter from Form V to Form VI, indiscriminately of whether the cocoa butter was tempered or not (Ray et al., 2012). Accelerated phase transition is often associated with the development of fat bloom, a negative quality attribute normally presenting itself as a colour change (whitish appearance) and loss of gloss.

As outlined in Chapter 1, bloom describes the whitish appearance of the surface of a chocolate (Lonchampt and Hartel, 2004) which may originate from the migration of sugar and/or fat from the centre to

the surface and recrystallization at the surface. In light of evaluating the impact of limonene on bloom formation only fat bloom was considered here. Fat bloom is due to the migration of less stable fat crystal polymorphs to the surface of chocolate during the transition to more stable crystal polymorphs. It often occurs as a result of exposing chocolate to temperatures above the melting temperature of some of the fat crystals, as may happen in the distribution chain or the consumer's home. The melted fats will diffuse to the surface of the chocolate and recrystallize to form a higher polymorphic form of crystal. Bloom can be largely prevented through correct tempering during the chocolate manufacture process ensuring the cocoa butter crystallises in Form V however, even a well-tempered chocolate can develop bloom as a result of slow transition from Form V to Form VI during prolonged storage (Kinta and Hatta, 2012).

The aim of the research reported in this thesis chapter was to evaluate the impact of limonene on the bloom of cocoa butter and on the bloom of tempered chocolate where seed crystal addition was chosen as the method of tempering. Chocolates were prepared with cocoa powder as cocoa ingredient instead of the commercially more commonly used cocoa liquor. This approach was chosen for ease of accessibility of cocoa powder and formulation design with regard to substituting cocoa butter with limonene. Chocolates were formulated at 30 g cocoa butter/ 100 g product and substitution level was restricted to 2 g limonene/ 30 g cocoa butter in cocoa butter and chocolate samples

otherwise the intensity of the orange flavour would be too strong to be commercially relevant. Samples were exposed to temperature cycling during storage to initiate bloom faster than during conventional storage. Development of bloom was quantified by measuring the whiteness index on the surface of the cocoa butter and chocolate samples. State of temper after sample preparation and changes in the melting temperature during storage was quantified through differential scanning calorimetry (DSC). Storage induced changes in the polymorphic form of the cocoa butter crystals were identified through X-Ray Diffraction analysis (XRD).

To the best of the author's knowledge, no research paper on the effect of limonene addition on the bloom of cocoa butter and chocolate have thus far been published and it was the objective of the research reported here to close this gap. The outcome of this study is essential if one were to consider the commercial application of this fat reduction strategy for chocolate and chocolate based confectionery.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Cocoa butter, cocoa powder and sunflower lecithin were supplied by ADM (Hull, UK) while soy lecithin and MyCryo Form V cocoa butter seed crystals were donated by Barry Callebaut (Banbury, UK). Sunflower lecithin was used in cocoa butter while soy lecithin was used in chocolate. Food grade limonene (97% pure) was a gift from FD Copeland and Sons Ltd (London, UK). The sugar ingredient was selected to be icing sugar due to its smaller particle size compared with granular or castor sugar, and was bought from a local supermarket. All ingredients were of standard factory product quality.

2.2.2 Preparation of limonene containing untempered cocoa butter blends

Cocoa butter:limonene blends were prepared at the three different weight based ratios of cocoa butter:limonene of 30:0 (0% limonene), 29:1 (1% limonene) and 28:2 (2% limonene) taking into account that chocolate in this study was formulated at 30 g cocoa butter/ 100 g and that limonene was limited to 2 g/ 100 g chocolate as aforementioned.

Firstly, cocoa butter was melted at 50°C overnight using an oven to erase all thermal memory. Limonene was then added directly to the cocoa butter, mixed thoroughly with a spatula and immediately transferred into square chocolate moulds (35 mm x 35 mm x 5 mm) and rectangular XRD sample holders (10 mm x 15 mm x 1 mm). Samples with the ratio of 28:2 of cocoa butter:limonene were moulded into a small aluminium foil cup (about 30 mL of size) as the samples were otherwise too difficult to be de-moulded due to their fragile soft texture. All samples were immediately transferred to an incubator at 7°C and kept at this temperature for one hour before being de-moulded, wrapped in aluminium foil and placed into an airtight plastic container. Samples were then stored at -18°C for 5 days to stop any crystal growth. They were then thawed overnight at room temperature before being transferred into an incubator set to cycle temperature between 20°C and 29°C changing temperature every 12 hours. Analyses were carried out on the day the incubator storage started and then every seven days for further three weeks of storage. Samples are labelled "Week 0" and "Week 1, 2 or 3" respectively in the results section.

2.2.3 Preparation of limonene containing seeded chocolate blends

The addition of limonene into chocolate was based on the same ratio of limonene to cocoa butter chosen for the cocoa butter samples. Icing sugar was incorporated at 41.5 g/ 100 g, followed by cocoa powder at 20 g/ 100 g and lecithin at 0.5 g/ 100 g. All ingredients were mixed together at 50°C for 4 hours using a household food processor with temperature control (Thermomix, Vorwerk, Ascot, UK). While mixing, limonene was added immediately after the temperature controller was switched off. Once the temperature reached between 32 – 34°C, details below, the cocoa butter seeds crystals were added at 1 g/ 100 g chocolate and mixed continuously for 4 min at 200 to 300 rpm in order to ensure that the seed crystals were well mixed in. For the seeding temperature of the 30:0 blend, the seed crystal supplier's recommendation of 34°C for dark chocolate was followed and seed crystals were added between at 33 – 34°C. As the addition of limonene thins the chocolate mixture, the slightly lower temperature window of 32 – 33°C was chosen as the seeding temperature for the 29:1 and 28:2 blends. The seeded chocolate was then poured into square plastic moulds (38 mm x 38 mm x 8 mm) before being placed into an incubator (MIR-153, Sanyo Electric Biomedical Co., Bunkyoku, Tokyo, Japan) and kept at 10°C for 30 min to set. The temper status was evaluated immediately after setting using DSC as described in the Section 2.2.6. The chocolate was then de-moulded, sealed into an aluminium pouch and aged at 20°C for one week in order to accelerate the formation of higher stable polymorphs. After that, the same accelerated shelf life trial described for the cocoa butter samples

was carried out and the sample age of “Week 0” refers to the beginning of this storage stage.

2.2.4 Whiteness index measurement

The whitish appearance on the surface of the cocoa butter and the chocolate samples, or bloom, was quantified through the whiteness index (WI) as suggested in literature (Bricknell and Hartel, 1998, Sonwai and Rousseau, 2006, Sonwai and Rousseau, 2008). This method consists of measuring the surface colour and a Hunter Lab Ultrascan Colorimeter (Hunter Associates Inc., Reston, USA) was used after calibration with white and black glass standards. Two spots were scanned for each of four replicate samples to obtain the values of L, a and b, which were automatically calculated by the instrument’s software. L indicates lightness on a scale from 0 – 100 where 0 is black and 100 is white. The values of a and b do not have a scale. A positive value of indicates red and a negative value of a is green. Positive b is yellow and negative b is blue (HunterLab, 2012). WI is based on the values of L, a and b and defined by Equation 2.1 (Briones and Aguilera, 2005).

$$\mathrm{WI} = 100 - [(100 - L)^2 + a^2 + b^2]^{1/2} \qquad \text{(Equation 2.1)}$$

2.2.5 X-Ray powder diffraction

X-Ray powder diffraction (XRD) patterns were acquired using an X-Ray Diffractometer (D5005, Bruker, UK) at room temperature (20 – 22°C). The radiation was monochromated copper K alpha (CuKα) with a wavelength of 1.5418 Å. A slit focus reflection geometry was used and scans were run over 2θ values between 3 and 38° at 0.05° intervals with a scan time of 2.5 s per interval. This protocol has previously been applied to limonene containing cocoa butter (Ray et al., 2012).

While cocoa butter samples were directly scanned as moulded into the XRD sample holders, chocolate samples required additional sample preparation and a published method with slight modification was followed (Cebula and Ziegleder (1993). The following steps are necessary to remove the sugar from the chocolate sample as its presence would overlay with the diffraction pattern of the cocoa butter rendering data interpretation difficult, if not impossible. Utilising a knife the chocolate was chopped into small pieces, with largest dimensions of 0.5 – 1.5 mm or less, which were placed into cold water a ratio of at least 1:100 (w/v) of chocolate:water. The mixture was then mixed vigorously for about 5 minutes and left to stand for at least 2 hours for the sugar to dissolve. The mixture was then filtered to remove the water and the undissolved material was subsequently left at room temperature until most of the water was evaporated. Finally, the leftover material was pressed into rectangular XRD sample

holders (10 mm x 15 mm x 1 mm) and the surface levelled with a blade. The changes in XRD patterns were observed every week for the course of the three weeks of storage.

2.2.6 Differential scanning calorimetry (DSC)

DSC measurement was only carried out on the chocolate samples to evaluate the thermal behaviour of the chocolate during their storage and to check state of temper immediately after sample solidification. All DSC analyses were conducted using a Mettler Toledo DSC Model 823e calorimeter (Mettler Toledo, Zurich, CH).

The state of the temper of the seeded chocolate was evaluated based on the 30:0 chocolate blends since no published literature on the impact of limonene on the DSC melting pattern of cocoa butter in reference to the tempering status was found. Therefore, samples with limonene that showed the same pattern of DSC curves as the 30:0 chocolate blend were considered well-tempered. After setting at 10°C for 30 min the seeded 30:0 chocolate blend was evaluated based on the DSC melting profile following published protocol (Svanberg et al., 2013). Approximately 15 mg of sample was placed into an aluminium pan which was then hermetically sealed. An empty pan was used as

reference. Samples were loaded at 10°C, held for 3 min at this temperature and then heated to 50°C at 4°C/ min.

The thermal behaviour of the three chocolate blends during storage was evaluated on a weekly basis by means of DSC melting curves following published protocol (Fessas et al., 2005). About 15 mg of sample were hermetically sealed into an aluminium pan and loaded into the DSC at 20°C. The temperature was then lowered to 15°C at 10°C/ min, held at this temperature (15°C) for 5 min followed by an increase to 50°C at 2°C/ min. The melting curves were analysed to evaluate the impact of limonene on the melting behaviour of the chocolate samples.

2.2.7 Statistical Analysis

The results of the whiteness index were statistically analysed to compare the means of the WI between the weeks of storage for each limonene concentration using one-way ANOVA. Significant differences between samples were analysed using Tukey HSD (Honestly Significant Different) multiple comparisons test at 95% significant level. The software used for this statistical analysis was IBM SPSS Statistics 22.

2.3 RESULTS AND DISCUSSION

The impact of limonene on bloom formation was investigated on cocoa butter:limonene blends and on chocolate:limonene blends, after chocolate:limonene blends were evaluated for temper status to ensure all chocolates were well-tempered. To accelerate bloom formation, all blends were exposed to cycled temperature storage during which the samples were visually inspected on a weekly basis and the whiteness index measured to assess the amount of bloom formed. As the root cause of fat bloom, the samples were also inspected for polymorphic changes through thermal analysis and the acquisition of XRD patterns.

2.3.1 Temper status of the seeded chocolate:limonene blends

Tempering is the most crucial part in making good quality chocolate. In this research, in order to control the crystallization, samples were seeded to produce a well- tempered chocolate by providing nucleation sites for the rapid growth of crystals (Svanberg et al., 2013). Figure 2.1 shows the melting thermograms of the chocolate samples prepared at different percentages of limonene substitution. The evaluation of tempering was based only on the melting peak produced by the chocolate with no limonene substitution. As the seeding

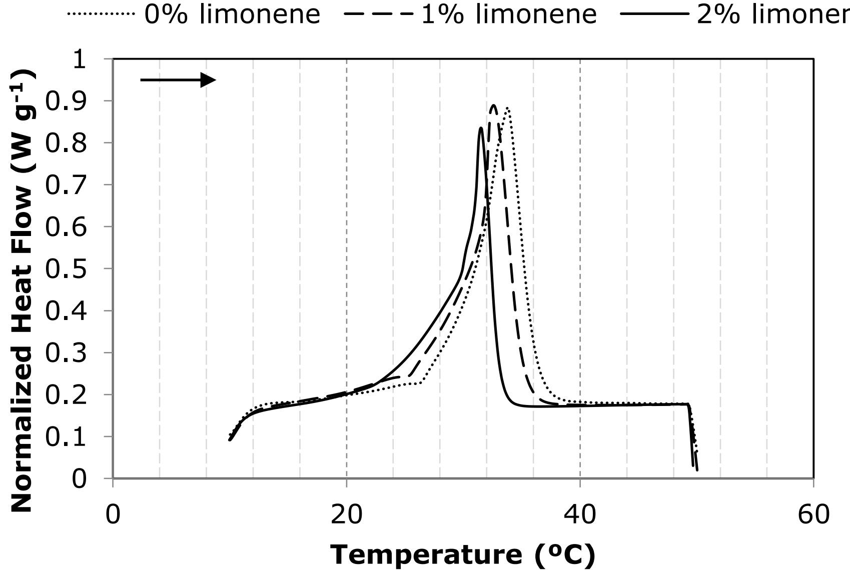

Figure 2.1 Thermograms of DSC showing the curves of tempering evaluation of chocolate-limonene based on the thermal peak of the Control sample when heating to 50°C at 4°C/ min

method by Svanberg et al. (2013) was followed, the sample with 0% limonene was considered well-tempered if the melting curve showed a slope similar to the slope of the curve reported by these authors as reproduced in Figure 2.2. A well-tempered seeded chocolate was described as being mostly Form V with a peak maximum temperature of 32.4°C (Svanberg et al., 2013). As indicated in Figure 2.1, the peak maximum temperature of the sample with 0% limonene was 33.9°C. Even though this was slightly higher than the temperature reported in the reference, it is within the melting temperature range of 33 – 34°C reported by Wille and Lutton (1966) for Form V of

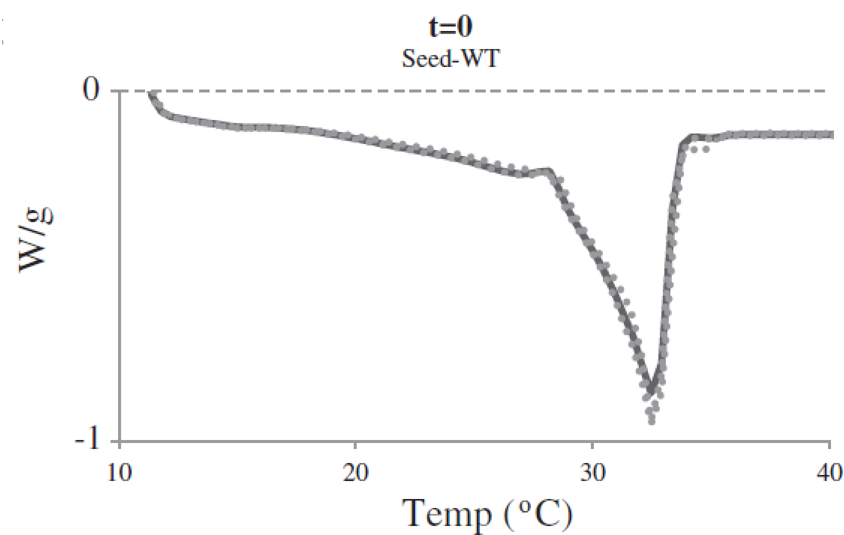

Figure 2.2 DSC melting curves of well-tempered seeded chocolate directly after solidification. The melting peak was at 32.4°C (Svanberg et al., 2013).

cocoa butter. Besides, the curve is also showing a similar, narrow pattern, which indicated that the samples were in a generally uniform polymorphic form (Form V). The melting curves of the chocolate samples with limonene substitution shown in Figure 2.1 are of similar shape but the peak melting temperature shifted to a lower value with increasing limonene substitution.

The temperature of the onset of melting also reduced as the limonene concentration increased. The onset of melting was easily identified for 0% and 1% limonene by the small "kink" when the curve started to increase with the temperature rise. However, it was difficult to

determine the onset of melting temperature for 2% limonene containing chocolate as no “kink” appeared. The smooth curve at the beginning of melting is similar to that found in under-tempered chocolate (Svanberg et al., 2013). However, the peak was not as broad as shown in under-tempered chocolate (Svanberg et al., 2013) showing that the crystals’ polymorphic form in the 2% limonene containing chocolate was uniformed and well-tempered (Svanberg et al., 2013). Nevertheless, the melting temperature, T_{onset} is defined by the extrapolated beginning of the curve, which is the point of intersection of the tangent with the point of maximum slope, on the principal side of the peak with the base line extrapolated (Araújo et al., 2010). Therefore, the onset of melting temperature for the chocolate containing 2% limonene was 20.61°C at the point of intersection of the tangent which was calculated by the computer software of the DSC.

The effect by limonene in lowering the melting temperature of chocolate may be closely related to the viscosity reducing effect shown by Do et al. (2008). The effect of reducing the viscosity of the cocoa butter:limonene mixture was reported to be due to the interaction of the low molecular weight hydrophobic compound (limonene) with the fatty acids (FAs) in the cocoa butter. As a result, the effect of limonene on the cocoa butter viscosity was seen as likely to be a simple dilution effect (Do et al., 2008). Evaluating the polymorphic behaviour of the cocoa butter:limonene system, Ray et

al. (2012) concluded that the lowering of the melting temperature was due to limonene decreasing the chemical potential of the crystal network in such a way that limonene would act as a “spacer” between triglycerides and fatty acids within those triglycerides.

2.3.2 Bloom formation in cocoa butter:limonene and chocolate:limonene blends

As a first assessment of bloom formation on the challenge stored cocoa butter and chocolate blends with limonene, the whiteness index was measured followed by inspection of the physical appearance of the samples. Figure 2.3 and Figure 2.4, respectively, show the development of the whiteness index of the cocoa butter:limonene and chocolate:limonene samples during storage. The whiteness index axis scale was chosen to be the same in both figures to facilitate comparison between the cocoa butter:limonene and chocolate:limonene sample sets. Numerical values of the plotted data and their standard deviations are given in Table 2.1.

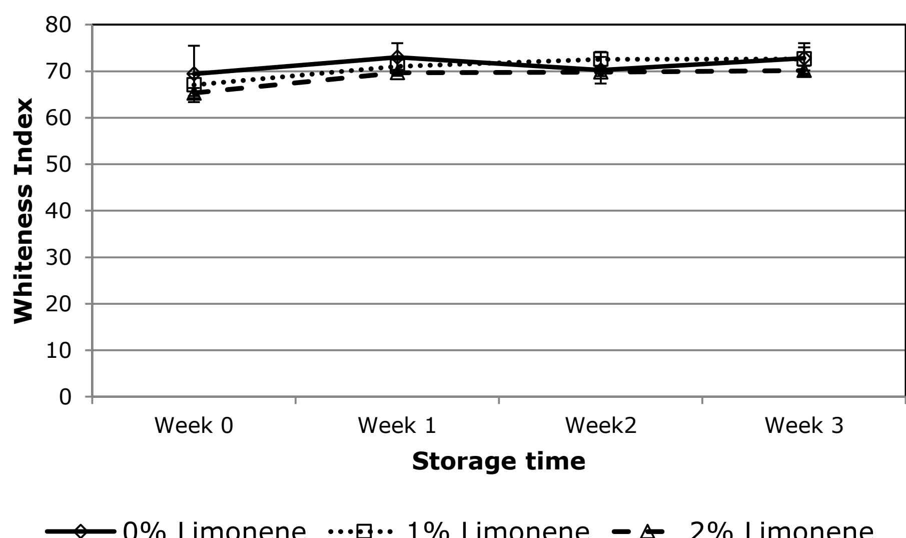

Figure 2.3 Whiteness index of cocoa butter:limonene during the storage at cyclic temperature

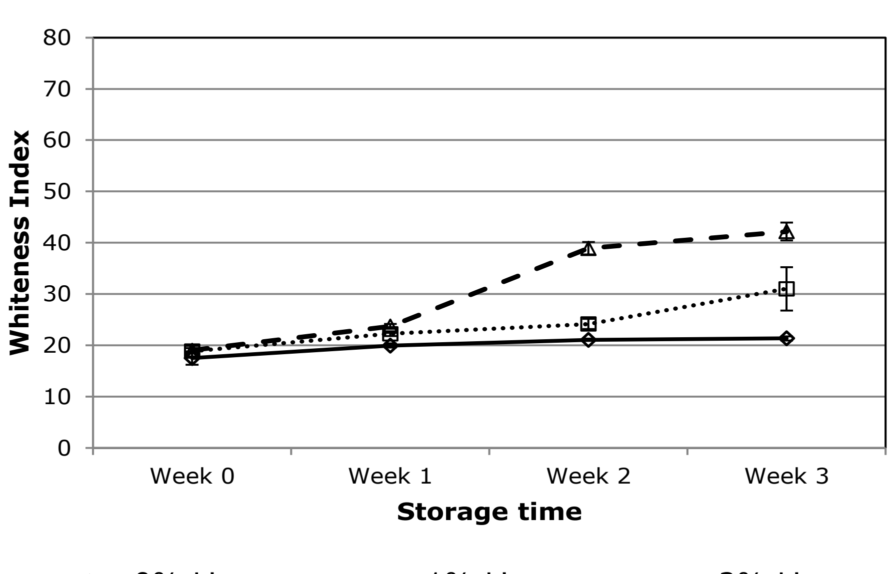

Figure 2.4 Whiteness index of chocolate during the storage at cyclic temperature

Figure 2.3 demonstrates that limonene concentration and storage period under cyclic temperature conditions, had little impact on the whiteness index of the cocoa butter samples, although a slight increase in whiteness index between Week 0 and Week 1 was found for all three blends. Lack of colour difference in whiteness index does not preclude bloom formation, however, since crystallised cocoa butter appears white and therefore the detection of bloom formation through whiteness index measurement is hampered. In Week 0, the 30:0 cocoa butter:limonene blend had the highest value but without significant difference (P>0.05) with other samples. Replacing some amount of cocoa butter with limonene slightly decreased the value of whiteness index whereas the impact was larger for a higher amount of limonene substitution. A decrease was not expected since fat bloom is generally recognised as a lighter appearance of the chocolate surface and therefore the decrease could be due to the yellowish colour of limonene added at increasing concentration. This effect only becomes obvious in the cocoa butter:limonene blend due to the absence of the cocoa powder component present in chocolate. The significant figures for the cocoa butter samples, especially for the control sample, was slightly higher due to the white colour of the cocoa butter itself and the colour was not evenly scattered on the surface of the cocoa butter as can be seen in Figure 2.5.

Table 2.1 Whiteness index (means of 4 measurements ± standard deviation) of cocoa butter:limonene and chocolate:limonene samples during storage trials

Ratio of CB to limonene (g)	Cocoa butter (CB)				Chocolate			
	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 0**	**Week 1**	**Week 2**	**Week 3**
30:0	69.42 ± 6.05^{Aa}	73.01 ± 3.03^{Aa}	70.21 ± 2.84^{ABa}	72.77 ± 3.25^{Aa}	17.49 ± 1.30^{Bc}	19.95 ± 0.41^{Cb}	21.08 ± 0.20^{Ca}	21.35 ± 0.41^{Ca}
29:1	67.05 ± 2.38^{Ab}	71.03 ± 1.77^{ABa}	72.58 ± 1.58^{Aa}	72.65 ± 2.47^{Aa}	18.86 ± 1.02^{Ac}	22.25 ± 0.48^{Bb}	24.15 ± 0.99^{Bb}	31.01 ± 4.22^{Ba}
28:2	65.30 ± 1.12^{Ab}	69.67 ± 0.64^{Ba}	69.80 ± 0.90^{Ba}	70.15 ± 0.98^{Aa}	19.00 ± 0.43^{Ad}	23.70 ± 0.48^{Ac}	38.92 ± 1.20^{Ab}	42.18 ± 1.73^{Aa}

Means ± standard deviations are in four replicates. Values with the same capital letter in the same column do not differ significantly (P>0.05). Values with the same letter in the same row do not differ significantly (P>0.05).

Even though it was difficult to differentiate the colour changes in cocoa butter, samples with 28:2 cocoa butter:limonene showed a dramatic change in colour between Week 0 and Week 3 (Figure 2.5). This indicates bloom may have formed on the samples of 28:2 cocoa butter:limonene. Also, the 28:2 cocoa butter:limonene sample appeared to be softer in texture and could not be de-moulded perfectly so was prepared in an aluminium foil cup. The softening effect of limonene was previously reported by Do et al. (2008) who observed a reduction in hardness of chocolate by 80% in presence of 13% limonene. This was explained as a weakening of the crystal network of the cocoa butter due to the presence of limonene.

In the case of the chocolate samples, it was easier to detect the development of bloom via the whiteness index measurement. Figure 2.4 shows the impact of limonene on the whiteness index of chocolate samples during storage at cycled temperature conditions as described in Section 2.2.2. As early as Week 0, the chocolate:limonene samples showed a significantly ($P<0.05$) higher whiteness index compared to the Control sample. At Week 1, the whiteness index had increased significantly ($P<0.05$) for all three chocolate blends. The whiteness index value continued to increase until the end of the storage, where the 28:2 chocolate:limonene blend showed the highest value, followed by the 29:1 and 30:0 chocolate:limonene blends. Figure 2.6 shows the visual inspection of bloom formation on the

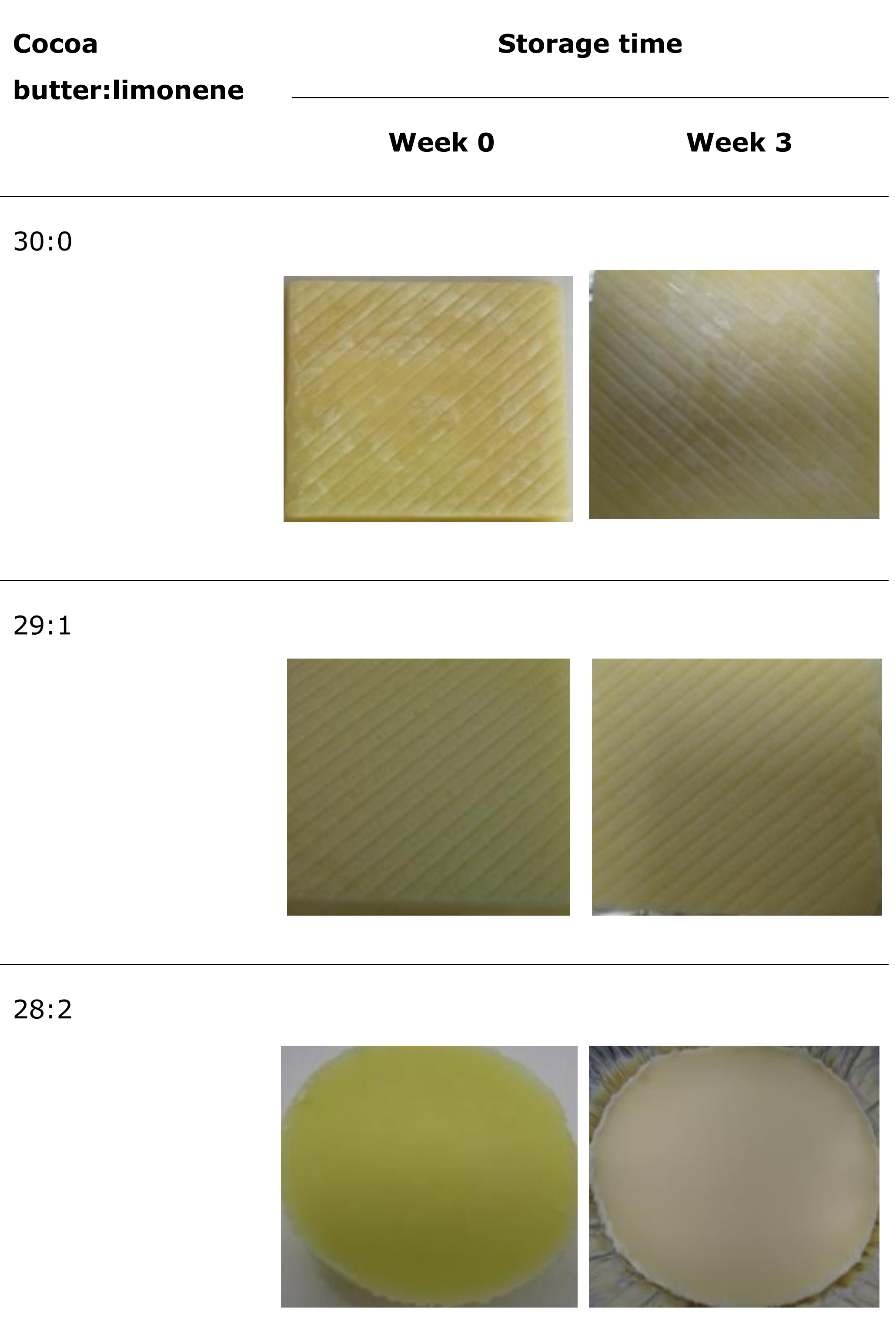

Figure 2.5 Physical appearance of cocoa butter: limonene blends throughout the three weeks of storage cycling temperature between 20°C and 29°C every 12 hours

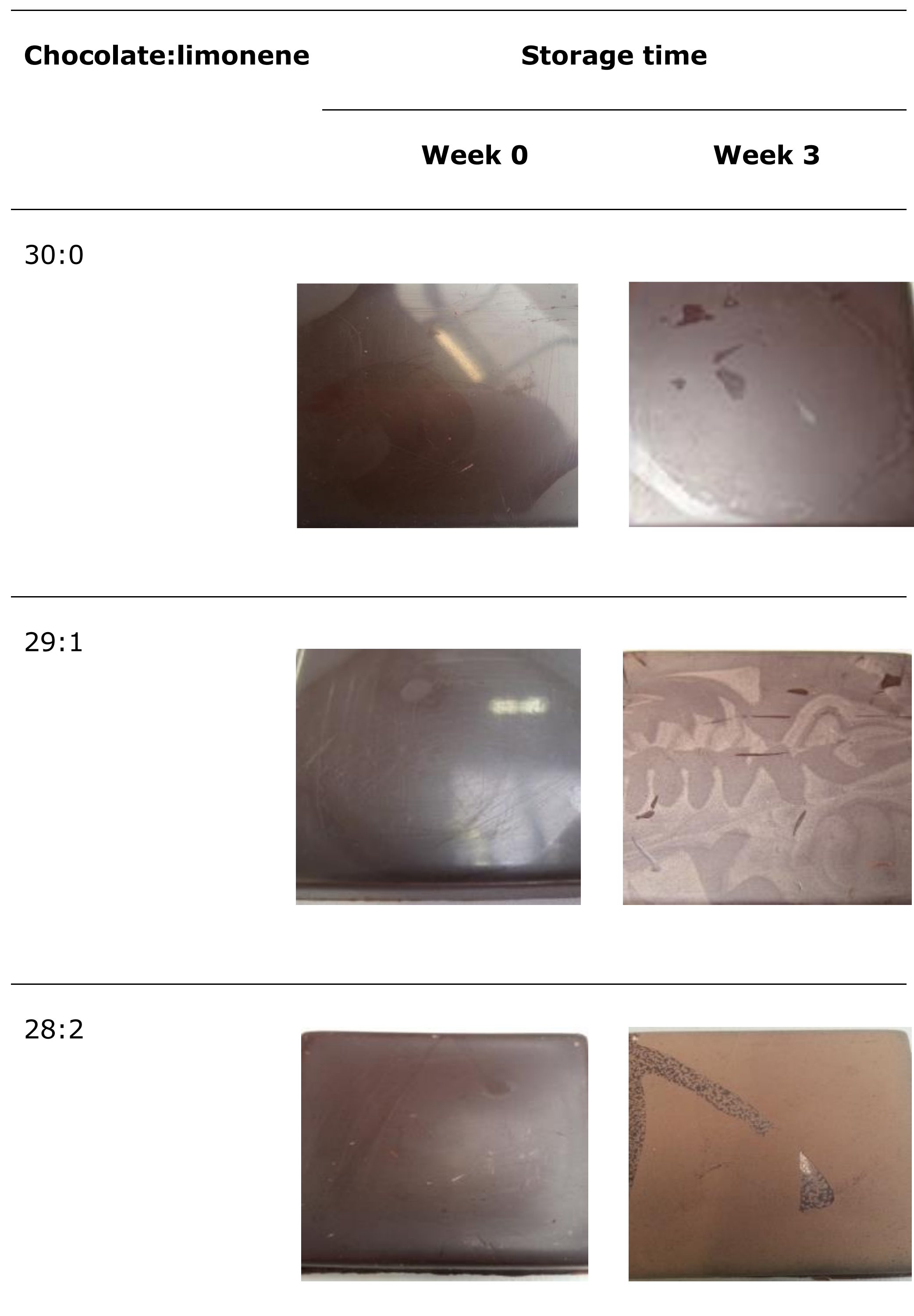

Figure 2.6 Physical appearance of chocolate: limonene blends throughout the three weeks of storage cycling temperature between 20°C and 29°C every 12 hours

chocolate:limonene blends. The sample of 30:0 chocolate:limonene was glossier than the other samples which corresponds with the lowest whiteness index value measured across the sample set. The matte surface increased the light scattering which is echoed by the higher whiteness index value as seen in the chocolate:limonene samples. At the end of the storage, a thick layer of bloom was clearly visible on the surface of the samples containing limonene.

2.3.3 Polymorphic forms

The transformation of the polymorphic form of the cocoa butter crystals in the cocoa butter:limonene and chocolate:limonene samples was analysed using XRD diffraction; the results are shown in Figure 2.7. The identification of the polymorphic forms was undertaken by comparison to published XRD patterns as reproduced in Figure 2.8 (van Malssen et al., 1999), besides comparing with the value of d-spacing which previously reported by Sonwai and Rousseau (2006) and also AOCS method in the article by Peyronel and Marangoni (2017). According to Sonwai and Rousseau (2006), freshly tempered cocoa butter (at t = 0 week), was in the Form V polymorph with a very strong diffraction peak at 4.58 Å and four smaller peaks at 3.99, 3.87, 3.75 and 3.68 Å. After a period of time, the diffraction peak at 3.99 Å began to shift toward larger d-spacings with a reduced peak height while the intensity at 3.68 and 3.87 Å continued to

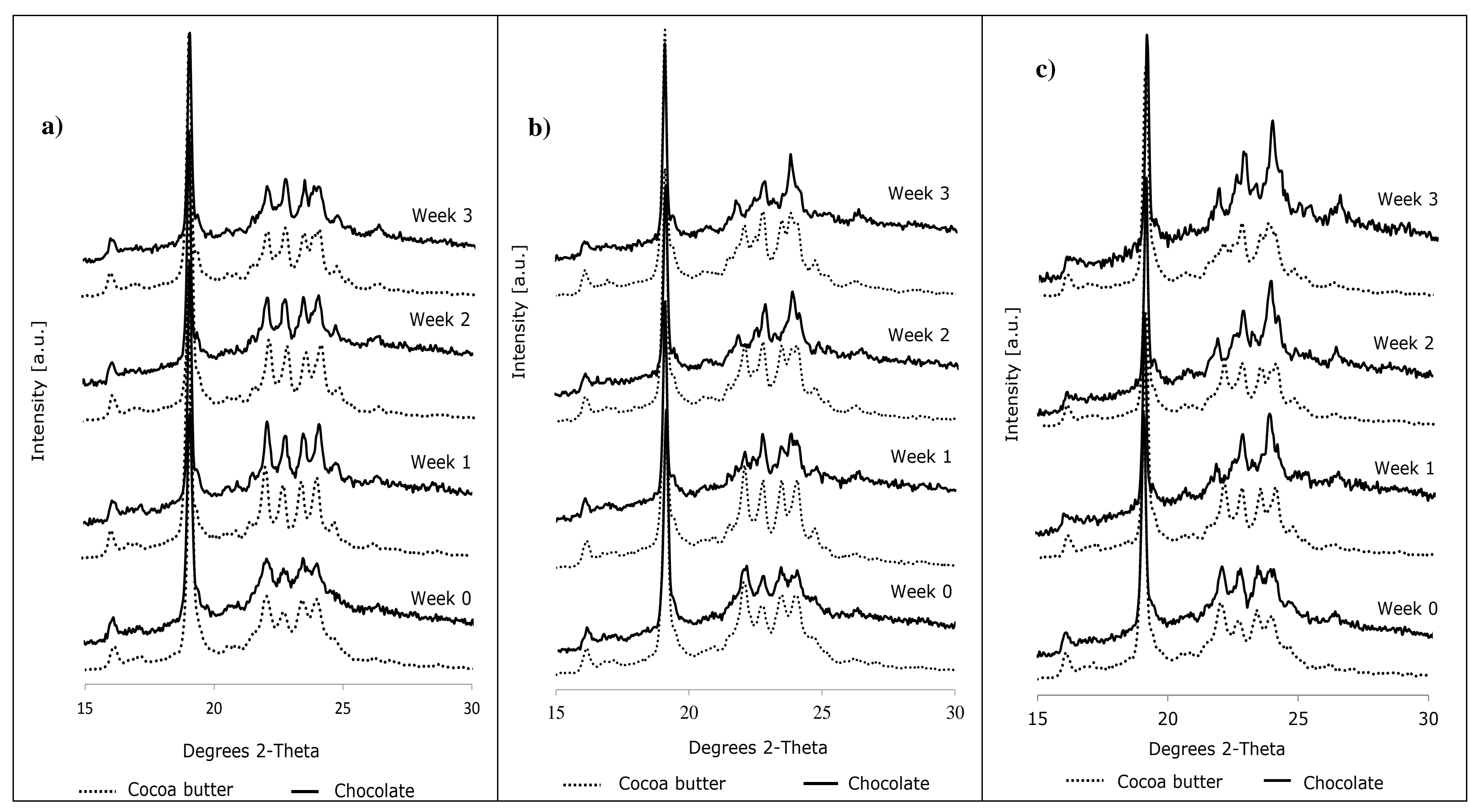

Figure 2.7 XRD patterns of cocoa butter and chocolate samples acquired on a weekly basis during cyclic temperature storage at 20 and 29°C shown for the 3 different blends as follows: a): 30:0; b): 29:1; c): 28:2.

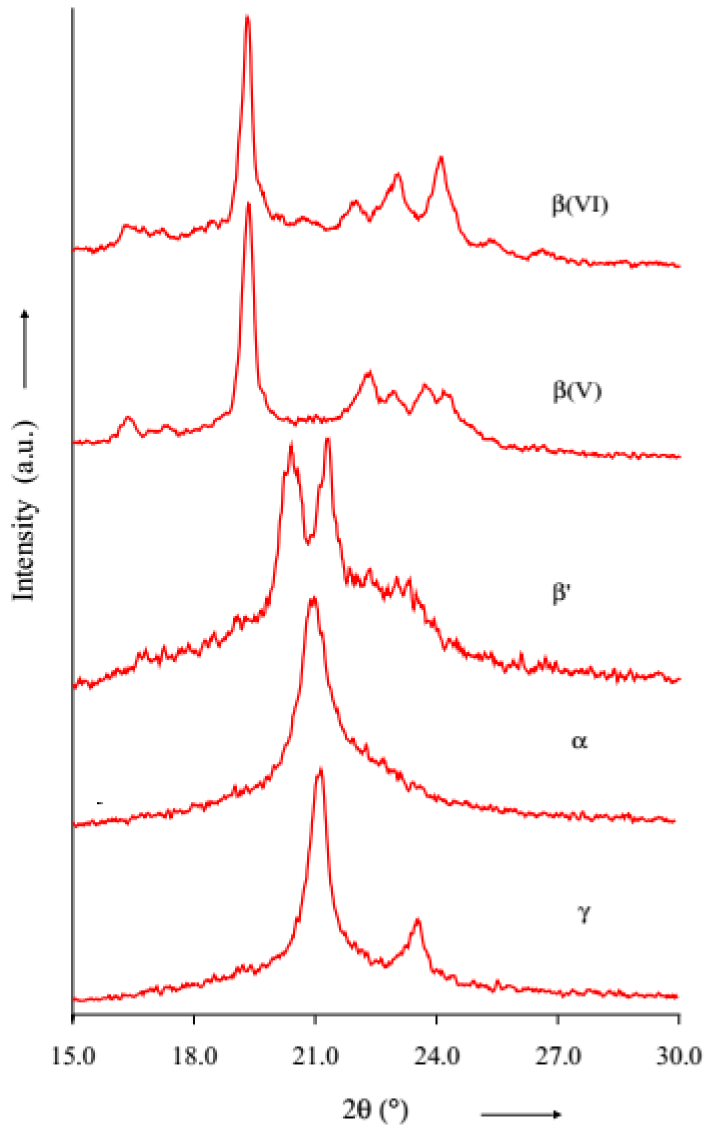

Figure 2.8 XRD pattern of cocoa butter polymorphic form which is following the work of van Malssen et al. (1999), where showing the β′ as the combination of Form III and IV (melting points is in the range of 20 – 27°C).

increase. The phase transition from Form V to VI continued with time and after 26 weeks of storage, Form VI was the sole polymorph. The Form VI has short spacings at 4.04, 3.86 and 3.70 Å which was also evidenced by Wille and Lutton (1966).

The cocoa butter:limonene and the chocolate:limonene at 0% limonene were shown to have Form V crystals. It was expected that chocolate:limonene would have Form V crystals due to tempering, but unexpected to find this polymorphism for the untempered cocoa butter:limonene sample. Usually, untempered samples show Form IV crystals. However, Form V also can be the result of chocolate exposed to a cooling tunnel (Talbot, 2009). The method used involved a cooling step at 7°C for 1 h and this could be the reason that the cocoa butter was in Form V rather than the expected Form IV for untempered cocoa butter. The polymorphic state of Form V was retained until towards the end of the storage period where both samples may show evidence of the presence of Form VI.

In the case of both the cocoa butter:limonene and chocolate:limonene blends, Form VI was detected at the earlier stages of the cyclic temperature storage. At the lower level of limonene incorporation, that is the 29:1 blends, Form V was identified from XRD analysis in Week 0. Analysing the samples after one week of cyclic temperature storage indicated a mixture of polymorphic Forms V and VI in the

chocolate sample. In the case of the cocoa butter sample, the rate of polymorphic transition appeared to be slower since the first evidence of Form VI was only detected in Week 2. The crystals in the chocolate sample, on the other hand, had fully transitioned into Form VI by Week 2. At the end of the storage period (Week 3), the cocoa butter sample was still showing a mixture of Form V and Form VI crystals.

The higher level of limonene incorporation in the 28:2 blends accelerated the polymorphic transition for the chocolate:limonene blend. The starting of the transition from Form V crystals to Form VI crystals were detected as early as Week 0 in case of the chocolate blend. After one week at cyclic temperature storage, the polymorphic transition appeared to be completed. However, the cocoa butter blend maintained exclusively Form V crystals until Week 1. During Week 2, Form VI polymorphs were detected based on the XRD analysis and, as at the lower level of limonene incorporation, the transition was not complete even after three weeks of cyclic temperature storage.

The higher rate of polymorphic transition in chocolate compared with cocoa butter may be due to the presence of “foreign” matter (sugar and cocoa powder), as previously reported for chocolate tempered with seeded cocoa butter (Svanberg et al., 2011). The “foreign” particulate chocolate ingredients provide additional nucleation sites thus accelerating the crystallization process (Svanberg et al., 2011).

2.3.4 Thermal behaviour of chocolate during storage

The melting curves acquired with the chocolate:limonene blends during the accelerated storage are shown in Figure 2.9. The curves shown for Week 0 samples indicate that addition of limonene has shifted the melting peak to a lower temperature. However, no trend was shown with the onset of melting temperature as the limonene content increased. The addition of 1% limonene had shifted the T_{onset}

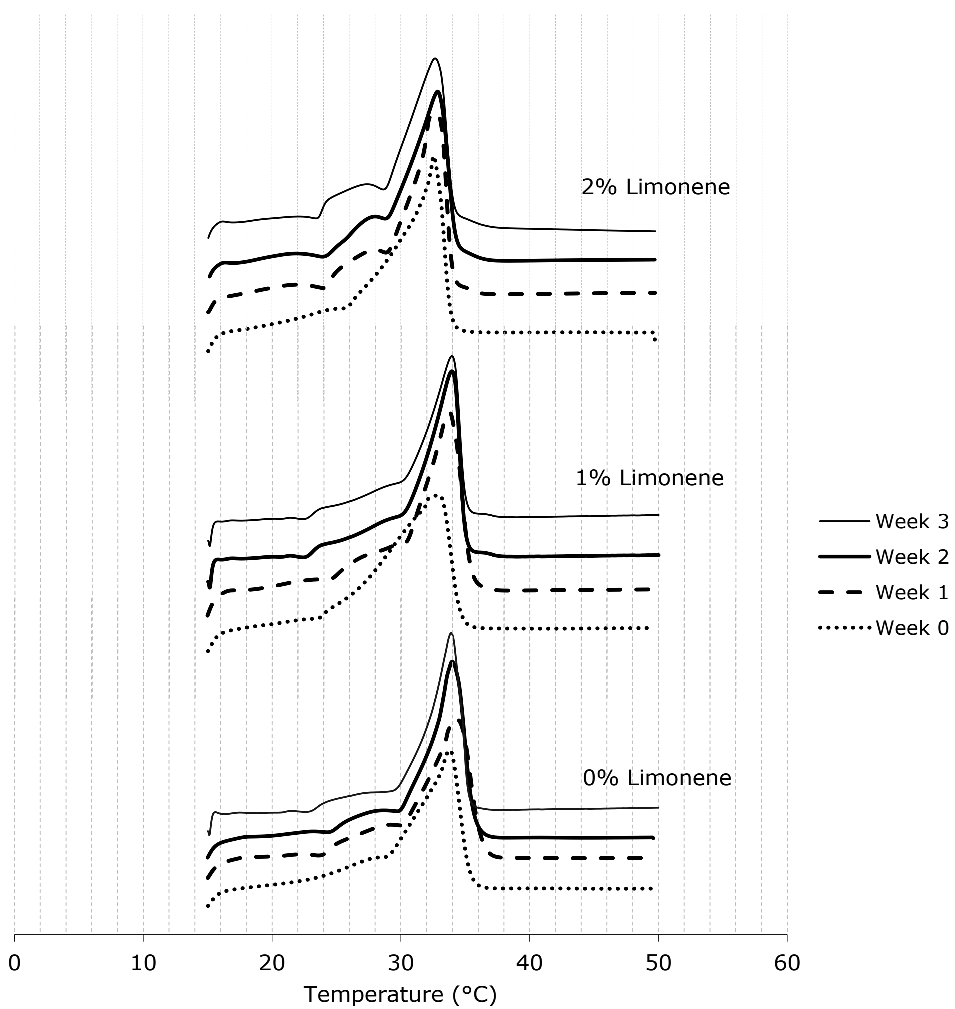

Figure 2.9 Melting curves of DSC for chocolate samples during accelerated temperature

of melting to a lower temperature but it increased again to higher temperature with the addition of 2% limonene. Nevertheless, the T_{onset} of melting limonene for chocolate containing limonene was generally shifted to a lower temperature compared to the sample without of limonene. There were also a small "lump" at the beginning of the melting curves and would be interesting to analyse this using XRD in the future in order to know the particular polymorphic phase of crystal at that particular temperature (around 27°C).

After one week of storage, the temperature at which the whole sample had melted, known as the end of melting temperature, T_{end} for all samples shifted to a higher value, signposting the occurrence of crystallization events in all of the sample and indeed the formation of a higher polymorphic form. The shift in the end of melting temperature from Week 0 to Week 1 was less obvious for the 28:2 chocolate:limonene blend compared to the 29:1 and 30:0 chocolate:limonene blends. This is possibly due to the formation of Form VI crystals in the mixture of Form V crystals as early as Week 0 in the 28:2 chocolate:limonene blend, which was identified by XRD (see Section 2.3.3). Thus, the subsequent storage time had not increased the end of melting temperature of the 28:2 chocolate-limonene blend by much, showing that the sample had reached a stable polymorphic form early on.

Limonene's effect in cocoa butter/chocolate to lower the melting temperature while driving the fat suspension to higher polymorphic form of crystals was expected due to several reasons. It does not crystallize with TAGs (Do et al., 2008), but the solubility effect of limonene could be a reason for the observed lowering of the melting temperature. Incorporating the low molecular weight hydrophobic compound solubilizes the solid crystals that had formed in the mixture, as reported in the study of Wright et al. (2005) and Kaufmann et al. (2012). The higher proportion of liquid due to the addition of limonene reduced the proportion of solid crystals in the mixture which is normally determined by the value of solid fat content (SFC) (Wright et al., 2005, Kaufmann et al., 2012). This effect was believed to be due to the solubility effect of limonene which was reported to reduce the amount of unstable fat crystals (Rigolle et al., 2016) by solubilisation into liquid oil and thus reducing the amount of solid crystals in the mixture (Kaufmann et al., 2012). This was also reported in the study of Do et al. (2008) where the addition of limonene to cocoa butter had decreased the solid fat content (SFC) by over 50% at temperatures above 25°C. As a result, limonene softens the chocolate texture and reduces the melting temperature of chocolate. Therefore, it could be expected that limonene would stay in liquid form between the solid crystals of cocoa butter, solubilize the unstable crystals of cocoa butter and make the chocolate softer in texture (due to a less compact crystal network of cocoa butter).

The impact of limonene in accelerating the crystallization rate of chocolate can be explained by the following process. The presence of liquid, limonene in this study, has altered the crystallization kinetics of cocoa butter and caused a slower nucleation rate (crystallization at lower temperature) as reported by Kaufmann et al. (2012) and Wright et al. (2005). At slower nucleation rates, fewer crystals are produced. The smaller amount of crystals formed in cocoa butter-limonene compared to pure cocoa butter was reported by Rigolle et al. (2016). The presence of liquid (limonene) has increased the rate of polymorphic transition due to the increased nucleus mobility (Perez-Martinez et al., 2007). The oil-mediated (or liquid-mediated) transformation of crystals is described as either initiated by spontaneous nucleation in liquid or by heterogeneous nucleation at the surfaces of existing crystals (Sato and Koyano, 2001), as illustrated in Figure 2.10. At higher temperature (29°C) the partial melting of cocoa butter has increased the amount of liquid in the sample where TAG molecules are detached from dissolving crystals of Form V and form nuclei of Form VI crystal through volume diffusion in the oil matrix (Sato and Koyano, 2001). Then at lower temperature (20°C), heterogeneous nucleation of Form VI may also occur but its rate is raised at the elevated temperature (Sato and Koyano, 2001). The growth rate of crystals was observed to be many times faster than the nucleation rate in the presence of liquid (Kaufmann et al., 2012) hence, a lesser number of large crystals are expected to developed (higher polymorphic crystals but in small quantity). Evidence of crystals of higher polymorphic form (Form VI) was also

observed in the study of Ray et al. (2012) where cocoa butter-limonene blend showed a higher crystal form than the one without limonene.

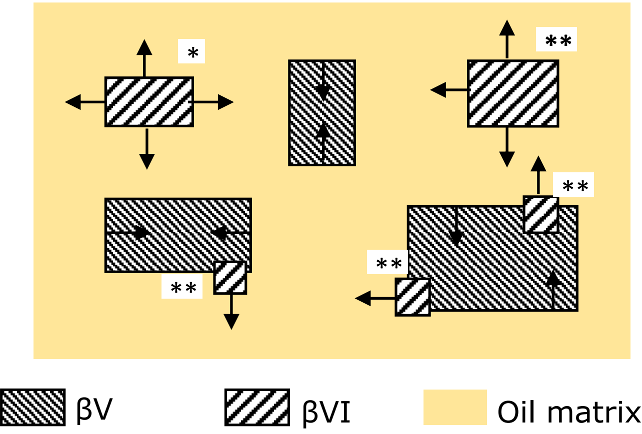

Figure 2.10 Basic models of fat bloom formation by oil-mediated transformation (spontaneous nucleation shown by *, and heterogeneous nucleation shown by **) (Sato and Koyano, 2001).

2.4 CONCLUSIONS

The analysis of bloom formation, polymorphism and thermal behaviour of cocoa butter:limonene and chocolate:limonene blends revealed that the presence of limonene in these fat matrices accelerates the rate of bloom formation. At the same time it was confirmed that, in the presence of limonene, more stable cocoa butter polymers are formed more quickly during temperature cycled storage. This apparent contradictory impact of limonene is explained by the solubility effect where the unstable cocoa butter crystals was

solubilized in the liquid of limonene which co-exists in the cocoa butter fat crystal network. Therefore, while based on research limited to DSC and XRD analysis of cocoa butter and chocolate containing limonene may suggest that this is a commercially interesting additive to formulate chocolate at lower cocoa butter addition without compromising the viscosity properties. However, the demonstrated accelerated bloom formation makes this a less attractive ingredient for moulded chocolate bars. It can be hypothesised that other terpenes will similarly impart these undesired product attributes. On the other hand, application in chocolate fillings or white chocolate bars may still be commercially relevant since bloom will not be visible due to the absence of an exposed chocolate surface, in the case of fillings, and the whitish appearance, in the case of white chocolate.

3 POLAR LIPID COMPOSITION OF SPINACH LEAVES AND CHLOROPLASTS, AND THEIR POTENTIAL AS A NATURAL SURFACTANT

This chapter focusses on the composition analysis of lipids from spinach leaves and chloroplasts in order to provide an understanding of the mechanism of the functional behaviour of those lipids which will be used in Chapter 4. The surface activity of both sources of lipids was also investigated and compared to the commercial surfactants.

3.1 INTRODUCTION

Natural surfactants are of great interest to the consumer goods industries since shoppers demand increasingly more natural ingredients in manufactured products. In the case of the research in this Ph.D., it is the replacement of synthetic surfactants with naturally surface active materials. A suitable alternative may be protein or lipid in nature, unless particulate surface active materials are sought after. The focus of this research, in regards to natural surfactants, were polar lipids. Probably the most commonly known class of surface active polar lipids are phospholipids, which are important biochemical intermediates in the growth and functioning of cells in plants and animals (van Nieuwenhuyzen, 2010b). As an industrially applied surfactant, phospholipids are typically encountered as a mixture in lecithin, rather than individual phospholipids, and due to their high

surface activity are widely used as a food emulsifier (van Nieuwenhuyzen, 2010a). Lecithins generally consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) and minor amounts of lysophosphatidylcholine (LPC) (van Nieuwenhuyzen, 2010b). Lecithins can be obtained as a by-product of oil seed processing after the degumming process (van Nieuwenhuyzen, 2010b).

Galactolipids represent another class of polar lipids of interest in the context of natural surfactants. While poor in phospholipids, photosynthetic plants are rich in galactolipids (Dörmann, 2013). In fact, galactolipids are considered to be the most abundant lipid class in nature because of their abundancy in photosynthetic organisms such as plants and algae in the biosphere (Dörmann, 2013). As molecules, galactolipids are glycolipids with galactose attached to the glycerol lipid molecule. They were first discovered in wheat about 50 years ago (Carter et al., 1956). Galactolipids play a very important role in the establishment of photosynthetic membranes and are highly abundant in the thylakoid membranes of chloroplasts (70% of the total membrane lipids) (Dörmann, 2013). In photosynthetic plants, the two most common galactolipids are monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) (Dörmann, 2013). In leaves, MGDG makes up approximately 50% of total glycerolipids while DGDG constitutes approximately 20% of total glycerolipids (Douce and Joyard, 1990).

In this research, the particular interest in natural surfactants was to explore the replacement of the synthetic surfactant polyglycerol polyricinoleate (PGPR) as the viscosity reducing agent in chocolate, motivated by a patent published some 25 years ago (Evans et al., 1991). In this patent, comparable performance of oat lipids with lecithin and polyglycerol polyricinoleate (PGPR) in reducing the viscosity of chocolate was claimed and explained by the glycolipids with digalactosyl residue fraction in the oat lipid (Evans et al., 1991). Oat lipids have also been shown to possess good emulsifying properties although, if added as the sole stabiliser, they do not prevent creaming in oil-in-water emulsions. Compositional analysis data in literature suggest that oat lipids consist of MGDG, DGDG, steryl glycoside, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and a minor fraction of phosphatidyl inositol (PI) (Kaimainen et al., 2012, Doehlert et al., 2010).

Spinach (*Spinacia oleracea* L.) is a leafy photosynthetic vegetable that is high in glycolipids especially MGDG, DGDG and sulfoquinovosyldiacylglycerol (SQDG) (Christie, 2015). As galactolipids are highly concentrated in the chloroplast envelope, most of the studies are related to the spinach chloroplast (Nishimura et al., 1976, Wolf et al., 1961, Lichtenthaler and Park, 1963) rather than spinach leaves. The average lipid content in spinach chloroplasts was reported to be about 30.9% of the dry weight of chloroplasts (Menke, 1938) and the thylakoid membrane of spinach chloroplasts

has been demonstrated to be capable of stabilising emulsions (Rayner et al., 2010). The lipolysis of these emulsions was then shown to be prolonged compared to emulsions stabilised with non-glycolipid based emulsifiers leading eventually to increased secretion of satiety hormones (Ostbring et al., 2015). This is of interest in the context of formulating healthier foods to combat obesity.

In view to applying spinach lipids as a natural surfactant in chocolate, the objective of the work reported in this thesis chapter was to analyse the composition of lipids extracted from spinach leaves and spinach chloroplasts and to assess their surface tension at the water/oil interface (water as a hydrophilic liquid modelling the hydrophilic sucrose in chocolate). Even though the galactolipids are concentrated in the spinach chloroplasts, it is also useful to assess the potential of lipids from whole spinach leaves in reducing the surface tension at the water/oil interface. This is because such data have not yet been reported in literature and in view of industrial application where fewer extraction steps would be required which may balance a potentially reduced activity with the cost of production.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Fresh spinach leaves (*Spinacia oleracia* L.) and sunflower oil (Sainsbury's) were bought from a local supermarket. PGPR and soy lecithin were provided by Danisco (Kettering, UK) and ADM (Hull, UK), respectively. The following analytical grade solvents and chemicals were used for lipid extraction: chloroform (Sigma-Aldrich, USA), methanol (Fisher Scientific, USA), isopropanol (Fisher Scientific, USA), sodium chloride (Sigma-Aldrich, USA) and sucrose (Sigma-Aldrich, USA).

3.2.2 Moisture analysis

Moisture analysis was followed according to the official method of AOAC (Association of Official Analytical Chemists) (Horwitz et al., 1975). Spinach leaves were chopped into small pieces and about 5 g was weighed into a dish. The leaves were then dried in a convection oven (Sanyo, Japan) at 105°C for 24 h. All samples were prepared as three replicates. The moisture content was determined according to Equation 3.1;

$$\text{Moisture content (wet basis)} = \frac{Fresh\ sample - dried\ sample}{Fresh\ sample} \times 100\%$$

(Equation 3.1)

Moisture analysis of the chloroplast fraction was not carried out with the method applied to spinach leaves. The chloroplasts obtained may vary significantly in the moisture content due to the addition of sucrose solution during grinding and isolation. Therefore, obtaining the dried weight of the chloroplast fraction was only an approximation of data from the result of freeze drying however, the data were needed in further analysis. Drying was carried out for three to five days until the weight remained constant. Oven drying was not used as it would destroy the chloroplast samples and they could not be used for further analysis.

3.2.3 Total lipid extraction from spinach leaves

Generally, the extraction of lipids from spinach leaves was performed to the established lipid extraction procedure devised by Folch et al. (1957). The mixture of chloroform-methanol (2:1) used in that procedure has been accepted by lipid analysts as it can extract lipid thoroughly from animal, plant and bacterial tissues (Christie, 1993a). The capacity of chloroform to associate with water molecules by weak hydrogen bonds was assumed to be the key factor that makes the chloroform-methanol (2:1) such a good lipid extractant (Schmid, 1973, Fishwick and Wright, 1977). Prior to lipid extraction, it is important to prepare the sample using appropriate pre-treatment methods in order to ensure the lipids are efficiently extracted.

Hydrolytic enzymes such as phosphatases, lipases and sulfolipase are easily activated if plants cells are ruptured (Benson, 1964). Extraction with methanol can enhance the action of phospholipase D in attacking phospholipids which can be detected by the presence of phosphatidic acid indicating enzymatic degradation during isolation and extraction (Benson, 1964, Christie, 1993a). The typical treatment to overcome this problem is by extracting with pre-heated or boiling isopropanol prior to extraction with chloroform-methanol (Kates and Eberhardt, 1957). Hot isopropanol is considered safest as it strongly inhibits the action of phospholipase D (Fishwick and Wright, 1977). Phospholipase D can catalyse a reaction with alcohol such as ethanol in which it replaces the base unit of phospholipids with ethanol. For example, phosphatidylmethanol was found to be produced by phospholipase D-catalysed transphosphatidylation during extraction of developing soybean seed with chloroform-methanol (Roughan et al., 1978). The reaction does not happen if isopropanol is the extractant. Therefore, hot isopropanol is classified as the most efficient solvent for enzyme denaturation (Fishwick and Wright, 1977).

Total spinach lipids were extracted as follows. The isopropanol was pre-heated at 80°C following the method of Yao et al. (2012). One hundred grams of spinach leaves was weighed and three volumes of pre-heated isopropanol added before chopping in a household blender for 1 min. The mixture of spinach-isopropanol was then decanted into a bottle with a small hole in the lid and kept at 80°C while stirring at

400 rpm on a hot plate magnetic stirrer for 20 min. The lid with hole was used to release pressure build up in the bottle during heating. The mixture was then filtered with at least three layers of cheese cloth. The residue was collected and re-extracted with 240 ml of chloroform:methanol (2:1) by stirring at 400 rpm for another 20 min. The extract was filtered again with cheese cloth and both extracts were combined in a round bottom flask before being transferred into a rotary evaporator to evaporate the solvent. The evaporation took place at 40°C until the extract was almost dry. The extract was then reconstituted with at least 24 ml of chloroform:methanol (2:1) and transferred into a separatory funnel. Another 6 ml of 0.9% sodium chloride solution was added into the mixture to adjust the ratio of chloroform:methanol:NaCl to 8:4:3 and shaken vigorously. This step followed the method of Folch et al. (1957) where any contaminant other than lipids will be in the polar phase (upper phase) while lipids will be in the non-polar phase (chloroform). The mixture was then allowed to stand 1 h until a complete separation of two layers was seen. It is critical to keep the ratio of chloroform:methanol:NaCl at 8:4:3 in order to develop a biphasic system so that any contaminating material in the lipids can be removed completely (Christie, 1993a). It may be necessary to apply another wash to the lower phase with chloroform:methanol:NaCl (8:4:3) to ensure that all non-lipid contaminants are eliminated. However, even washing just once was reported to be enough to eliminate all of the non-lipid contaminants (Folch et al., 1957).

The presence of the salt in water solution helps to move the polar lipids from the upper phase to the lower phase and the mechanism by which this happens was explained by Folch et al. (1957). The polar lipids in the extracts are in the dissociated form of salts of either cations Na, K, Ca or Mg in the polar upper phase. When salts of either of those cations are added, the salts would decrease the dissociation of the polar lipids and transfer the polar lipids by a mass action effect to the lower non-polar phase.

The lower phase was then collected in a pre-weighed round bottom flask and evaporated at 40°C with a rotary evaporator (Büchi Labortechnik AG, Switzerland). The flask with the dried lipid extract was weighed and the recovery of the lipids was recorded as yield. If the lipids were to be stored for further analysis, the lower phase of the separatory funnel would be directly transferred into a screw cap glass bottle and stored at -80°C.

3.2.4 Total lipid extraction from spinach chloroplasts

The method of extracting lipids from chloroplasts was akin to that devised by Whitaker (1986) except for the chloroplast isolation step. Before extraction, the chloroplasts were isolated from the leaves as follows. Seventy grams g of spinach leaves were weighed into a

household blender and mixed with 210 ml of 0.3 M sucrose solution for 1 min. The slurry was then filtered with at least three layers of cheese cloth. The filtrate was subjected to centrifugation at 4°C with rotation of 1500 g for 20 min to isolate the chloroplasts. The chloroplast fraction was then collected in a pre-weighed screw cap bottle with a small hole in its lid.

Now that the chloroplasts had been isolated, the extraction procedure could start, as follows: about three volumes of pre-heated isopropanol (80°C) were added into the chloroplast fraction and the mixture was kept at 80°C for another 20 min while stirring on a hot plate magnetic stirrer. Filtration with cheese cloth was then carried out to collect the extract. The waste was re-extracted to maximise the yield by mixing it with chloroform:methanol (2:1) for another 20 min. The combined extracts were transferred into a round bottom flask and evaporated with a rotary evaporator at 40°C until almost dry. The lipid extract was reconstituted with at least 24 ml of chloroform:methanol (2:1) and transferred into a separatory funnel. To obtain a ratio of 8:4:3 of chloroform:methanol:NaCl, 6 ml of 0.9% NaCl was added followed by vigorous shaking. The mixture was left to stand for 1 h until a complete separation of two liquid phases was seen. The lower phase was then collected in a pre-weighed round bottom flask and evaporated at 40°C with a rotary evaporator. The recovery of the lipid yield was obtained after re-weighing the round bottom flask.

3.2.5 Lipids analysis

Lipids class analysis was carried out by an external lab (Mylnefield Lipid Analysis at James Hutton Limited, Dundee, UK). To send the samples, the extracts were packed in a polystyrene box filled with dry ice to maintain sample temperature of -80°C. 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 (GC). The following descriptions of the procedures are based on information obtained from the external lab.

Thin Layer Chromatography (TLC)

The lipid classes were separated on 1-dimensional glass HPTLC (high performance thin layer chromatography) Silica gel 60 F_{254} plates (Merck, Germany) 20 cm x 10 cm in size. The polar lipid classes were then separated on 2-dimensional glass HPTLC Silica gel 60 F_{254} 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 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. 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 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 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) was used followed by a mixture of chloroform/methanol/acetic acid/water (80:12:15:4) 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.

Gas Chromatography (GC)

The sample preparation for GC 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 for the quantitation of the fatty acids compounds.

Lipid fractions removed from the 2-D TLC plate were MGDG, DGDG, SQDG, 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.

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 oil.

3.2.6 Sunflower oil purification

Sunflower oil was used in the measurement of the interfacial tension in this study. Purification of sunflower oil is required to remove any surface active impurities that naturally occur in sunflower oil. This removal of surface active impurities aids interpretation of the interfacial tension data acquired on the added emulsifiers in this study as well as ensuring that their interfacial adsorption is not affected by unknown components/ impurities. The purification of sunflower oil was conducted by mixing with 4% activated magnesium silicate (Florisil®, Sigma, U.S.) at 600 rpm for 30 min on a magnetic stirrer. The mixture then was centrifuged at 1700 g for 25 min to remove the silicate.

3.2.7 Addition of surface active lipids into sunflower oil

Purified sunflower oil containing surfactant was prepared as follows. A known amount of spinach or chloroplast lipids in chloroform was added to a known amount of purified sunflower oil in a pre-weighed

round bottom flask. The mixture was agitated for at least 5 min until completely mixed as evaluated by visual observation, followed by evaporation with a rotary evaporator. It is important to ensure that all of the chloroform has been completely evaporated by deducting the sum of weight of the flask, lipids and oil. The concentration of lipid needed in oil was then adjusted as desired by appropriate dilution of stock spinach lipids with purified oil and stirring on a magnetic stirrer for at least 24 h before use. The concentration of lipids in oil is reported as g lipid / g sunflower oil.

3.2.8 Interfacial tension determination at water/oil interface

Interfacial tension at the water/oil interface was measured as a function of time with a Drop Shape Tensiometer (DST) (Figure 3.1) (PAT-1, Sinterface, Berlin, D). The oil phase was added to a cubic glass cuvette and a water drop was formed in the oil sample. This technique of measurement is called pendant drop (Figure 3.2). The drop shape was formed in less than one second at the tip of a straight capillary of 2 mm outer diameter and was continuously monitored at room temperature (20ºC) by a video-camera coupled to a computer for 900 s. Under gravity, the drop assumes a shape which minimises the total energy of the system. The cross-sectional drop area was kept constant at 30 mm^2. This shape depends on the interfacial tension and on the density of the two fluids (Miller and Liggieri, 2009).

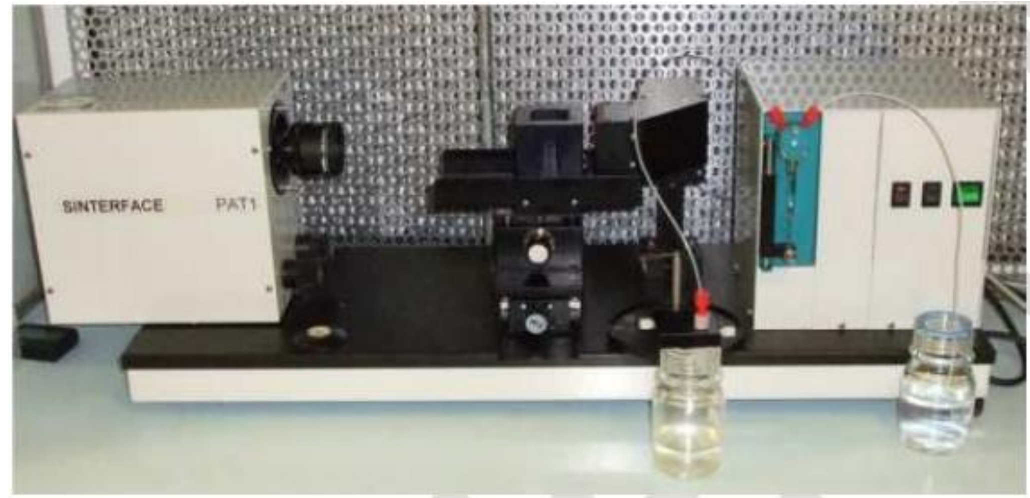

Figure 3.1 Drop shape tensiometer (PAT1 Sinterface)

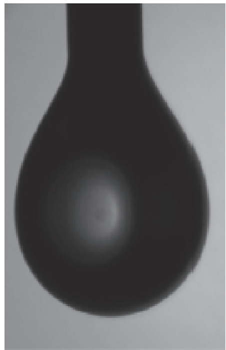

Figure 3.2 A pendant drop of water in bulk oil (Berry et al., 2015)

The instrument's software determines the interfacial tension by fitting the Gauss-Laplace equation (Equation 3.2) to the coordinates of the drop.

$$\gamma\left(\frac{1}{R_1}+\frac{1}{R_2}\right) = \Delta P_o + \Delta\rho g z \qquad \text{(Equation 3.2)}$$

where R_1 and R_2 = main radii of drop curvature, ΔP_o = pressure difference between the two liquids in a reference plane, $\Delta\rho$ = density between the two liquids, g = acceleration due to gravity and z = vertical height of the drop measured from the reference plane.

The water phase was kept constant as purified water whereas the oil phases included purified sunflower oil, purified sunflower oil with added spinach lipids, chloroplast lipids, lecithin or polyglycerol polyricinoleate (PGPR). The data reported is the average of three readings.

3.3 RESULTS AND DISCUSSION

3.3.1 Lipid extracts from spinach leaves and chloroplasts

Before extraction could take place, spinach leaves require a pre-treatment with hot isopropanol to limit the variations in lipid composition due to enzymatic degradation, and to ease the process of blending the leaves. Blending leaves directly with chloroform:methanol was not feasible in a household blender as the solvent can dissolve the plastic parts of the blender. Pre-treatment on the chloroplast fraction was carried out after the isolation of intact chloroplasts.

The moisture content of the leaves was 94 ± 0.2 g / 100 g leaves. About 1 g (dry weight basis) of chloroplasts were extracted from 100 g of fresh weight of leaves. Therefore, in 100 g of dried leaves, 16.7 g of dried chloroplasts were extracted.

The amount of lipid extracted from leaves and chloroplast are reported in Table 3.1 as g lipid yield per 100 g samples. The amount of lipid extracted was higher in the chloroplasts than the leaves as most lipids are highly concentrated in the chloroplasts (Nishimura et al., 1976). Leaves on the other hand has lots of other components such as cuticle and cell wall, thus the percentage of lipid in the leaves is lower. However, the amount of lipids extracted from the leaves was comparable to published values reported by Fricker et al. (1975) and Yunoki et al. (2009). On the other hand, the 24% lipid yield from the chloroplasts in this extraction was slightly lower than the 37% lipid

Table 3.1 Total lipids in leaf and chloroplast (n=4)

	Leaves	**Crude chloroplast preparation**
Lipids (g / 100 g dried weight samples)	14.90 ± 4.48	24.00 ± 4.55
Lipids (g/ 100 g of fresh parent leaves)	0.89 ± 0.27	0.24 ± 0.05

chloroplasts reported by Menke (1938). Since the lipid extraction from chloroplasts required an isolation of crude chloroplast tissue first, some lipids may have been lost due to structural damage of some chloroplasts during the leaves grinding and the centrifugation step (Nishimura et al., 1976).

Based on the fresh weight of the parent leaves, lipid recovery from the leaves was higher than lipid recovery from the chloroplasts. This is because in 100 g of fresh parent spinach leaves, only about 1 g dried chloroplast was collected; therefore the percentage of lipids from the chloroplasts in the basic of parent leaves were low. Lipid from other components (not chloroplasts) such as phospholipids are also reported to be higher in leaves (Wintermans, 1960).

3.3.2 Lipid classes and fatty acid composition of spinach leaves and chloroplasts

Lipid classes from leaves and chloroplasts were separated on TLC and are shown in Figure 3.3. Lipid classes were separated on 1-dimensional TLC (1D TLC) while polar lipid classes were separated on 2-dimensional TLC (2D TLC) due to their more complex structure. The lipid classes separated on 1D TLC were neutral lipids (triacylglycerol),

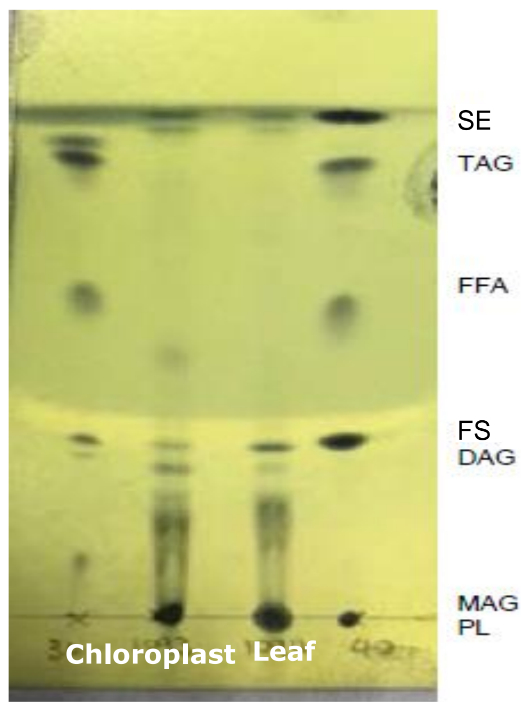

Figure 3.3 Separation of lipid classes from spinach leaf and spinach chloroplasts lipids; SE, TAG, FFA, FS, DAG, MAG, PL

polar lipids (phospholipids and glycolipids), free fatty acids, and cholesterol. The complex lipids like polar lipids remained at the origin (Christie, 2011) and this is a commonly found phenomenon as the polar lipids absorb strongly to the stationary phase while non-polar lipids elute and appear at the end of the chromatogram. The intensity of the polar lipid spot was much higher than other lipids' spots.

Polar lipids were separated on 2D TLC to identify the composition of each polar lipid class. The TLC plate results from chloroplasts and leaves are depicted in Figure 3.4 and 3.5, respectively. Both lipids from chloroplasts and from leaves had similar polar lipid classes but the intensity of spots was higher in lipids from chloroplasts. The main glycolipids identified on the plate were two major galactosyl diglycerides (MGDG and DGDG) and the sulfolipid (SQDG). The spot near the origin was suspected to be TGDG but no standard was available for this lipid to confirm the component. The presence of a spot at the bottom of the plate near to origin indicates that the lipid in the spot was more polar. GC analysis of this spot verified that this component contained fatty acids and it can be hypothesised that the relatively high polarity of TGDG (three polar head groups of galactose) led to strong attachment to the plate and not elute. However, this has to remain speculation until a standard for TGDG could be identified.

Other polar lipids detected were some phospholipids such as phosphatidylcholine (PC) and phosphatidylglycerol (PG). Phosphatidylethanolamine (PE) has never been found in plant chloroplasts (Mazliak, 1977b) so was used as internal standard in this study.

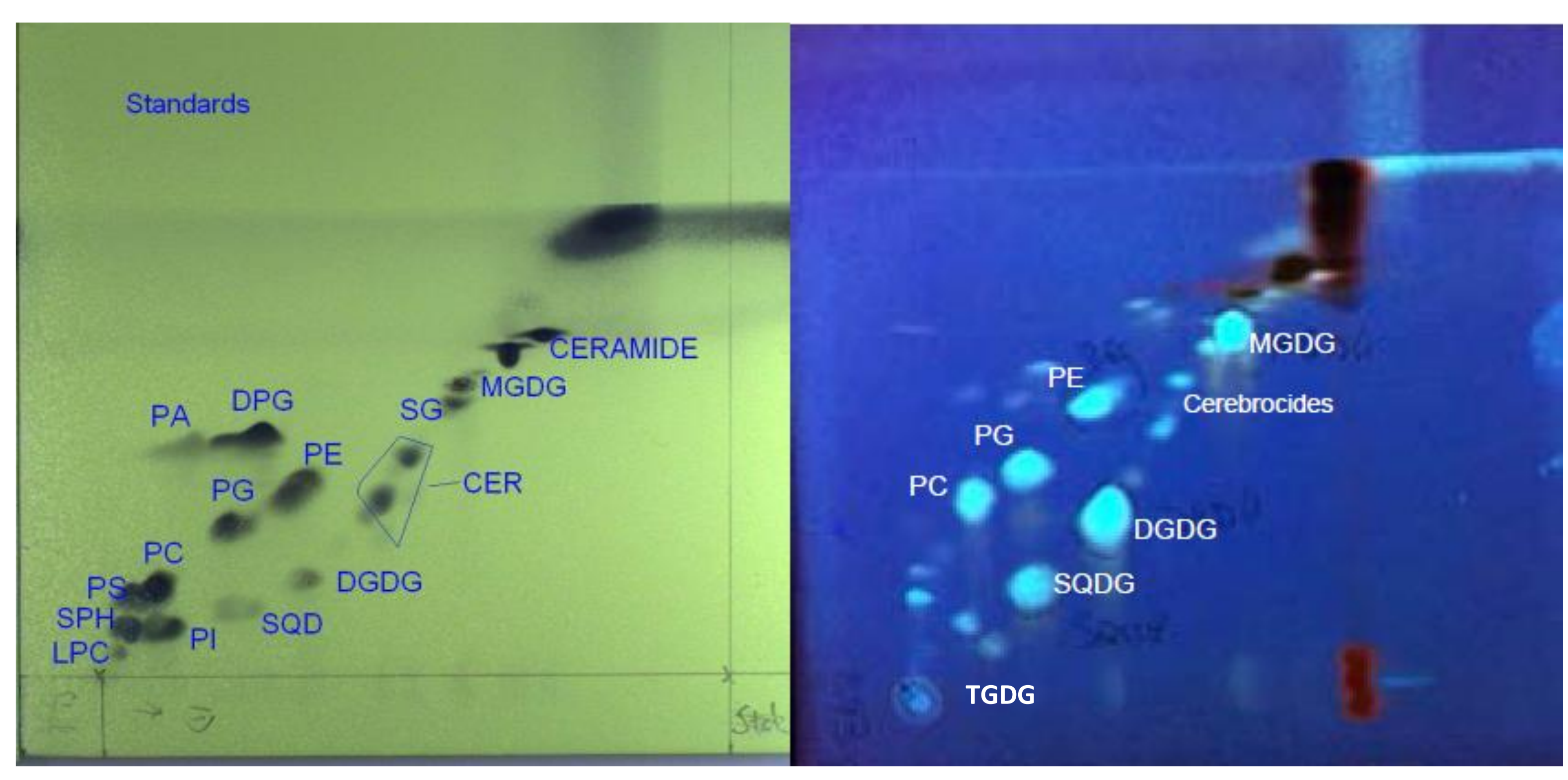

Figure 3.4 Polar lipids separation in chloroplast lipids; PC, PG, PE, MGDG, CER, DGDG, SQDG and TGDG

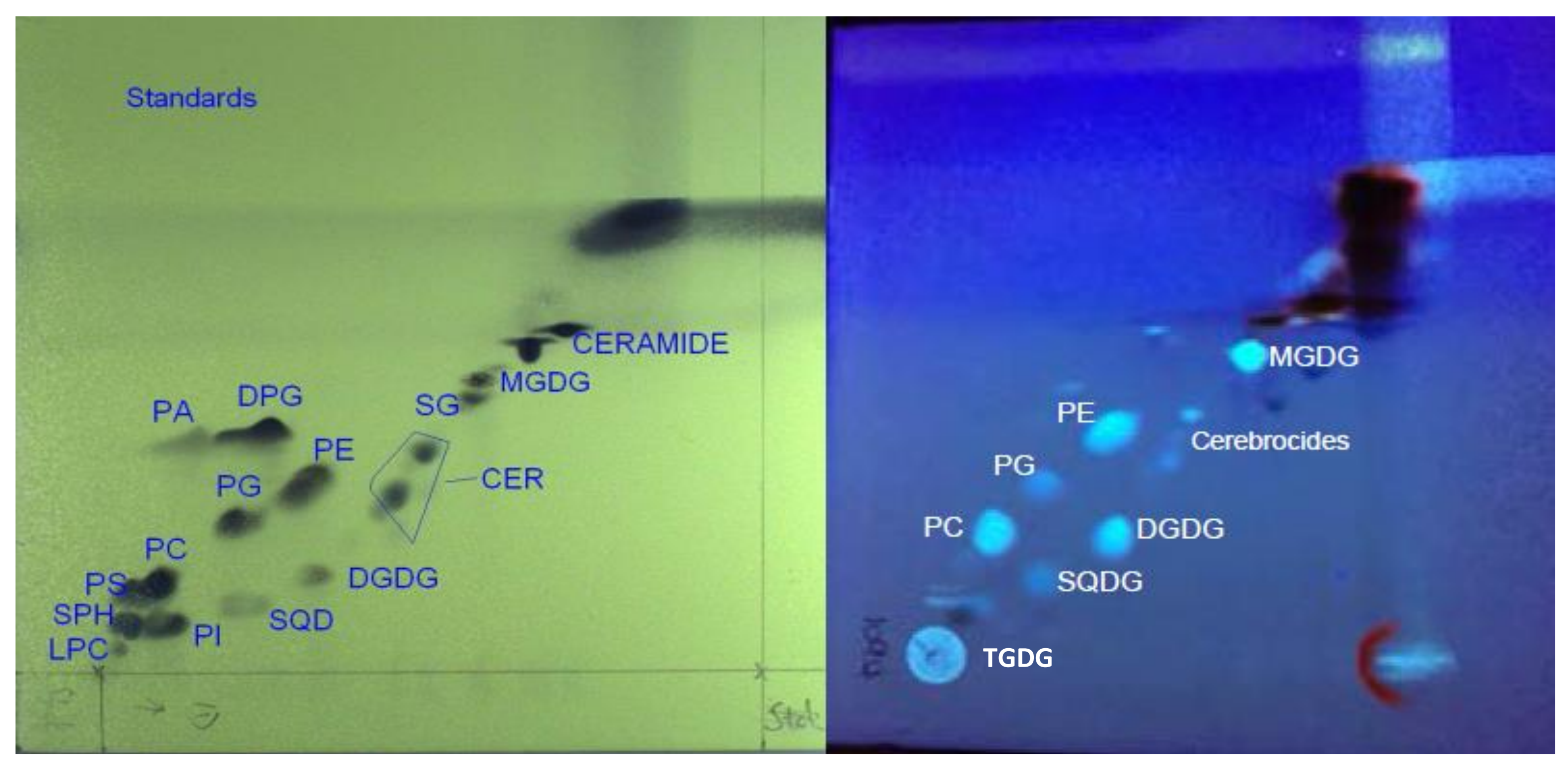

Figure 3.5 Polar lipids separation in leaves lipids; PC, PG, PE, MGDG, CER, DGDG, SQDG and TGDG

The quantitation of the polar lipid classes was carried out using GC and for quantifying the total amount of fatty acids, see Table 3.2 for the results. Since the analysis was carried out at a price by an external laboratory, samples were analysed only once. However, the results for spinach leaves extract analysis reported in this thesis are similar to those obtained for an independently prepared batch of spinach leaves extract following the same protocol and analysed at the same external laboratory (Chen, 2016). The ratio of phospholipids to glycolipids found was comparable to that reported by Dörmann and Benning (2002), where about 70% glycolipids content was detected in the chloroplast. The phospholipids ratio was higher in leaves than in the chloroplast, which was similar to the study of Wintermans (1960).

Table 3.2 Polar lipids compositions of spinach leaf and chloroplast (mg/ g lipids)

	Total phospholipids (PhL)	Total glycolipids (GL)	Ratio PhL:GL	Individual glycolipids				Ratio M:D:T:S
				MGDG (M)	DGDG (D)	TGDG (T)	SQDG (S)	
Chloroplast	181.8	418.9	30:70	202.4	124.2	53.2	39.1	48:30:13:9
Leaves	201.6	240.7	46:54	113.7	79.6	24.8	22.6	47:33:10:9
Leaves (Chen, 2016)	198.3	224.4	47:53	101.4	92.1	4.4	26.5	45:41:2:12

Yunoki et al. (2009) have reported the amount of glyceroglycolipids in spinach leaves but ignoring the presence of TGDG, thus they obtained a higher percentage of MGDG from the total glycerolipids which was at 57%. The amount was just slightly higher than in this study which was 53% when TGDG was ignored in the calculation. The percentage of DGDG and SQDG were also comparable with the study of Yunoki et al. (2009).

MGDG is highly important for the function of photosynthesis, and thus, is concentrated in the chloroplast. As photosynthesis takes place in the thylakoid of the chloroplast, MGDG is abundant in the thylakoid and the inner envelope of a chloroplast while DGDG and PC are abundant in the outer envelope of the chloroplast (Dörmann, 2013). DGDG is also high in non-photosynthetic tissue, for example fruits, roots, flower and stem (Block et al., 1983).

The fatty acid composition of lipids found in spinach chloroplasts and leaves fractions can be seen in Table 3.3. Generally, all polar lipid classes in spinach have a high amount of polyunsaturated fatty acids. The two main trieonic acids in spinach are hexadecatrienoic acid and α-linolenic acid, and the latter is highly abundant. MGDG and DGDG were seen to be made up of more than 75% α-linolenic acid and this was similar to that observed in previous work

Table 3.3 Fatty acid composition of polar lipids from spinach leaves and chloroplasts (% total lipids)

Fatty acids	Total Polar Lipids		MGDG		DGDG		TGDG		SQDG	
	CH	**L**	**CH**	**L**	**CH**	**L**	**CH**	**L**	**CH**	**L**
14:0 (myristic)	0.1	0.1	-	0.1	0.1	0.2	4.2	2.0	0.5	0.7
15:0 (pentadecylic)	-	0.1	-	-	-	-	-	-	-	0.4
16:0 (palmitic)	6.8	12.5	0.7	0.6	4.3	5.8	24.1	15.0	41.6	45.6
16:1 (palmitoleic)	3.8	3.0	0.3	0.1	1.1	0.2	25.2	8.3	3.4	0.6
16:2 (polyenoic)	0.1	0.1	-	0.1	0.2	0.2	6.7	2.0	0.7	0.7
17:0 (margaric)	-	-	-	-	0.1	0.1	-	-	-	-
16:3(n-3) (hexadecatrienoic)	11.1	0.8	18.6	20.6	3.3	3.3	-	-	0.6	1.0
18:0 (stearic)	0.4	0.6	0.3	0.2	0.8	1.1	13.9	16.3	2.5	2.3
18:1(n-9) (elaidic)	1.0	2.7	-	0.1	0.4	0.9	2.9	4.4	2.9	0.6
18:1(n-7) (vaccenic)	0.7	1.0	0.4	0.4	1.6	1.4	-	-	0.5	0.6
18:2(n-6) (linoleic)	4.6	11.3	0.9	0.9	1.1	2.0	3.3	2.1	5.3	3.2
18:3(n-3) (α-linolenic)	70.7	58.2	78.7	76.3	86.1	83.2	-	3.1	40.3	42.4
20:0 (arachidic)	0.1	0.4	-	0.1	0.2	0.2	4.4	21.3	0.5	0.5
20:1 (gondoic)	0.1	0.4	-	-	-	-	-	-	-	-
20:3(n-3) (mead)	0.2	0.1	-	0.1	0.3	0.4	-	-	-	-
22:0 (behenic)	0.1	0.4	-	0.1	0.2	0.2	4.3	20.9	0.5	0.5
22:1 (erucic)	-	-	-	0.2	-	-	-	-	-	-
24:0 (lignoceric)	0.2	0.9	-	0.2	0.3	0.8	11.1	4.6	0.6	1.1

(Gounaris et al., 1983a, Melo et al., 1995). The high content of polyunsaturated fatty acids in chloroplast lipids is important so as to maintain their biological function at low temperatures (Andersson and Dörmann, 2009). The amount of hexadecatrienoic acid was quite high in MGDG and comparable to what has previously been reported for solanaceous leaves (Whitaker, 1986). The presence of hexadecatrienoic acid (16:3) classifies spinach as a 16:3 plant (Andersson and Dörmann, 2009). This classification is given as only certain plants have this fatty acid (Andersson and Dörmann, 2009).

Saturated fatty acids like palmitic acid are also present in quite considerable amounts in TGDG and SQDG. In contrast to the other two galactolipids, TGDG was low in polyunsaturated fatty acids like α-linolenic acid, but had a relatively high amount of saturated fatty acids especially in the leaves fraction. The fact that an equal amount of 16:0 and 18:3 fatty acids were found in the composition in SQDG was also reported by Siebertz et al. (1979). This characteristic distinguishes SQDG from galactolipids and phospholipids (Siebertz et al., 1979). The total polar lipids found in leaves have a slightly higher amount of saturated fatty acids in general in comparison to the lipids found in chloroplasts. Even though heating was applied in the extraction method, the results obtained from the lipid analysis show that this extraction method was reliable as all the lipid class associated to thylakoid membrane were detected and the data

reported was comparable to the previous literature such as Dörmann and Benning (2002) and Yunoki et al. (2009).

3.3.3 Adsorption of spinach lipids at water/oil interfaces

The interfacial tension was measured at the water/oil interface utilising the pendant drop technique where a water drop was formed at the tip of a capillary and suspended into the oil phase. The oil phase was either purified sunflower oil or purified sunflower oil/ surfactant solution. The purified sunflower oil/ surfactant solutions were first prepared by mixing the oil with the surfactant at a maximum concentration of 0.005% (w/w). The concentration had to be low because of the deep green colour of spinach lipids. At higher concentrations, the colour would have interfered with the droplet imaging capability of the equipment to analyse the droplet shape and interfacial tension. Measurement was carried out for 900 s and the interfacial tension values acquired during the period are shown in Figure 3.6. Each graph includes reference data at 0% added surfactant in the oil phase with an equilibrium interfacial tension value of 30 mN/m ± 1 mN/m which is comparable to previously reported values (Gülseren and Corredig, 2012, Gaonkar and Borwankar, 1991).

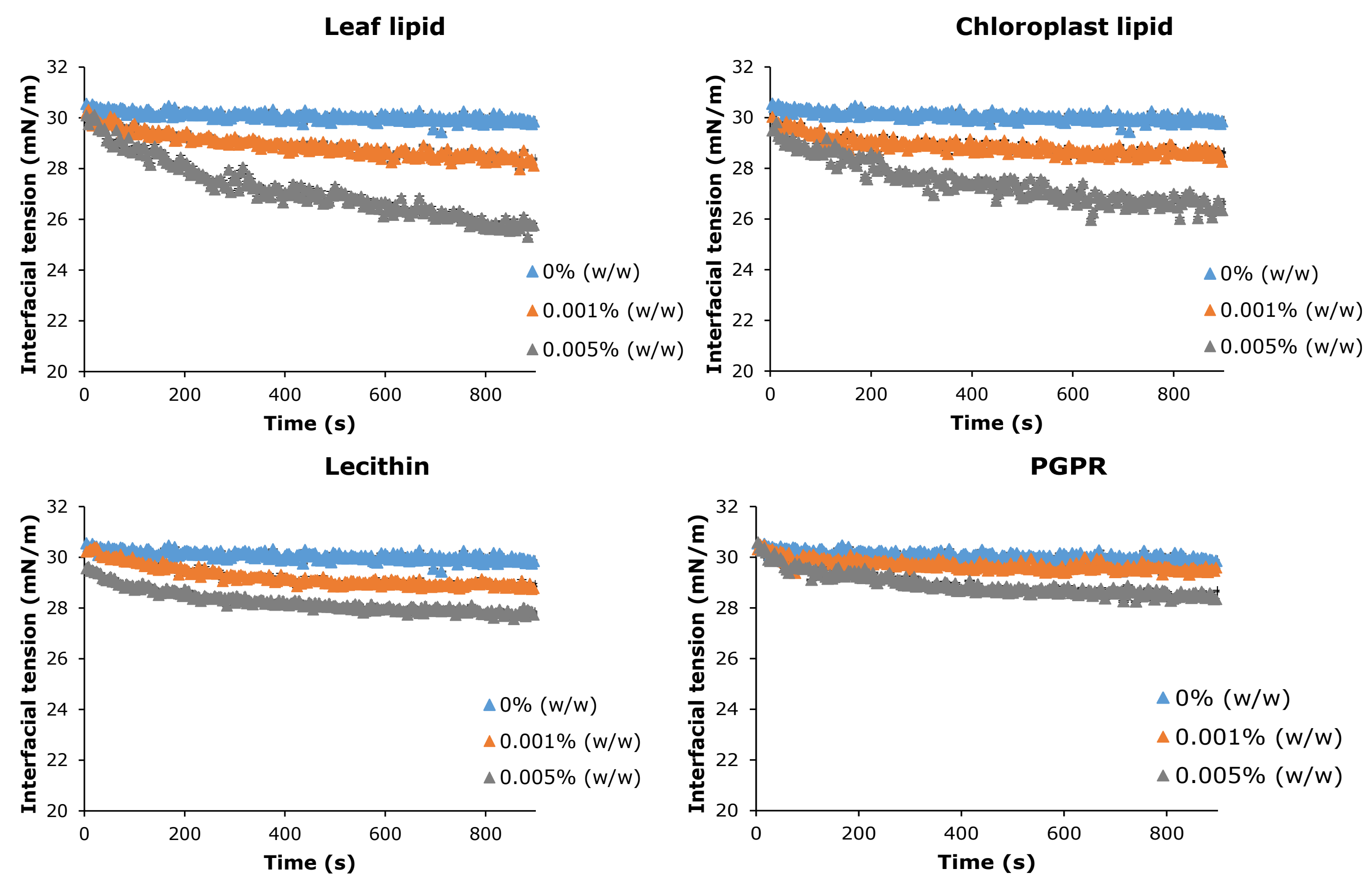

Figure 3.6 Surface tension at the water-oil interface of added surfactant either with leaf lipid, chloroplast lipid, lecithin or PGPR. Data plotted as means ± 1 standard deviation for n=3

Generally, lipids from both spinach chloroplasts and leaves were seen to be more interfacially active than lecithin or PGPR in the narrow range of concentrations applied in this research. The data show that all surfactants tested tend to lower equilibrium interfacial tension values although it appears that a plateau has not quite been reached at the end of the 900 s measurement period for lecithin and leaves lipid. The results validate the proposed interfacially active nature of the lipid extracts under investigation in this research which, based on their composition, should not be surprising. The results also suggest that the galactolipids are more interfacially active than phospholipids although the phospholipid composition of lecithin will be different from the phospholipid composition of the spinach lipid extracts, which might be the reason behind this observation.

When comparing all of the oil/ surfactant mixtures analysed PGPR appears to be the least interfacially active. Even with the highest concentration applied, PGPR reached its plateau before the end of the 900 s which shows that PGPR has reached the limit of interfacial reduction. On the other hand, spinach leaves and chloroplast lipids were still decreasing the interfacial tension and therefore it can be hypothesised that, due to the complex composition of these two systems, long term re-arrangements are still ongoing. Hence, interfacial tension should be followed for longer in any future tests. It may also be possible that some of the molecules de-sorb into aqueous phase, adding to the complexity of what is going on at the

interface. The partitioning coefficient should be acquired as a fundamental parameter to support the correct interpretation of the adsorption kinetics data, because a surfactant solubility in water (due to polarity of the surfactant) always exists, causing a transfer across the interface during the dynamic process (Ravera et al., 1997).

In order to be able to fully understand the results, the different fractions of the spinach lipid extracts would need to be individually considered for their potential as a surfactant and the lecithin would need to be analysed for composition. PGPR is an excellent surfactant for water-in-oil emulsions (Wilson et al., 1998), thus one would maybe expect a noticeable lowering of the interfacial tension value. This has indeed been reported by Gülseren and Corredig (2012) who observed a significant decrease from 30.5 mN m^{-1} (0% PGPR) to about 25 mN m^{-1} (0.001% PGPR) at an interface of soy oil/ water. However, Bahtz et al. (2016) reported a similar result to that shown in the research presented in this thesis where the presence of PGPR at 0.001% in MCT (medium chain triglyceride) had no impact on the interfacial tension in water phase. The pure MCT/water system was reported to have a value of 21.0 mN/ m. These variations may be due to the source of the PGPR used (Gülseren and Corredig, 2012).

The high surface activity of spinach lipids was also supported by Chu et al. (2009). MGDG was reported to lower the interfacial tension of the oil to the extent that the oil drop did not stay at the tip of the needle long enough to allow any measurement. Spinach lipids are composed of a complex mixture of membrane lipids which consist of MGDG, DGDG, SQDG and PG. Each of these may play an important role in reducing the interfacial tension.

3.4 CONCLUSIONS

The research reported in this chapter comprised of the extraction of polar lipids from spinach leaves and spinach chloroplasts including yield, compositional characterisation and interfacial tension at water/oil interface. Based on the weight of parent leaves, a higher amount of polar lipids was recovered from the leaves fraction compared to the chloroplast fraction. The ratio of the composition of the lipids in the glycolipid class were comparable in both spinach leaves and chloroplasts. The ratio of the amount of phospholipids to glycolipids present however was higher in leaves than the chloroplasts, showing that chloroplast lipids were concentrated with glycolipids. The amount α-linolenic acid was relatively high in MGDG and DGDG compared to other fatty acids in both spinach leaves and chloroplasts. However, chloroplast lipids showed a higher amount of α-linolenic acid in their composition than the leaves fraction, while

the lipids in leaves showed a higher amount of palmitic acid. This shows that leaves lipids are more saturated than the chloroplast lipids. A lower interfacial tension measurement at the water/oil interface of both spinach leaves and chloroplast lipids when compared to the commercial surfactants suggest that both lipids have at least a comparable performance with the commercial surfactants as a surface active ingredient. Even though lipids from both fractions showed a comparable reduction in the interfacial tension, slight variations of the ratio of phospholipids to glycolipids highlight the reason to investigate the performance of both lipids as a flow enhancer. This was carried out as next steps in the current PhD research and the results are reported in the subsequent two major chapters. Should performance indeed be similar, leaves lipids would be commercially more interesting due to the higher yield and simpler extraction process.

4 SPINACH LIPIDS FROM LEAVES AND CHLOROPLASTS AS A FLOW ENHANCER IN SUGAR/OIL SUSPENSIONS

In the previous chapter, the results from the compositional analysis of spinach lipids from leaves and chloroplasts were reported. Also reported were the interfacial tension values at the water/sunflower oil interface when the lipids were added to the oil phase in comparison to, lecithin and polyglycerol polyricinoleate (PGPR) which are commonly applied to chocolate. The results showed promise for spinach lipids to be tested as a natural flow aid in chocolate formulations, and the next step would be to test their performance in sugar/oil suspensions as a model system often used for chocolate. The corresponding results with brief introduction, material and methods sections relevant to this part of the PhD research, are presented in this chapter.

4.1 INTRODUCTION

Spinach lipids have been reported to have a high surface activity due to the abundance of galactolipids in their membrane lipids composition (Rayner et al., 2011a, Rayner et al., 2011b). This was confirmed in this research by the data shown in Chapter 3 where spinach leaves were used as a representative of green leaves tissue and the functionality of their extracted lipids as flow enhancer investigated. Should this research confirm flow enhancing properties

then one would hypothesise that other green leaf tissue lipid extracts would perform similarly well and one could consider using waste green leaf material, for example pea vine.

In these experiments, the polar lipid fraction extracted from whole spinach leaves as well as the polar lipid fraction extracted from spinach chloroplasts were tested. If the whole leaf extract was to show equivalent performance as a flow enhancer, it would be more economical to use this than having to extract the chloroplast first and then the polar lipid fraction. In Chapter 3, it was shown that the lipid yield from the leaves was higher than from the chloroplasts. However, the concentration and type of lipid varied, so both the whole leaf and the chloroplast extracts were included in the investigation of the impact of the rheological properties of sugar/oil suspensions.

An investigation into the functionality of spinach lipids as a flow enhancer in lipid continuous suspensions has not yet been presented in literature. An indication that this functionality is likely to exist is given by the previously cited patent where oat lipids were demonstrated to have the ability to reduce the low shear rate viscosity of chocolate albeit less than lecithin but comparable with PGPR (Evans et al., 1991). This effect was claimed to be due to the DGDG within the oats with its high amount of unsaturated fatty acids (Evans et al., 1991).

Chocolate represents a suspension of sugar particles and cocoa solids dispersed in a continuous phase of cocoa butter containing a lipid surfactant and, sometimes, small amounts of other fats. The combined amount of solids usually represent about 0.68 volume fraction (Taylor et al., 2009), which means that chocolate is a highly filled suspension of high viscosity. A useful model that describes the concentration dependence of the relative viscosity of concentrated suspensions is the Krieger and Dougherty model (Equation 4.1).

$$\eta_r = (1 - \frac{\phi}{\phi_m})^{-[\eta]\phi_m} \qquad \text{(Equation 4.1)}$$

where η_r is the relative viscosity (viscosity of suspensions over viscosity of continuous phase), ϕ_m is the maximum packing fraction of particles by volume and $[\eta]$ is the intrinsic viscosity. The maximum packing fraction is when the particle concentration approaches a dense packing arrangement of the particles where there is no longer sufficient fluid to lubricate the relative motion of the particles, and the viscosity rises to infinity (Metzner, 1985). The value for $[\eta]$ is 2.5 for rigid spheres (Barnes, 2000), assuming there is no slip between the liquid and the particle surface, which is well fulfilled for isolated particles. The increase of the viscosity in chocolate is due to the amount of fat immobilized on the particle surfaces (Johansson and Bergenstahl, 1992a) and so the fat is no longer is available to aid the flow process. At the same time the yield stress rises (Beckett, 2009a).

Being able to finely adjust the flow properties of chocolate is important when considering its processing and sensory properties. A chocolate with high yield stress will complicate moulding processes as these involve low shear rates while high viscosity leads to difficulty in pumping. High viscosity also affects the sensory properties of chocolate resulting in a harder texture and a pasty taste in mouth making the chocolate difficult to swallow (Beckett, 2008). To overcome these challenges, a small quantity of surfactant is normally added to the continuous cocoa butter phase with lecithin and PGPR being the most commonly used surfactants in chocolate. It has been reported that the use of lecithin at 0.1-0.3% can reduce the viscosity up to 10 times more than the same weight of cocoa butter (Beckett, 2008). Therefore, the use of a surfactant causes a reduction in the amount of cocoa butter in a chocolate bar which leads to economic benefits to the manufacturer and consumer as cocoa butter is the most expensive ingredient in the formulation.

However, the issue of lecithin increasing the yield stress when added at more than 0.5% has required the incorporation of PGPR at least 0.2% to reduce the yield value. PGPR has a special property where it can reduce the yield stress of chocolate without significantly affecting the viscosity (Rector, 2000a, Schantz and Rohm, 2005). But, even though PGPR is considered to be safe by FDA (FDA, 2006) its use is restricted to 2.6 mg kg^{-1} of body weight per day (Wilson et al., 1998)

and, as it is not a natural surfactant, it has a negative consumer perception.

The research reported and discussed in this chapter was carried out to evaluate the potential of spinach lipids as a natural surfactant in a model system that is closely related to chocolate. In Chapter 3 of this thesis, it is shown that spinach lipids are more surface active than lecithin and PGPR. However, the adsorption of surfactant at the oil/water interface may be different to the adsorption of the same surfactant at the solid/liquid interface and, in this case, at the sugar/oil interface. This study was designed to evaluate the functional properties of spinach lipids in comparison to soy lecithin and PGPR, and an attempt was made to explain the mechanistic molecular interactions of spinach galactolipids at sugar/oil interfaces in affecting the flow properties of sugar/oil suspensions.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Sugar/oil suspensions were prepared from icing sugar and sunflower oil which were bought in a local supermarket. Icing sugar is frequently used as the sugar component in a study of the flow behaviour of model chocolate suspensions (Arnold et al., 2014, Arnold et al.,

2013). The particle size of icing sugar is lower than that of other commercially available sugar and is comparable to the size of the sugar particles in chocolate. In traditional chocolate manufacture, sugar is pre-crushed into powdered sugar before being mixed with the cocoa mass, milk powder and other ingredients (Krüger, 2009). Icing sugar is highly hygroscopic due to the effect of grinding which possess amorphous surface layers and absorbs moisture at lower relative humidity than in the case of crystallized parts of sugar (<65% relative humidity) (Krüger, 2009). Thus, the icing sugar was dried at 60°C for 24 h using a vacuum oven (Gallenkamp, Fistreem International, Loughborough, UK) and then left to cool in a desiccator prior to suspension preparation.

Before being used as a suspension medium, the sunflower oil was freed from surface active impurities as described in Section 3.2.6. Presence of these impurities would render as challenging the interpretation of the suspension rheology data acquired to assess the performance of spinach lipid extract as flow enhancer in comparison to the commercially applied lecithin and PGPR. Both the spinach leaf lipid extract and spinach chloroplast lipid extract prepared as described in Section 3.2.3 and 3.2.4, respectively, were tested to assess their abilities as a flow enhancer. Their performance was compared to the commercially applied lecithin (LEC) and PGPR; both surfactants were the same as used for the earlier reported interfacial tension measurements (see Section 3.2.1 for their specifications).

4.2.2 Icing sugar particle size distribution

While the particle size of the icing sugar was not a variable of the sugar/oil suspension system in this research, it was quantified due to the impact of particle size distribution on the flow behaviour of suspensions. Indeed, this has previously been demonstrated for icing sugar applied in sugar/oil suspensions (Arnold et al., 2013, Arnold et al., 2014). The particle size of the icing sugar was determined using laser diffraction equipment (Beckman Coulter LS13320, Meritics, Wycombe, UK) fitted with a dry powder module (Beckman Tornado Dry Powder System, Meritics, UK). Measurement was carried out on three replicates and the averaged (mean) volume based particle size distribution is shown in Figure 4.1. The distribution appears to be tri-modal where the peak at the highest particle diameters may be representative of particle aggregates while the peak at the smaller particle diameters may indicate the presence of sugar dust.

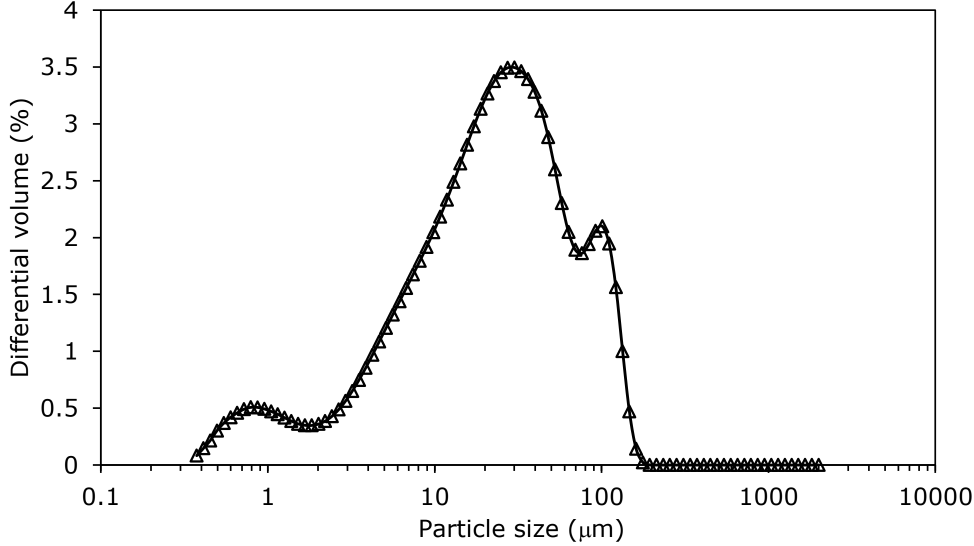

Figure 4.1 Particle size distribution of the icing sugar ingredient

Since the calculation of characteristic values for particle size distributions assumes the distribution to be monomodal, the three populations apparently present in this sample of icing sugar were separated and characteristic values calculated for each using the equipment's software. The results are shown in Table 4.1. Volume (%) shows the total amount in percent of particles in that particular group of particle size. The volume based diameter D(4,3) was also included in describing the particle size and calculated as a volume weighted mean diameter. The volume based characteristic particle sizes for which 10, 50 and 90% of the particles were smaller than D_{10}, D_{50} and D_{90} were also calculated.

Table 4.1 Characteristic size distribution values for the three particle populations of the icing sugar sample used in the sugar/oil suspension system

Size boundary	**0.375 – 1.832 µm**	**1.832 – 76.43 µm**	**76.43 – 194.2 µm**
Volume (%)	6.43 ± 0.22	80.47 ± 0.06	13.10 ± 0.30
D(4,3) (µm)	0.97 ± 0.01	26.05 ± 0.16	107.33 ± 3.12
D_{10} (µm)	0.53 ± 0.01	5.59 ± 0.07	81.70 ± 0.40
D_{50} (µm)	0.91 ± 0.01	21.83 ± 0.25	104.43 ± 2.87
D_{90} (µm)	1.55 ± 0.01	54.20 ± 0.18	137.07 ± 5.34

4.2.3 Bulk density determination of sugar

The density of sugar was determined based on the bulk density by the following steps. About 50 g of sugar was dispersed into 50 g of sunflower oil. The dispersion was carried out with a digital impeller stirrer (IKA Werke, Staufen, D) at the rotational speed of 1000 rpm for 60 min. The dispersion was then poured into a pre-weighed cylinder and the weight of 50 ml dispersion was determined. The step was carried out for at least three replicates. Therefore, the volume of 100 g dispersion can also be determined.

Since the purified sunflower oil has a density of 0.9197 g/cm^3 from the measurement using a density meter (Anton Paar, Germany) the density of sugar can be calculated from the Equation 4.2;

$$\text{Vol. of 100 g dispersion} = \text{vol. of 50 g sugar} + \text{vol. of 50 g oil} \qquad \text{(Equation 4.2)}$$

Density is equal to mass over volume and thus the density of sugar can be determined. The density of icing sugar used was calculated to be 1.5513 ± 0.04 g/cm^3 from the bulk density determination. The density of icing sugar obtained was comparable to the density of icing sugar from literature which was reported to be 1.58 g/cm^3 (Arnold et

al., 2013). The volume fraction of each sugar concentration can then be calculated and is shown in Table 4.2.

Table 4.2 Volume fraction of sugar of the model suspensions

Sugar mass fraction (%)	Weight of sugar (g)	Weight of oil (g)	Volume of sugar (cm^3)	Volume of oil (cm^3)	Volume of suspens-ion (cm^3)	Sugar volume fraction, Φ
40	40	60	25.78	65.24	91.02	0.28
45	45	55	29.00	59.80	88.8	0.33
50	50	50	32.23	54.37	86.6	0.37

4.2.4 Sugar/oil suspension preparation and composition

Sugar/oil suspensions were prepared by initially dissolving the surfactant into the oil phase (purified sunflower oil) at the desired concentration. The procedure applied for both the spinach leaf lipid and the chloroplast lipid as surfactant was the same as described in Section 3.2.7. Lecithin or PGPR was added into the purified sunflower oil by weighing the required amount of surfactant into a pre-weighed amount of sunflower oil in a glass bottle followed by mixing on a magnetic stirrer for 24 h. Glass containers were used to avoid any chemical reaction of the surfactant with the material of the container, as could be the case for plastic.

Sugar/oil suspensions to act as the chocolate model system were then prepared by dispersing the required amount of icing sugar into the oil phase containing the dissolved surfactant. Sugar mass fractions used in this research were 0.4, 0.45 and 0.5 which is relevant to chocolate application with sugar contents of typically between 30% and 55% (Wohlmuth, 2009). These particular concentrations were chosen since a lower amount of sugar would potentially not show up the flow enhancing properties due to the decreased level of interparticle interaction and since the typical amount of sugar in dark chocolate is 44.5% (Wohlmuth, 2009). The dispersion of the sugar was carried out with a digital impeller stirrer (IKA Werke, Staufen, D) by applying a rotational speed of 1000 rpm for 60 min as in the reference published by Arnold et al. (2013). The suspensions were then allowed to gently mix for 24 h using an overhead shaker (Reax 2, Heidolph, Schwabach, D) prior to rheological assessment.

The surfactant concentrations in the final suspensions were 0 (Control), 0.1, 0.3, 0.5 and 0.7% (w/w). This concentration range was chosen as commercial chocolate contains between 0.3 – 0.5% (w/w) lecithin and 0.2 – 0.3% (w/w) PGPR (Beckett, 2009c).

4.2.5 Rheology

To assess the functionality of the spinach lipid extracts as a flow enhancer in comparison to the commercially applied surfactants lecithin and PGPR, steady shear viscosity curves of the sugar/oil suspensions were acquired using a rotational shear rheometer (MCR 301, Anton Paar, Graz, A) fitted with concentric cylinder geometry with a bob diameter of 26.66 mm, a cup diameter of 28.92 mm and a bob length of 39.99 mm (CC27, Anton Paar, Graz, A). Published protocol (Arnold et al., 2013) was followed and slightly modified by starting the measurement with a pre-shear at 10 s^{-1} for 50 s. The pre-shear was carried out in order to reach equilibrium in the interaction between the particles and therefore to reduce the likelihood of slip occurring during the measurement since, according to Servais et al. (2003), a smooth bob and cup geometry has high risk of producing slip at low shear rates. Shear rate was then increased from 0.01 s^{-1} to 1000 s^{-1} in a logarithmic ramp within 990 s. After maintaining shear at 0 s^{-1} for 120 s, the shear rate was decreased from 1000 s^{-1} to 0.01 s^{-1} (logarithmic ramp, 990 s). Fifty-one data points were taken in each logarithmic ramp with the measurement time logarithmically decreasing from 100 s at 0.01 s^{-1} to 0.5 s at 1000 s^{-1} and then increasing again to 100 s at 0.01 s^{-1} for the decreasing shear rate ramp. The measurement temperature was 22°C.

The results are reported as viscosity curves and as apparent viscosity and yield value taken as the viscosity at 40 s^{-1} and shear stress at 5 s^{-1} from the increasing shear rate ramp. This method was introduced by Servais et al. (2003) as the most appropriate method to measure and report apparent viscosity and yield value for chocolate systems using a bob and cup geometry.

4.2.6 Statistical analysis

All results were analysed statistically using one way ANOVA to compare means. Significant difference between samples was analysed using Tukey HSD (Honestly Significantly Different) multiple comparisons test at 95% significance level. The software used was IBM SPSS Statistics 22.

4.3 RESULTS AND DISCUSSION

This section starts with the presentation of the impact of surfactants on the flow behaviour of sugar/oil suspensions. Viscosity curves show the implication on the viscosity and stress during the increasing shear. To understand the impact of surfactants on the properties measured in real chocolate, the recommendation by The International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC) was

followed and shown in the second section. The value of apparent viscosity of the suspensions were determined at 40 s^{-1} and the value of yield was the shear stress at 5 s^{-1}. A molecular approach to the impact of polar lipids is then discussed based on the result of flow behaviour and the properties on the yield value and apparent viscosity.

4.3.1 Viscosity curves

The viscosity curves of the sugar/oil suspensions as affected by the four different surfactants investigated are shown in Figures 4.2 - 4.4. Four different concentrations of the surfactants were applied to each of the mass fractions of sugar (40%, 45% and 50%). The data correspond to the relative viscosity values measured on the increasing shear rate ramp. However, in order to see the effect on shear behaviour, the data were plotted as a function of increasing stress. The curve then was evaluated by looking at the minimal shear stress that caused a reduction of the viscosity during the increasing stress. The minimal stress is subsequently also referred to as "the critical stress". In the steady shear experiment, as in the case of this study, the critical stress is defined as the stress required for the sample to start flowing (Taylor et al., 2009). Since the addition of surfactant had no impact on the viscosity of the sunflower oil and showed a Newtonian behaviour at all of the concentrations of

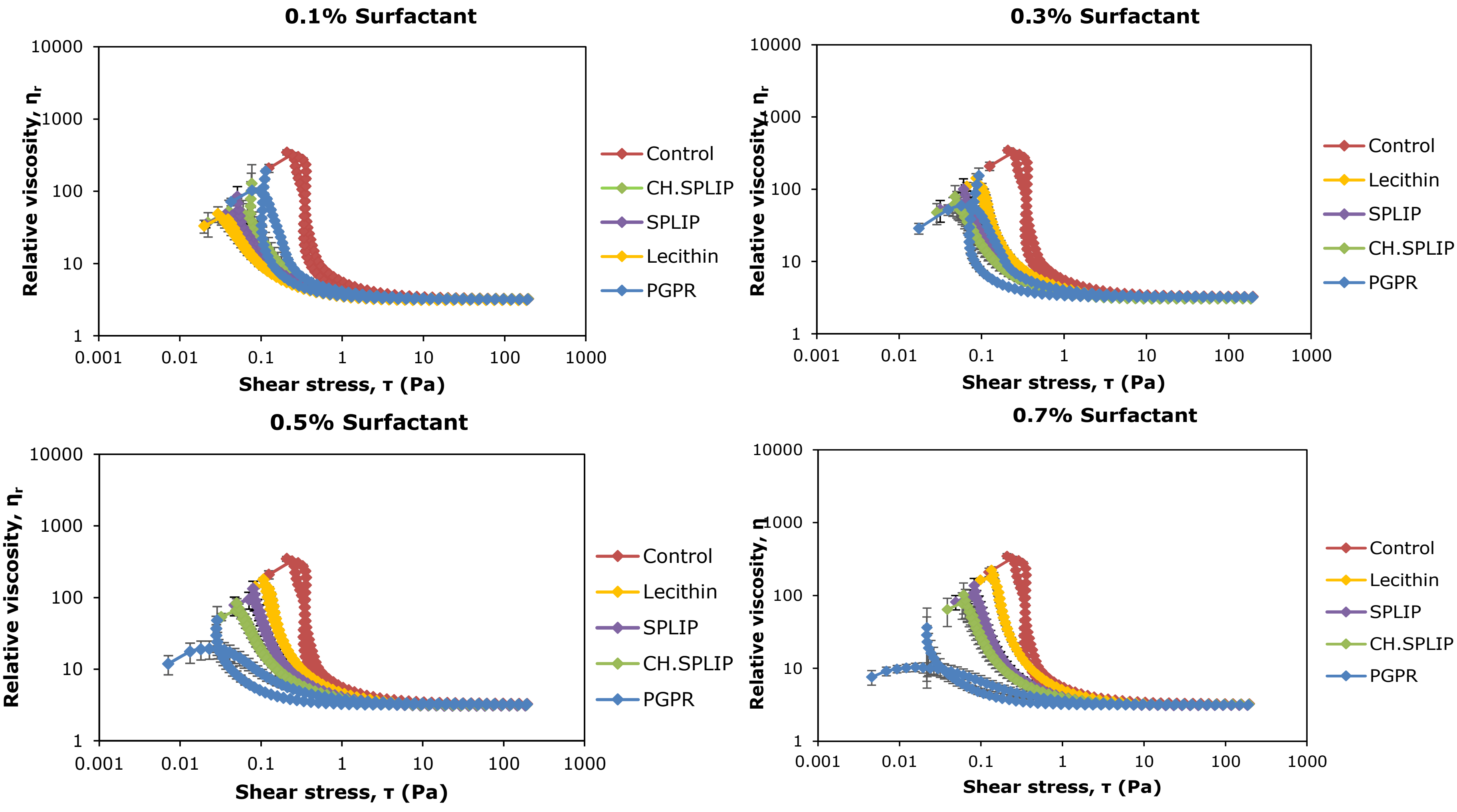

Figure 4.2 Viscosity of sugar/oil suspensions at 40% sugar content as affected by the type and concentration of surfactant at 22 ºC (means ± 1, n=4).

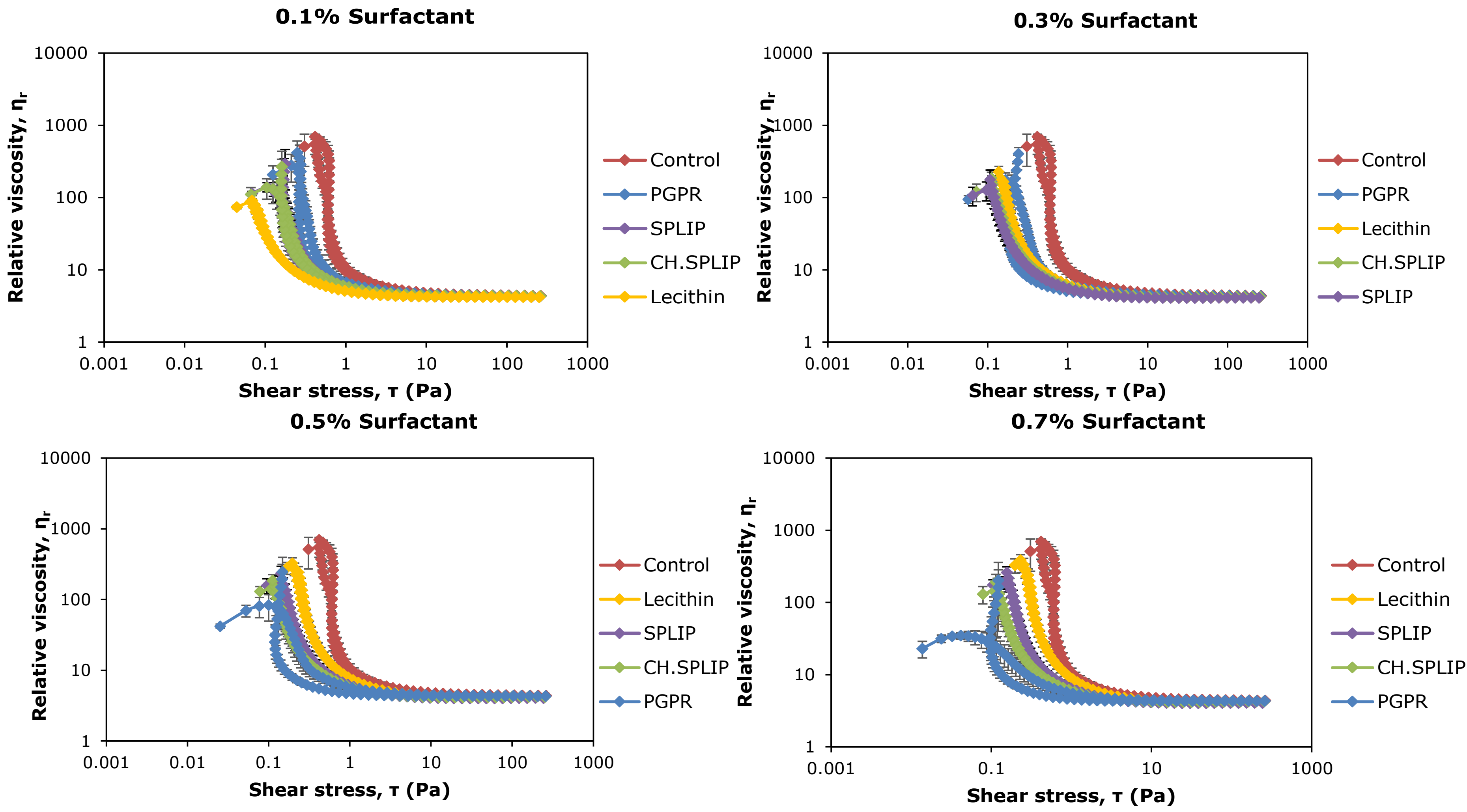

Figure 4.3 The flow curve of viscosity of sugar/oil suspensions at 45% sugar content as affected by the concentration of surfactants (means ± 1.0, n=4).

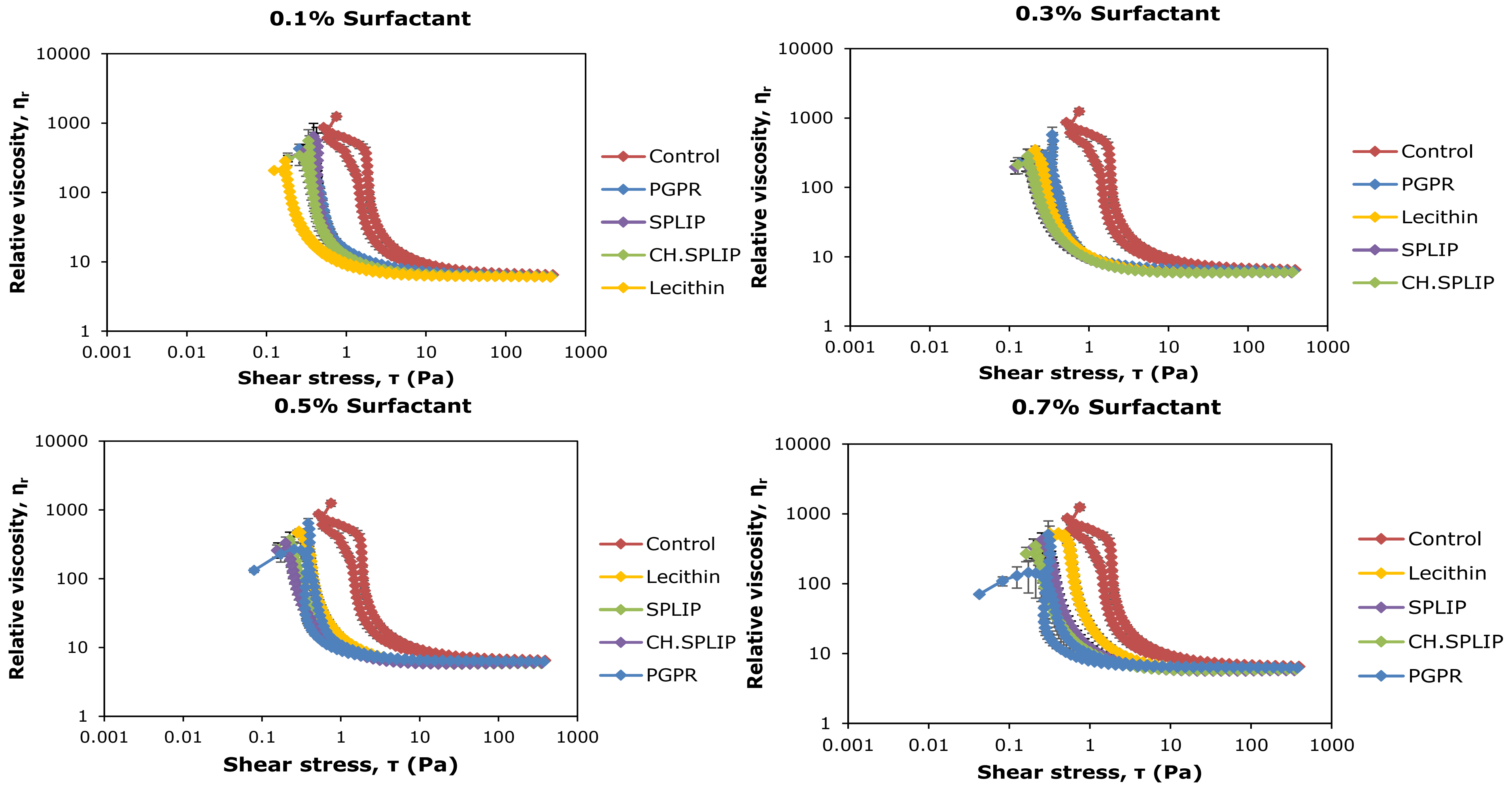

Figure 4.4 The flow curve of viscosity of sugar/oil suspensions at 50% sugar content as affected by the concentration of surfactants (means ± 1.0, n=4).

surfactant, the viscosity of all of the medium phases was taken as an average of 0.060 ± 0.002 Pa.s. The behaviour of the suspension was only observed for the curve of ascending rate.

The sugar/oil suspensions generally showed shear thinning behaviour and a transition to a Newtonian plateau at high shear stress, irrespective of surfactant type and concentration. The control sample showed the highest value of viscosity at its critical stress value. The critical stress of the control sample with 40%, 45% and 50% sugar was around 0.5 Pa, 0.85 Pa and 1.35 Pa, respectively. Application of surfactants into the sugar/oil suspensions reduced the critical stress value of the suspensions. The value varied depending on the surfactant used. The viscosity value followed the trend of the critical stress value. The lower the critical stress shown by a surfactant, the lower the viscosity value.

Lecithin exhibited the lowest value of critical stress when used at the lowest concentration which was 0.1% (w/w). This was followed by both spinach lipids and PGPR. The pattern was observed for all sugar concentrations. Since the only polar particle used in this study was sugar, lecithin adsorbed at the surface of the sugar and caused the suspensions to have lower viscosity and stress values. The strong effect of lecithin on sugar particles was also discussed in the published work of Ziegler et al. (2003). Increasing the concentration of lecithin

however increased the critical stress as well as the viscosity value of the suspensions. The impact was largest at the lowest sugar concentration (40%). The low amount of sugar caused the excessive lecithin to self-assemble to form a bilayer around the sugar (Beckett, 2008). Hence, the polar head of the second layer lecithin faced the lipophilic medium (oil) and caused the suspension to increase its critical stress value.

PGPR showed a very low stress at the start of the measurement. The stress then increased for several measurement points and decreased again. The point where the stress value started to decrease again during the increasing shear was defined as the critical stress. The low stress showed initially was most likely due to effect of the pre-shear at 10 s^{-1} for 50 s. PGPR was not as effective at low concentration as the higher concentrations. The impact was only efficient at 0.3% concentration or more, depending on the amount of sugar in the suspension. At the 40% sugar concentration, an addition of 0.3% PGPR led to a lower critical stress value than lecithin but higher than the suspensions with either of the spinach lipids. However, at higher sugar concentrations, PGPR showed the highest critical stress value as compared to the other surfactants. The impact of PGPR was very strong when it was added at the high concentrations. This can be seen in the suspension with the lowest amount of sugar. The addition of 0.7% of PGPR had dramatically reduced the critical stress of the suspension and caused the suspension to have a near Newtonian

behaviour. For the descending curve of PGPR, the viscosity was seen to keep increasing at the lowest shear shown. The mechanism behind this observed behaviour however was not fully understood. Nevertheless, PGPR was known to have a great impact in reducing the yield stress of chocolate while at the same time not greatly influencing the viscosity of chocolate (Rector, 2000b, Schantz and Rohm, 2005, Middendorf et al., 2015).

Spinach leaf and chloroplast lipids showed a comparable impact on the behaviour of the suspensions at all concentrations used (sugar and surfactant). At the 0.1% addition, spinach lipids generally reduced the critical stress of the suspension to a level higher than the suspension with lecithin but lower than the suspension with PGPR. As the concentration was increased to 0.3%, the critical stress of the suspensions with spinach leaf and chloroplast lipids was further reduced to the relatively lowest level compared to the other surfactants. Further increasing the concentration of the spinach leaf and chloroplast lipids in the suspension to 0.5% and 0.7% had no additional impact and results looked similar to those at 0.3% addition. At 0.7% addition, the suspension with the chloroplast lipid showed a lower critical stress value than the suspension with leaf lipid. The value would need to be evaluated in terms of significant differences to make it more meaningful. The recommended measurement of chocolate quality to calculate yield is at a shear rate of 5 s^{-1} (IOCCC, 2000). Therefore, the significant differences between spinach leaf and

spinach chloroplast lipids were only determined for the yield measured at the shear rate of 5 s^{-1}, as shown in the next section.

4.3.2 Apparent viscosity and yield value

As previously explained, it is common to report the flow properties of chocolate as apparent viscosity (η_{40}) and yield (τ) measured at shear rates of 40 s^{-1} and 5 s^{-1}, respectively. These values were subsequently extracted from the viscosity curves to demonstrate the potential value of spinach lipids as a flow enhancer alternative to lecithin and PGPR. The results are shown in Figure 4.5.

Generally, all surfactants significantly reduced the viscosity and the yield (5 s^{-1}) of the sugar/oil suspensions. At 0.1% surfactant, lecithin reduced the viscosity and yield (5 s^{-1}) to a value lower than the other surfactants at all sugar concentrations. However, the impact of lecithin was not significantly different ($P>0.05$) on the viscosity and yield (5 s^{-1}) shown by the leaf and chloroplast extracts, except for the viscosity with the highest sugar concentration. PGPR did not really lead to a pronounced viscosity reduction compared to the other surfactants and showed no significant difference ($P>0.05$) between

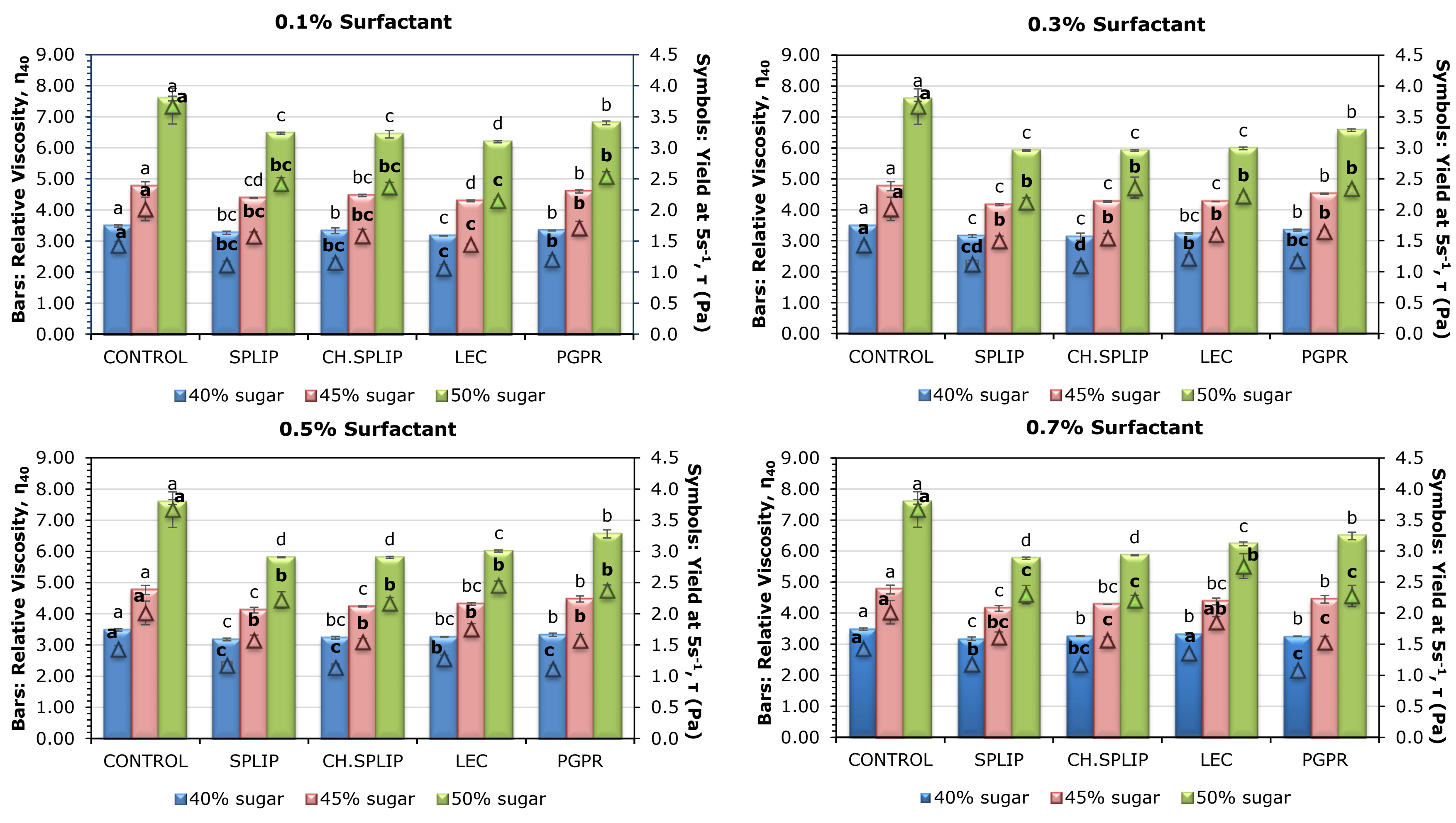

Figure 4.5 The apparent viscosity and yield value of sugar/oil suspensions. Letters indicate statistically significant difference (P<0.05) between samples of same sugar content. Bold letters refer to yield value.

it and both spinach lipids looking at the yield (5 s^{-1}) value. The behaviour of PGPR was in line with literature where it was shown that PGPR can reduce the yield (5 s^{-1}) value of chocolate without reducing the viscosity significantly (Rousset et al., 2002, Schantz and Rohm, 2005, Middendorf et al., 2015).

At 0.3% addition, all surfactants generally reduced the viscosity and the yield (5 s^{-1}) of the suspensions. Chloroplasts lipid showed significantly lowest ($P<0.05$) yield (5 s^{-1}) than lecithin and PGPR at 40% sugar concentration. While, looking at 45 and 50% sugar concentrations, both spinach lipids and lecithin showed similar impact on the flow properties of the suspensions. PGPR on the other hand was only impact similarly on the yield (5 s^{-1}) with leaf lipid and lecithin, but significantly ($P<0.05$) showed higher viscosity than the viscosity shown by other surfactants.

Increasing the concentration of surfactants from 0.3% to 0.5% resulted in no significant changes of the viscosity and yield (5 s^{-1}). This was apart from the values at the 50% sugar concentration, where both spinach lipids decreased the viscosity of the suspension slightly.

At the 0.7% surfactant addition, lecithin led to an enhanced viscosity value and significantly increased ($P<0.05$) the yield (5 s^{-1}) value compared to the other surfactants. For the spinach leaf lipid, a slight increase in the yield (5 s^{-1}) value was observed. Spinach lipids at this concentration generally reduced the viscosity of the suspensions with 45% and 50% of sugar to a value lower than lecithin and PGPR. The yield (5 s^{-1}) values were also low but not significantly different ($P>0.05$) to PGPR. Therefore, the behaviour shown by the spinach lipid extracts in this first research into their properties as a flow enhancer is promising in view of their potential future application as a natural surfactant in the chocolate industry.

4.3.3 Mechanistic model

It has been demonstrated that the addition of spinach lipids into the sugar/oil suspensions reduced the viscosity and yield (5 s^{-1}) value of the suspension to a lower value than lecithin and PGPR. To understand the behaviour shown by each surfactant and to compare their performance, it is important to consider their molecular structure as depicted in Section 1.10 and how this interacts with the surface of the sugar particle.

The action of lecithin in influencing the flow properties of oil based suspensions was described as a head-tail emulsifier; this has been discussed extensively in the literature (Beckett, 2008, Middendorf et al., 2015). The hydrophilic phosphatidyl groups of the phospholipid molecule interact with the hydrophilic surface of the sugar particles while the fatty acid chains are oriented into the continuous fat phase (Weyland and Hartel, 2008). Therefore, the sugar surface becomes coated with a phospholipid monolayer and acquires a nonpolar character (Dedinaite and Campbell, 2000).

PGPR, on the other hand, was reported to not follow the principle of a head-tail emulsifier like lecithin (Ziegler et al., 2003). However, while several hypotheses have been brought forward to explain the functionality of PGPR as a flow enhancer in chocolate, it was only recently that data substantiating one of these have been published. Middendorf et al. (2015) conducted a study based on atomic force microscopy (AFM) including the measurement of the adsorption forces to understand the interactions of PGPR with sugar in the medium of cocoa butter. As seen in Figure 4.6, PGPR was seen to interact with cocoa butter immobilized on the surface of sucrose forming pillow-like deposits between the individual sucrose particles, thereby separating these by steric hindrance

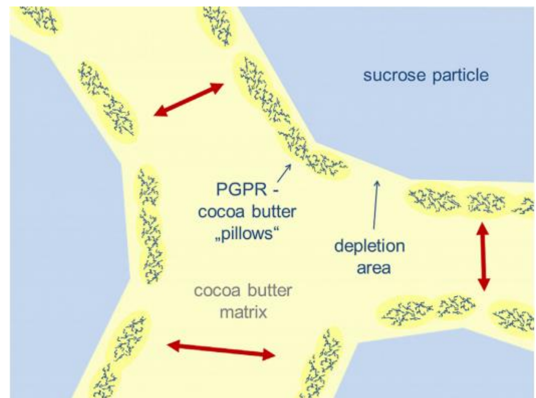

Figure 4.6 Illustration on the interaction of PGPR with the cocoa butter from the surrounding of sucrose and acts as pillow to restrict the interaction between sugar particles (Middendorf et al., 2015)

(Middendorf et al., 2015). The interaction of the immobilized cocoa butter and PGPR produces a space (depletion area) which needs to be filled by the cocoa butter from the bulk, thus increasing the amount of immobilized fat. As a result, PGPR does not affect the viscosity significantly. The pillow-like structures effectively reduce interaction between sucrose particles so they are able to repel each other and less structure is built up which subsequently has to be broken to induce flow. Consequently, the yield value is dramatically decreased (Middendorf et al., 2015).

Spinach lipids are a complex mixture of MGDG, DGDG, SQDG, PG and some PC as the polar lipids (Mazliak, 1977a). Each of these polar lipids have a different molecular structure that shows different behaviour when dispersed in pure water (Bottier et al., 2007, Chu et al., 2009). While MGDG and DGDG are neutral lipids (no charge), SQDG and PG carry a negative charge on their polar head group. MGDG has one galactose in the structure and thus appears to have a smaller head group than DGDG. Due to the small head group of MGDG, and with the bulk of lipophilic tails, MGDG molecules aggregate in the form of an inverted rod like structure; not forming bilayers but adopting the hexagonal-II (HII) phase with the polar head group facing toward the centre of the rod (Dörmann, 2013). On the other hand, DGDG has a larger sized polar head group so it adopts a cylindrical shape which in turn forms bilayers in pure water (Dörmann, 2013). Similar to DGDG, SQDG and PG also adopt a cylindrical shape and form bilayers when dispersed in pure water (Kobayashi, 2016).

The different structures of the polar lipids complicate the hypothesis about how they behave at the interface of sugar/oil. Specifically, total polar lipids found in chloroplasts contain about 20 mol% acidic lipids (15 mol% SQDG and 5%PG), 28 mol% DGDG and 50 mol% MGDG (Gounaris et al., 1983b). Even though the MGDG forms a non-bilayer structure,

there is no evidence showing the non-bilayer structure of spinach polar lipids in membranes (Webb and Green, 1990). The anionic lipids SQDG and DGDG have been demonstrated to play an important role in maintaining the bilayer structure of neutral lipids where screening out the lipids by increasing the cations or lowering the pH has resulted in the fusion of the vesicles and the appearance of non-bilayer structures (Gounaris et al., 1983b).

The non-bilayer phase of MGDG, however, is understood to be a thermotropic mesophase where, at relatively low temperatures, the MGDG can form a bilayer structure however, on heating it can transform into a non-bilayer structure (Quinn, 2012). The phase separation of the non-bilayer lipids was also reported in the thylakoid membrane of chloroplast when exposed to a high temperature (above 45°C) and formed aggregates of cylindrical inverted lipid micelle (Gounaris et al., 1984). It was suggested that this was due to the damage of chlorophyll-protein which is important in the grana stacking of chloroplasts. The heat-induced aggregation of thylakoid membranes were further studied by Ostbring et al. (2015) with regard to their emulsifying properties. Heating to above 55°C has facilitated the formation of larger droplets of oil-in-water emulsions which was suggested to be due to the degradation

of the chlorophyll molecules and the reorganization of the hydrophilic/hydrophobic parts inside the thylakoid membranes.

The spinach leaves were also heated in this study. To be specific, during the extraction of the lipids, the leaves or the chloroplasts were pre-heated with hot isopropanol (80°C) prior to the lipid extraction. Therefore, a non-bilayer structure of MGDG is could also be present in the lipids extract. However, understanding the behaviour of the polar lipids at the interface of sugar/oil is quite complicated as no previous studies have reported on the effect of heating on the emulsifying properties of chloroplast lipids in water-in-oil emulsions, which mimic the suspensions of sugar-oil. Nevertheless, since DGDG, SQDG and PG aggregate into a cylindrical form like the phospholipids, the lipids are expected to behave like lecithin, where at high concentration form a bilayer structure at the interface of sugar/oil and cause the polar head to face the oil phase as is found with lecithin. This hypothesised bilayer structure however was not shown to increase the yield measured at 5 s^{-1} (Figure 4.5) like lecithin. This is most probably due to the action of the MGDG where at high concentrations it would appear as an inverted micelle and help to maintain the low yield value of the suspensions. The negative charged lipids, PG and SQDG also play an important role in avoiding particle aggregation by the electrostatic repulsion reported by

Webb and Green (1990). However, the leaf lipid was shown to increase the yield (5 s^{-1}) value slightly when the concentration rises to more than 0.3%. This behaviour may due to the higher amount of phospholipids in the composition of leaves than chloroplasts.

4.4 CONCLUSIONS

Based on the rheological studies of sugar/oil suspensions, it was shown that both spinach leaf and chloroplast lipids affect the flow properties of the chocolate model suspension. Spinach lipids were seen to have a similar impact as lecithin does when added at least a 0.3% addition. Increasing the concentration of spinach lipids however does not increase the yield (5 s^{-1}) of the suspension as in the case of lecithin. Spinach leaf lipids and chloroplast lipids showed a comparable functional behaviour even though there was a slight difference in the lipid compositions (Chapter 3). Therefore, further investigation of spinach lipids in chocolate would reveal the potential of spinach lipids as a flow enhancer. Due to the higher recovery of lipids from the basic of parent leaves (Chapter 3), spinach leaf lipid was chosen to be applied in chocolate.

5 APPLICATION OF SPINACH LIPID INTO CHOCOLATE AS NATURAL FLOW AID AGENT

In the previous chapter of this thesis, it has been shown that lipids from leaves and chloroplasts have a similar effect on the flow properties of sugar/oil suspension. The higher recovery of lipids from leaves compared with the amount of lipids recovered from chloroplasts considering the volume of leaves used at the start of extraction meant that the leaf lipid method were chosen to be applied into real chocolate. The verification of the functional behaviour of the leaf lipids on real chocolate is important as this now involved the interaction of the lipids not only with the sugar and cocoa butter but also with the cocoa powder.

5.1 INTRODUCTION

Chocolate is a highly filled food suspension that requires a surfactant as part of the formulation to enable the material to flow or be of sufficiently low viscosity to be processed and eaten without difficulty. Due to their amphiphilic characteristic, the surfactant molecules adsorb with their hydrophilic moiety to the surface of the hydrophilic sugar particles thus aiding their dispersion in the lipophilic cocoa butter matrix (lipophilic tails pointing into the fat phase). Besides using lecithin which is the commonly

applied surfactant in chocolate, PGPR can also be added together with lecithin to reduce the yield stress produced when lecithin is added at more than 0.5% (Beckett, 2008, Ziegler et al., 2003, Schantz and Rohm, 2005).

Sugar and cocoa butter are the key ingredients in chocolate by which we can describe its suspension system. However, chocolate also contains cocoa solids. Since sugar is by far more polar than cocoa solids (Ziegler et al., 2003), sugar is normally used as the polar substance in a model system of chocolate suspensions (Babin et al., 2003, Babin et al., 2005, Arnold et al., 2013) as the impact of sugar particles on the flow properties of chocolate is more pronounced than the impact of cocoa particles. Nevertheless, studying a sugar/oil suspension model system is not enough to fully investigate the real impact of a surfactant on the flow properties of chocolate.

In this chapter, the impact of spinach leaf lipids was tested on the flow properties of commercially made chocolate and compared to the impact of lecithin and PGPR added to a sample of the same chocolate. The variables compared between samples with surfactant were the apparent viscosity and the yield (5 s^{-1}) of the chocolate.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Cocoa butter and ready-made dark chocolate (32% fat) were received from Barry Callebaut (Banbury, UK). The chocolate was stored at 45°C for at least 24 h as recommended by Barry Callebaut to ensure it was fully molten before proceeding to be used in analysis. The method of spinach lipid extraction was explained in detail in Chapter 3. The lipid extract (in chloroform) was then mixed with an amount of cocoa butter and evaporation was carried out at 50°C for 1 h.

5.2.2 Spinach leaves lipid extraction

Spinach leaves lipid was extracted following the procedure described in Section 3.2.3.

5.2.3 Preparation of surfactant into cocoa butter

Two concentrations of spinach lipids in cocoa butter were prepared to investigate the impact of the surfactant addition on the flow properties of chocolate; 0.3 and 0.6%. A concentration of 0.3% was used to mimic the amount of surfactant that is commonly added into chocolate and 0.6% was chosen as a slightly higher quantity than the common amount of lecithin used in chocolate. Lecithin at more than 0.5% will show an adverse effect which will increase the yield stress of the chocolate (Beckett, 2008).

Before the experiment was carried out, the chocolate and the cocoa butter were left at 45°C for at least 12 hours, as recommended by Barry Callebaut. The blank sample had no surfactant ingredient and was prepared by adding 4 g of cocoa butter into 96 g of chocolate. The concentration of surfactant was prepared in the 4 g cocoa butter by changing the surfactant to cocoa butter ratio. In order to produce 0.6% surfactant based on whole chocolate, the amount of surfactant in the 4 g of cocoa butter should be 15% (15 g surfactant + 85 g cocoa butter). On the other hand, to produce 0.3% surfactant in the formulation, the amount of surfactant in the 4 g of cocoa butter should be 7.5% (7.5 g surfactant + 92.5 g cocoa butter).

First, the 15% surfactant in cocoa butter samples was prepared; the concentration of surfactant per g of cocoa butter was 0.1765 g surfactant /g of cocoa butter. The formulations of surfactant into cocoa butter, based on a spinach lipid extract of 1.67 g, were as shown in Table 5.1. The spinach lipid (after extraction) needed to be dissolved in chloroform first before mixing with cocoa butter. An amount of cocoa butter was then added into the spinach lipids and evaporation took place at 50°C for 1 h to remove all of the chloroform. Four grams of each cocoa butter-spinach lipids mixture were then added into 96 g of chocolate and stirred manually by hand for 5 min. The chocolate mixture was then allowed to stand at 45°C for at least 24 hours before proceeding to the rheological analysis.

Table 5.1 Formulations of surfactant (15%) into cocoa butter

	Control	**PGPR**	**Lecithin**	**SPLIP**
Surfactant (g)	0	3	3	1.67
Cocoa butter (g)	20	17	17	9.46
Concentrations	0.1765 g surfactant/ g cocoa butter			

5.2.4 Rheology

The impact of the surfactant on the flow properties of chocolate was conducted using the same rheological procedure as for the sugar/oil suspensions described in Chapter 4. However, the measurement was taken at 40°C as recommended by the International Confectionery Association (ICA), due to the properties of cocoa butter which is solid at room temperature. The result of the experiments were taken as apparent viscosity at 40 s^{-1} and yield measured at 5 s^{-1} from the increasing shear rate ramp.

5.2.5 Statistical analysis

All results were analysed statistically using one way ANOVA to compare means. Significant difference between samples was analysed using Tukey HSD (Honestly Significantly Different) multiple comparisons test at 95% significance level. The software used was IBM SPSS Statistics 22.

5.3 RESULTS AND DISCUSSION

The impact of spinach leaf lipid extract on the flow behaviour of chocolate was assessed at a 0.3 and 0.6 % addition and benchmarked against the performance of lecithin and PGPR at the same concentrations. As a control sample, chocolate without added surfactant was included; the results are shown as viscosity curves in Figure 5.1 and 5.2 for 0.3% and 0.6% addition, respectively. The measurements were conducted as an up-and-down shear rate controlled test where the shear rate was initially increased and once the highest shear rate of 1000 s^{-1} was reached the shear rate was decreased again. To highlight the yield behaviour which is of high importance in chocolate suspensions, the results were plotted against shear stress in relation to the increase of shear rate. As in Chapter 4, the curve was also evaluated based on the critical stress shown by each type of surfactant. The closed symbols showed an ascending shear curve while the open symbols showed a descending shear curve.

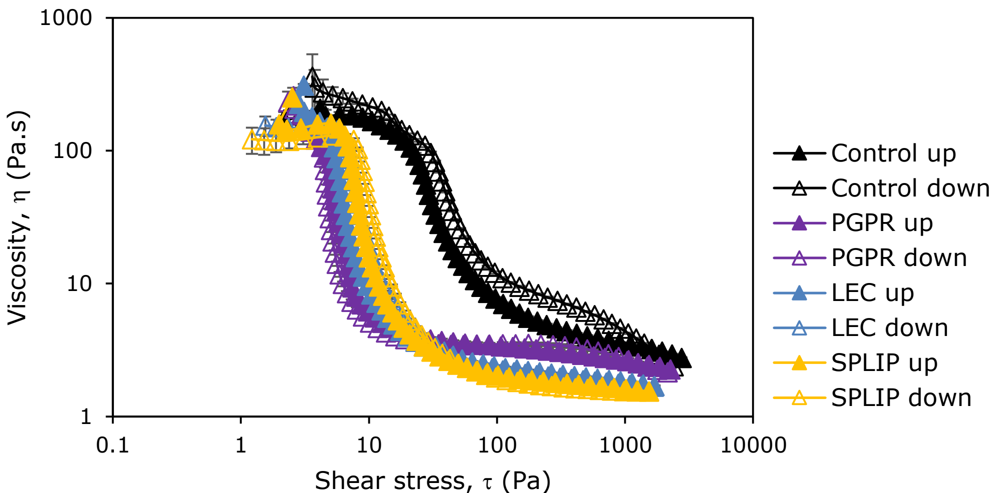

Figure 5.1 Viscosity behaviour of chocolate as affected by different surfactants at 0.3% addition (mean ± 1.0, n=4).

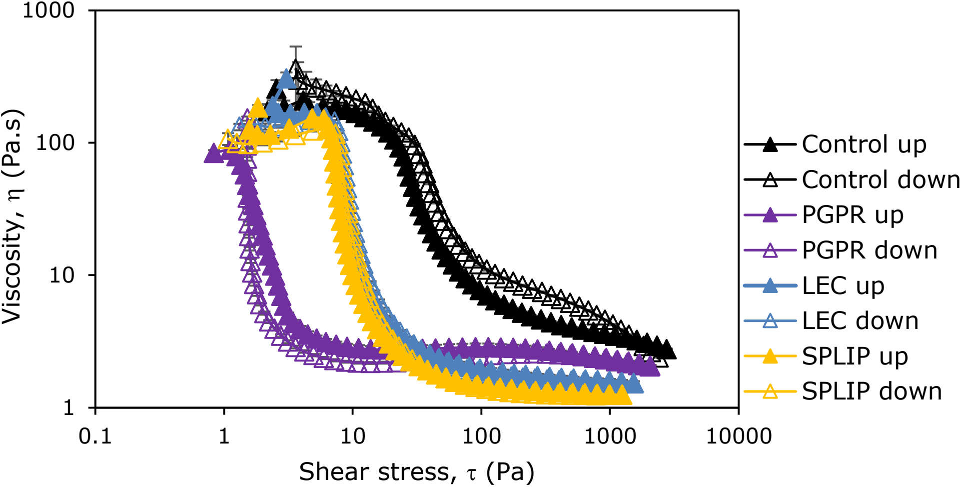

Figure 5.2 Viscosity behaviour of chocolate as affected by different surfactants at 0.6% addition (mean ± 1.0, n=4)

The data reveal that all chocolate samples displayed shear thinning behaviour; that is viscosity decreased with increasing shear stress. Hysteresis was not observed for any of the samples. However, the control sample showed a slight increase in viscosity in the descending curve at the start of high shear (1000 s^{-1}) after a 120 s period of rest. This is probably due to the fact that the rate applied for the start of the shear after the rest was too high for the highly concentrated chocolate, thus showing a slight difference in the viscosity behaviour. Without surfactant in the suspension, the interaction between particles in a highly concentrated chocolate may be strong enough to quickly rebuild the internal network structure thus showing a slight increase in viscosity at the start of descending curve if the rate applied was too high. The International Confectionery Association (ICA) however has recommended that the stress and viscosity of chocolate is measured between 2 s^{-1} and 50 s^{-1} (Afoakwa et al., 2009a). Since this study was carried out to validate the results of Chapter 4, the same measurement method as in Chapter 4 was applied.

The addition of surfactant at a concentration of 0.3% into the chocolate suspension decreased the viscosity and the critical stress value. PGPR reduced the critical stress of the suspension to the lowest level of all the samples, followed by lecithin and SPLIP. However, the stress values

shown by all surfactants were comparable between 5 to 10 Pa. The viscosity at the critical stress value was seen to follow the trend shown by the critical stress where a suspension with lower critical stress value was seen to have a lower viscosity value as well. All surfactants at this concentration generally showed a comparable impact on the suspensions.

However, the data acquired on the chocolate with PGPR added at the higher concentration (0.6%) showed a dramatic reduction in the critical stress value. Lecithin and SPLIP were less effective and performed similarly in terms of critical stress and viscosity reduction.

In the high shear region (100 – 1000 Pa), spinach lipids showed a lower viscosity value than lecithin and PGPR suggesting that the spinach lipid was more effective in reducing the viscosity of the chocolate. PGPR is known not to largely affect the viscosity (Schantz and Rohm, 2005) and so it was not surprising that it showed the highest viscosity among the surfactants used. In this high shear region, the interaction between particles are of minor importance and it purely depends on the viscosity of the continuous phase. Therefore, the spinach lipid extract was seen to

be more effective at lowering the viscosity of cocoa butter compared to lecithin and PGPR.

To assess the rheology data for the presence of wall slip, Figures 5.3 and 5.4 show the data as shear stress versus shear rate. A "kink" in the curves at low shear rates with a large drop in stress would be indicative of wall slip. Indeed, wall slip occurred for all samples as clearly indicated by the shape of the curves at shear rates lower than 0.1 s^{-1}. Due to the nature of chocolate, wall slip is nearly always observed in a smooth bob and cup geometry and, as a rule of thumb, viscosity values are only correct at shear rates above 0.1 s^{-1} (Servais et al., 2003).

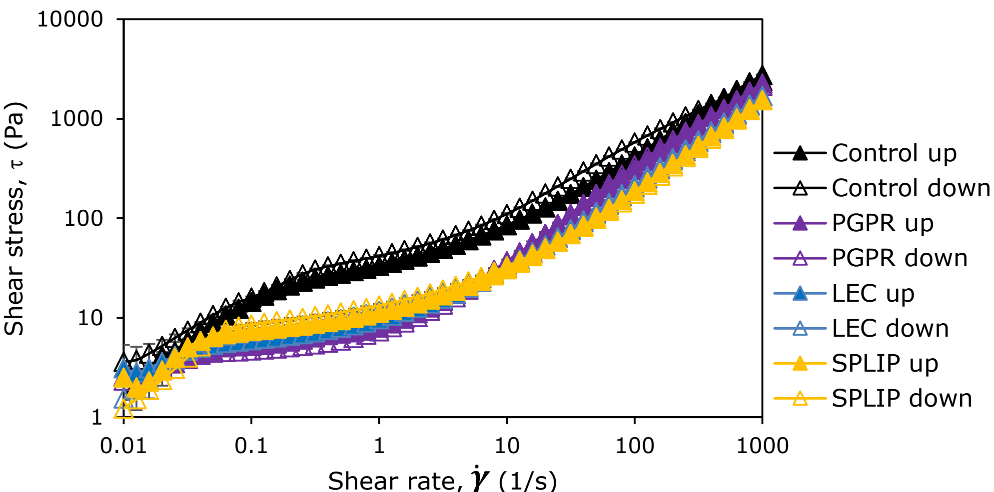

Figure 5.3 Stress curves of chocolate showing the effect wall slip at 0.3% addition of surfactants (mean ± 1, n=4)

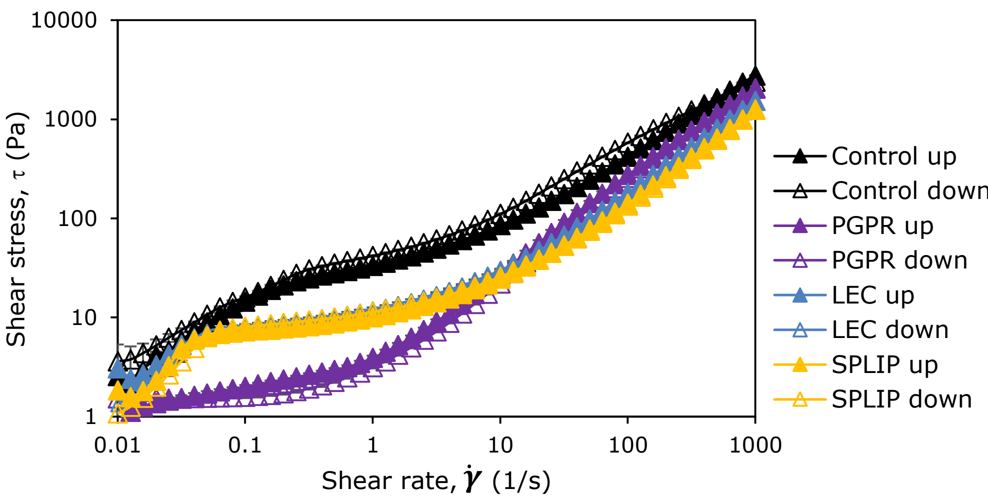

Figure 5.4 Stress curves of chocolate showing the effect of wall slip at 0.6% addition of surfactants (means ± 1, n=4)

To further illustrate the comparative performance between the surfactants, the apparent viscosity at 40 s^{-1} and the yield measured at 5 s^{-1} were isolated from the data, see Figures 5.5 and 5.6 for the 0.3 and 0.6% addition. Each figure shows yield (5 s^{-1}) as bars/columns and data points for viscosity at 40 s^{-1} taken from the up curve data.

At 0.3% addition, the yield (5 s^{-1}) obtained for all three surfactants showed a comparable impact in lowering the yield (5 s^{-1}) of the chocolate. In terms of the apparent viscosity (40 s^{-1}), SPLIP showed the lowest value and significantly ($P<0.05$) different with lecithin and PGPR.

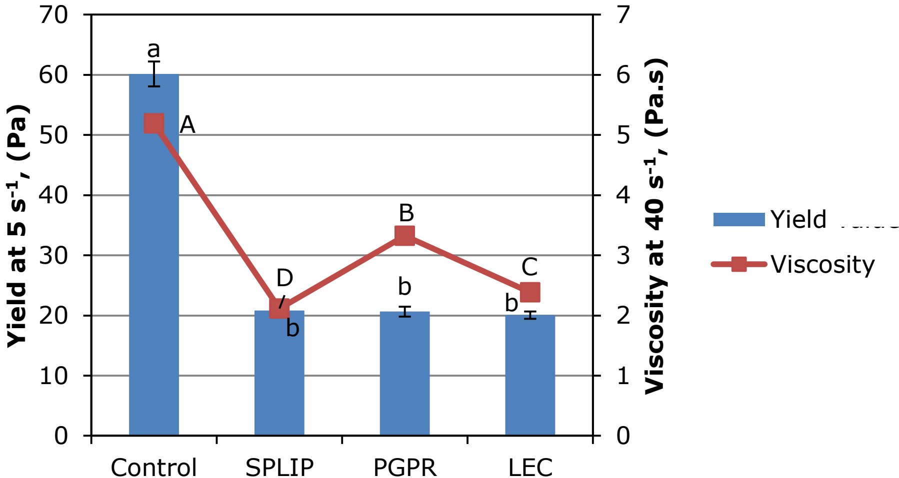

Figure 5.5 Impact of 0.3% surfactants concentration on the yield measured at 5 s^{-1} and apparent viscosity of chocolate. Small letters indicate significant different in the yield (5 s^{-1}) value while capital letters indicate significant different in the viscosity value.

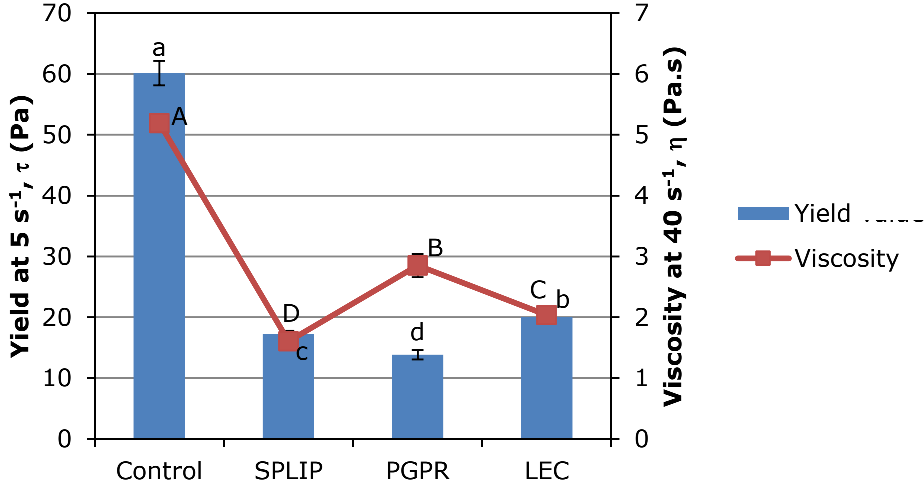

Figure 5.6 Impact of 0.6% surfactants concentration on the yield measured at 5 s^{-1} and apparent viscosity of chocolate. Small letters indicate significant different in the yield (5 s^{-1}) value while capital letters indicate significant different in the viscosity value.

PGPR was the only added surfactant which led to relatively high shear viscosity although the value is decreased compared to chocolate with no added surfactant. This observation holds for both levels of surfactant addition. This demonstrates why chocolate manufacturers adding PGPR to adjust the yield (5 s^{-1}) usually also include lecithin. Lecithin added at 0.6% showed a very similar yield (5 s^{-1}) value to that measured when only 0.3% was used. Meanwhile, in the case of SPLIP and PGPR there was a noticeable decrease. In terms of yield value alone, above a minimum concentration somewhere between 0.3 and 0.6% addition, PGPR is clearly the most effective of the three surfactants analysed. In the case of viscosity measured at 40 s^{-1}, SPLIP was lower compared to lecithin suggesting that SPLIP could be considered as a replacement for lecithin in the formulation of chocolate although, of course, there are more points to consider than simple viscosity reduction. These may include cost, flavour and impact on tempering behaviour to name but a few.

The results showed in this chapter, have validated the results of Chapter 4 where SPLIP shows a lower viscosity value than that shown by lecithin, and had a comparable yield (5 s^{-1}) value to the value shown by PGPR when they are added at 0.3%. However, when the surfactants are added at 0.6% the lowest yield (5 s^{-1}) value for the chocolate was that obtained

with PGPR. While the chocolate with SPLIP showed only a slightly reduction in the yield value, it was not comparable with PGPR in its effectiveness at lowering the yield (5 s^{-1}) value of chocolate. The finding that high concentrations of lecithin showed a detrimental effect in the both properties of viscosity and yield (5 s^{-1}) value in the sugar/oil suspensions were not carried through to the chocolate tests; the viscosity and the yield value of chocolate was not increased by the high concentration of lecithin. This phenomenon may have a strong relation to the adsorption of lecithin towards sugar particles and cocoa particles. As mentioned in the study of Ziegler et al. (2003), lecithin adsorbed strongly on the surface of sugar and cocoa particles while PGPR showed a lesser adsorption power than lecithin.

However, at the outset of this research the question was asked whether PGPR could be replaced with spinach lipid extract. The results are indeed promising, although it appears that SPLIP functions more like a combination of lecithin with a slightly sub-optimally performing PGPR. The fact that SPLIP shows performance properties of lecithin and PGPR is not surprising based on its composition which composed of MGDG and DGDG, and in combination of anionic lipids which helped to disperse solid particles by electrostatic repulsion.

5.4 CONCLUSIONS

The influence on the flow properties of the chocolate by spinach lipids at 0.3% concentration was similar to the flow properties of the model suspension in Chapter 4. However, the impact of spinach lipids in lowering the yield at 5 s^{-1} was not as good as with PGPR. Nevertheless, the action of spinach lipids when used at high concentrations to further reduce the viscosity and yield (5 s^{-1}) to a value lower than lecithin shows the potential of spinach lipids to act as a single surfactant.

6 OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

6.1 LIMONENE IMPACT ON BLOOM OF COCOA BUTTER

The objective of this study was to investigate the impact of cocoa butter substitution of the ratios 29:1 and 28:2 cocoa butter:limonene or chocolate:limonene on the crystallization of cocoa butter and chocolate and consequently the formation of bloom. Previous studies have shown a viscosity reduction in chocolate by limonene addition (Do et al., 2008) and the potential of limonene to enable a decrease in the amount of cocoa butter used in chocolate but to maintain a relatively low viscosity.

Clearly, this study has demonstrated that limonene substitution has softened the texture of cocoa butter where it is incorporated at the ratio of 28:2 cocoa butter:limonene which made the demoulding process difficult. Limonene substitution also increased the whiteness index value of the samples, which is clearly seen with the sample of chocolate; the cream colour of cocoa butter made the amount of bloom difficult to assess accurately using the whiteness index measurement. Therefore, this study showed that limonene substitution promotes the formation of bloom on chocolate.

The formation of bloom was believed to be due to the crystal phase transformation in the sample, thus it was investigated with XRD. Limonene substitution resulted in the formation of the most stable cocoa butter crystal (form VI crystal) as early as Week 1 in the chocolate sample while in the cocoa butter sample it first appeared in Week 2. It was suggested that the faster rate of crystal polymorphism transition in the chocolate sample was due to the incorporation of other foreign particles in the sample such as sugar and cocoa powder which may act as an additional nucleation site during crystallization. It is also thought to affect the oil mediated crystallization; limonene as liquid among the cocoa butter and chocolate microstructure. In the presence of a liquid, the growth rate of crystals is faster than the nucleation rate. As a result, the size of the cocoa butter crystals form in the presence of limonene was found to be larger as the amount of seed crystals formation were less.

Bloom formation will influence whether consumers will accept the chocolate even though the defect is not related to a microorganism. Since bloom was not obvious in the cocoa butter due to the light colour of the cocoa butter itself, limonene still has a potential to be used in white chocolate where the bloom will not be noticed. However, it is suggested that any limonene addition is restricted to 1% in order to

prevent the texture softening effect on the chocolate and the extensive smell of orange from the limonene.

6.1.1 Future work

For further investigation of the impact of limonene in chocolate, other aspects of study could be:

- Using solid fat content (SFC) analysis during the crystallization process of melted cocoa butter-limonene and chocolate-limonene blends to investigate the nucleation of cocoa butter crystals in the samples. This work can be verified from microscopic images together with the results of SFC.

6.2 SPINACH LIPIDS AS NATURAL SURFACTANT IN CHOCOLATE

The application of spinach lipids as a natural surfactant is a novel approach to adjust the rheological properties of low-fat chocolate and it was compared here to the impact of lecithin and PGPR. Most studies with chloroplast lipids investigate the emulsifying properties of the lipids at the interface of oil/water system, particularly during lipid digestion in the stomach (Chu et al., 2009, Rayner et al., 2011a, Rayner et al., 2011b, Ostbring et al., 2015). This study, however, focused on the ability of both leaf and chloroplast lipids to adsorb at the sugar/oil interface and to change the flow properties of the suspension.

The high percentage of moisture in spinach sample (around 94%) (Choe et al., 2001) meant that the method of choice for extraction was with chloroform-methanol, following to the method of Bligh and Dyer (Bligh and Dyer, 1959) as was reported to be the best method to be dealt with wet samples (Schmid, 1973). However, the use of chloroform-methanol caused enzyme-catalysed degradation of lipids (Christie, 1993b), thus a pre-treatment with hot isopropanol at 80°C was used. This heating was predicted to cause the MGDG separation from other lipids and act as a single lipid in the form of inverted micelle in the mixture. The extraction method was validated to be reliable by comparing to the previous study and, from the results of the lipid composition obtained, all the lipid class

expected in spinach lipids were extracted. Both spinach leaf and chloroplast lipid fractions were confirmed to have high amounts of MGDG and DGDG, which were claimed to be the most important polar lipids to give the functional behaviour of a surface active ingredient (Chu et al., 2009, Evans et al., 1991). However, the yield of glycolipids was more concentrated in the chloroplasts while leaves showed a higher percentage of phospholipids in the extracts. The total amount of lipid recovered was higher in spinach leaf extract than the chloroplast extract when taking the amount of parent leaf as the basic of calculation. Therefore, in terms of economics, the use of spinach leaf lipids in food applications would be preferred as long as the functional behaviour of lipids in the leaf extract is at least comparable with the chloroplast fraction.

The result of interfacial tension tests showed a comparable result between the spinach leaf and chloroplast lipids, a result which demonstrated a lower value compared to the commercial surfactants lecithin and PGPR at the concentrations used. Further investigation in the flow properties of the chocolate model with surfactants also showed a comparable behaviour in the function between the spinach leaf and chloroplast lipids.

The impact of surfactants on the flow properties of the suspensions were evaluated based on the viscosity curves and on the extracted value of apparent viscosity and yield (5 s^{-1}), measured at a shear rate 40 s^{-1} and 5 s^{-1}, respectively. The viscosity curves demonstrate the impact on the viscosity and the critical stress, which is where the viscosity starts to reduce. In the chocolate model tests, lecithin generally reduced the viscosity and the critical stress of the suspension to the lowest value of the four surfactants tested, at the lowest concentration applied. However, increasing the concentration of the surfactants increased the viscosity and critical stress of the suspension containing lecithin, while spinach lipids continued to reduce the viscosity and critical stress of the suspension. The reducing effect shown by both spinach lipids however only up to the concentration of 0.3%. Increasing more than 0.3% of concentration of spinach lipids showed no further impact on the viscosity and the critical stress of the suspension. PGPR had a major impact in reducing the critical stress but showed little effect on the viscosity. Determination of the apparent viscosity at a shear rate of 40 s^{-1} and the yield (5 s^{-1}) at a shear rate of 5 s^{-1} showed reduced viscosity and yield (5 s^{-1}) of the suspension containing spinach lipids. The value shown was lower than the viscosity shown by the suspension containing lecithin and a comparable yield (5 s^{-1}) shown by the suspension containing PGPR. Both spinach leaf and chloroplast lipids showed a comparable functional behaviour in affecting the flow properties of the model suspension. As a

result, spinach leaf lipids were chosen to be applied into real chocolate for further investigation in validating the results of the model suspension.

Applying spinach lipid into chocolate at a concentration of 0.3% and 0.6% (by weight) displayed a similar characteristic of both spinach leaf lipid and lecithin when looking at the viscosity flow curve. Spinach lipid displayed a lower value of high shear viscosity, suggesting that the spinach lipid is more effective in reducing the viscosity of the continuous phase. It is thought that this effect was closely related to the high amount of α-linolenic acids in the spinach lipids as lipids with high polyunsaturated fatty acids have higher solubility (Benson, 1964). Surfactants with limited solubility in oil have been reported to increase the viscosity of the oil when their concentration exceeds the solubility limits (Johansson and Bergenstahl, 1992b).

The impact on the yield and the apparent viscosity of chocolate at shear rate 5 s^{-1} and 40 s^{-1}, respectively, was also investigated. The addition of 0.3% surfactants into chocolate has resulted in a similar result in that it decreases the yield (5 s^{-1}), while the lowest viscosity value was shown by spinach leaf lipids, followed by lecithin and PGPR. Increasing the concentration of surfactants to 0.6% however, did not increase the yield

(5 s^{-1}) and the viscosity of chocolate with lecithin, as what have been observed in the Chapter 4. The chocolate with spinach leaf lipid extract showed a slight decrease in both characteristics to a lower value than lecithin. A dramatic drop in the yield value shown by the chocolate with PGPR showed that the spinach leaf lipid was not as good as PGPR in lowering the yield (5 s^{-1}). The different behaviour of chocolate with lecithin in this chapter with the suspension with lecithin in Chapter 4 implies that the lecithin has a strong adsorption on the surface of sugar and cocoa particles. Nevertheless, even though the ability in reducing the yield (5 s^{-1}) was not as strong as PGPR, the spinach lipid was still showing potential in decreasing the viscosity and yield value of the chocolate to a lower value than lecithin. Besides, PGPR would not be used as a single surfactant in chocolate due to its low impact on viscosity reduction.

6.2.1 Future work

As a continuation of this study, in relation to the use of spinach lipids in chocolate, further investigation could be:

- A validation of the ability of spinach lipids in influencing the rheological properties of a known amount of sugar and cocoa particles in chocolate in order to confirm the conclusion of the potential of spinach lipids as the flow enhancer.

- As heating caused MGDG separation in the lipids mixture, it would be valuable to further study a non-heated lipids extract which could be conducted by spray drying the spinach leaves, reducing the size of the dried leaves by mortar and pestle and then extracting directly with the chloroform-methanol. Solvent evaporation could be carried out by flushing with nitrogen gas. This would then compare the impact of heating on the ability of spinach lipids as the flow enhancer.

- A study on the impact of spinach lipids on the solid fat content (SFC) of the molten chocolate during the crystallization process. This study would need to combine information about the rate of polymorphism transformation of cocoa butter crystals with XRD and the impact on the melting behaviour of chocolate using the DSC. The study on the impact of spinach lipids on the texture, snap and gloss of chocolate would also provide constructive information. It would also be useful to study any bloom formation by measuring the changes on the whiteness index during a cyclic temperature storage, to ensure consumer acceptance.

- To fully assess consumer acceptability, it would be important to run a sensory test on the spinach lipids-chocolate. However, the lipids would need to be extracted using a non-toxic substance such as hexane/isopropanol (3:2, v/v) or other technology which could be solid phase extraction (SPE).

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APPENDIX

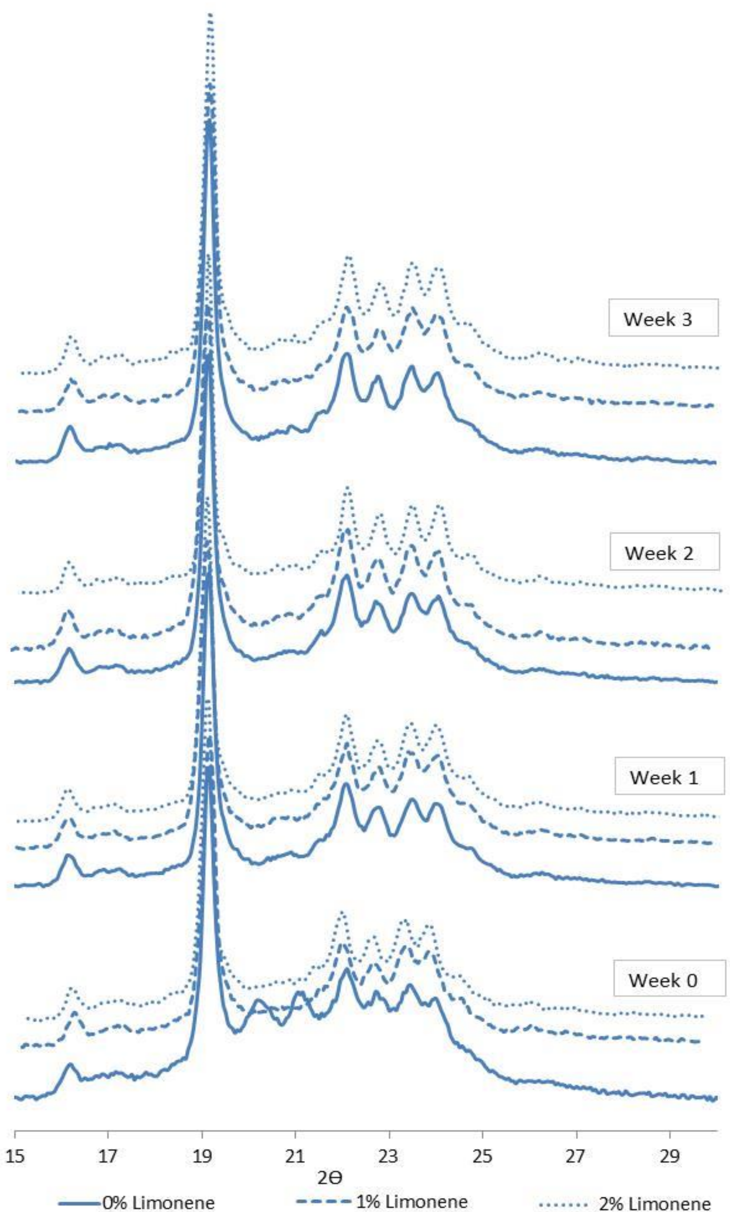

Figure A.1 Cocoa butter-limonene was incubated at 20 °C from the melt (60 °C), where limonene only increased the rate of crystallization of cocoa butter in Week 0.