APPLICATION OF RICE BRAN WAX ORGANOCEL TO SUBSTITUTE SOLID
FAT AND ENHANCE UNSATURATED FAT CONTENT IN ICE CREAM

by

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ABSTRACT

APPLICATION OF RICE BRAN WAX ORGANOGE L TO SUBSTITUTE SOLID FAT AND ENHANCE UNSATURATED FAT CONTENT IN ICE CREAM

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The objective of this study was to investigate the potential application of rice bran wax (RBW) organogel to replace solid fat content and create the fat network in ice cream. Ice creams with 10% fat or 15% fat were formulated with RBW organogel as the fat source, and two different emulsifiers were used: polmo, a commercial blend of emulsifiers which contains 80% mono- and diglycerides and 20% polysorbate 80, and Glycerol monooleate (GMO). Candelilla wax (CDW) organogel and carnauba wax (CBW) organogel were also tested for comparison. RBW organogel had the ability to form and sustain structure in 15% fat ice creams when GMO was used as the emulsifier. Transmission electron microscopy revealed that the RBW crystal morphology within the fat droplet, when GMO was used as the emulsifier, was characterized by the growth of crystals at the outer edge of the droplet which increased fat destabilization and network formation.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CBW</td>
<td>Carnauba Wax</td>
</tr>
<tr>
<td>CDW</td>
<td>Candelilla Wax</td>
</tr>
<tr>
<td>Cryo-SEM</td>
<td>Cryo – Scanning Electron Microscopy</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>GMO</td>
<td>Glycerol Monooleate</td>
</tr>
<tr>
<td>HOSO</td>
<td>High Oleic Sunflower Oil</td>
</tr>
<tr>
<td>ILS</td>
<td>Integrated Light Scattering</td>
</tr>
<tr>
<td>PKO</td>
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<td>Rice Bran Wax</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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1. INTRODUCTION

The risk associated with the consumption of trans- and saturated fat has been intensely discussed during the last few decades. The dietary intake of trans- and saturated fat has been associated with an increase in serum levels of low-density lipoprotein (LDL) cholesterol, also called “bad cholesterol”. At the same time, unlike the effect caused by the consumption of saturated fat, trans-fat intake has been associated with a decrease of blood levels of high-density lipoprotein (HDL), known as “good cholesterol”. Both effects are responsible for increasing the risk of coronary heart disease (Mensink and Katan, 1990; Hu et al., 1997, Mozaffarian et al., 2006). As a result of the epidemiologic investigation regarding the intake of trans-fat, the U.S. Food and Drug Administration has issued a regulation requiring the declaration of the content of trans- fatty acids in food labels (US FDA, 2003). Since then, many dietary recommendations have suggested the reduction (or elimination) of saturated fat and trans-fat to reduce the risk of cardiovascular diseases and other life-style associated disorders (Lichtenstein et al., 2006; USDA and US HHS, 2005; Health Canada, 2011). On the other hand, the consumption of oil as a dietary source of omega-3 and omega-6 fatty acids has been suggested since it provides numerous benefits to health including reducing the risk of developing heart disease (National Institute of Health, 2005).

Trans-fats are composed of unsaturated fatty acids with their double bonds in the trans-configuration instead of cis-configuration, while saturated fats are composed of fatty acids with no double bond. Trans- fat can occur naturally, in low concentration, in ruminate animal fat although it occurs more commonly as a product of the hydrogenation
of liquid fat, which is the main source of this type of fat. Sources of fat with a high concentration of saturated fatty acids are animal fat such as milk fat, tallow and lard, and some vegetable fats such as coconut oil and palm kernel oil (O’Brien, 2004).

The physical properties of saturated and trans- fat have been exploited by industry to create structure in food products. Hydrogenated fat was extensively used by the industry in the last century as a substitute for animal fat. As a product with high melting point, great oxidative stability and sourced from vegetable oils, hydrogenated fat was an economical source of saturated fatty acids for the industry. However, given the recent worldwide concern related to the consumption of high levels of saturated fat and trans-fat accompanied by changes in regulations and dietary recommendations, industries that traditionally use hydrogenated fats are encountering challenges in the reduction of saturated and trans- fat from fat-based food products.

The reduction of the concentration of solid fat in food cannot be accomplished by simply replacing the solid fat by liquid oil. The structure of fat-based foods usually requires a large concentration of solid fat to be achieved. The replacement of solid fat by liquid oils would have a large effect on the quality of the product. Organogelation, a novel structuring method, has been of great interest recently because of its ability to structure edible liquid oils, thus conferring features of a solid fat without the need of large amounts of saturated fat. Edible oil organogels are viscoelastic materials comprised of high concentrations of liquid oil. A three dimensional network, formed by an organogelator, immobilizes the liquid oil to create a hard material with the greatest part of its composition formed by liquid oil (Weiss and Terech, 2006). The potential application of organogels in food to substitute trans- and saturated fat arise from their
solid-like rheological properties. Because of that fact, these materials have the potential to confer structure and enhance textural quality of food products. Recently, some food grade organogelators have been suggested, however, the application of innovative organogelators and the progression in the application of organogels in food is still very limited.

Fatty acids, fatty alcohols, wax esters, monoacylglycerols, phospholipids, sorbitan esters, phytosterols and ceramides are some of the different types of food grade organogelators used to structure liquid edible oil (Pernetti et al., 2007; Rogers et al., 2011). The structuring potential of edible oil by wax esters has been recently investigated (Toro-Vazquez et al., 2007; Dassanayake et al., 2009, Morales-Rueda et al., 2009a,b) and its potential food applications were suggested.

Waxes are substances composed mainly of esters of fatty acids and fatty alcohols. However, hydrocarbons, fatty acids and fatty alcohols, sterol esters and ketones are also present in waxes at different concentrations (Wolfmeier et al., 2002). The origin of natural waxes can be animal, insects, plants, microorganisms or mineral (Warth, 1956). Nowadays, to meet consumer preferences and beliefs, marketers are demanding in preference products with a plant origin, rather than animal or mineral. Some examples of plant waxes with the potential to structure edible oil are candelilla (CDW), carnauba (CBW) and rice bran wax (RBW). Among them, RBW showed a better ability to structure edible oil and only a minimum of 0.5% wax was reported to be enough to structure olive oil compared to CDW (~1%) and CBW (~4%) (Toro-Vazquez et al., 2007; Dassanayake et al., 2009, Morales-Rueda et al., 2009a,b). The excellent structuring properties of RBW attracted the attention as a possible fat substitute in products that
contain a high concentration of saturated fat, whose fat structure is essential for texture formation in products such as ice creams.

Ice cream is a complex food composed by several structural elements that confer the desirable texture of ice cream. These structural elements consist of air bubbles, a fat globule network, ice crystals and an unfrozen serum phase. The whipping/freezing process is responsible for incorporating air bubbles that are constantly broken into smaller air cells, for freezing the water and creating uniformly shaped ice crystals and for applying intense shear that destabilizes fat droplets, thus creating a fat network that stabilizes air cells. Partial coalescence seems to be the most important form of fat destabilization in milk fat ice cream (Marshall et al., 2003). However, this fat destabilization mechanism seems to happen only in the presence of partially crystalline fat droplets. This impedes the application of high concentrations of liquid oil. The partial coalescence phenomenon occurs because fat droplets collide as a result of the intense shear applied by the rotation of the blade. Upon collision, the presence of fat crystals at the fat droplet interface causes the interpenetration of the crystals from one fat droplet to another fat droplet. This mechanism leads to the aggregation of two fat droplets that continue to aggregate forming large fat agglomerates in the form of chains or clusters. The presence of a minimum amount of crystalline fat is necessary to keep the round shape of the fat droplets even after destabilization (Marshall et al., 2003; Boode and Walstra, 1993). Therefore, the fat source seems to have an important role in the stability of the structure of ice cream, as well as flavour, creating its characteristic richness and smoothness.
Although the application of wax organogels have the potential to improve the nutritional quality in ice cream, the absence of high concentrations of crystalline particles can lead to challenges in the application of such organogels.

The application of high concentrations of liquid oil in ice cream can also have an effect in its legal definition. The standard definition of ice cream varies considerably between countries. In Canada, ice cream must contain a minimum of 10% milk fat. In Europe, on the other hand, the definition of ice cream is not based on the content of milk fat and the same definition is also applied for products containing non-dairy fat. In the present study, "ice cream" will be applied for products developed with dairy or non-dairy edible fat including organogel ice creams.

The purpose of this research is to investigate the potential application of RBW organogels in ice cream. The formation of an oil-in-water emulsion in which the fat phase is a RBW organogel was first evaluated followed by the creation of an ice cream mix using RBW organogel as the fat source. To accomplish the purpose of this research, the effect of different emulsifiers, concentrations of fat and freezing processes in the RBW organogel ice cream were also investigated. The standard ice cream measurements were performed for all the formulations tested and this includes overrun measurements, fat droplet size distribution and meltdown rate. Differential scanning calorimetry was a useful tool to investigate the crystallization of the RBW inside the fat droplets of ice cream emulsions. The microstructure of ice creams and the ultrastructure of organogel droplets were investigated using cryo-scanning electron microscopy and transmission electron microscopy.
Objectives

- To develop oil-in-water emulsions wherein the dispersed fat phase is in the form of organogel droplets instead of a liquid oil droplet.
- To generate desirable ice cream structure and stability while using wax organogels.
- To understand the mechanism of fat aggregation and structural formation in ice cream when wax organogels are used as solid fat replacers.
2. LITERATURE REVIEW

2.1. Ice Cream

Ice cream can be described as a frozen emulsion of dairy or non-dairy fat, sugars, proteins, stabilizers and flavours, which undergoes several manufacturing steps that confers the desirable smoothness and softness of ice cream (Marshall et al., 2003). However, the scientific understanding of ice cream is not as simple. Ice cream can be defined as a complex food foamed emulsion sustained by the combination of several components: ice crystals, fat network, air bubbles and a highly concentrated unfroze phase (Stanley et al., 1996).

The intense shear applied during the freezing process promotes the destabilization of the partially crystalline emulsified fat droplets that agglomerate into a unique form defined as partially coalesced fat. The partially coalesced droplets help stabilize the air incorporated during whipping. The unfrozen phase is formed by the creation of ice crystals; a process that concentrates the serum phase and reduces its freezing point. Under these transformations the structure of ice cream is formed. The sensory attributes of smoothness and softness result from the perception of the texture of ice cream (Goff, 2006).

2.1.1. Fat Sources

The choice of the fat used in ice cream is important for the optimal development and stabilization of structure. Some attributes should be taken into consideration when choosing the fat source in ice cream such as the ratio of saturated/unsaturated fat, the
melting and crystallization temperature of the fat, the rate of crystallization of the fat after emulsion formation, and the flavour of the fat (Goff, 2006).

2.1.1.1. Milk Fat

Traditionally, ice cream is formulated with milk fat. Milk fat has unique physical properties that strongly influence the development of the ice cream structure and confer a rich flavour and creamy texture to the ice cream. With an ideal solid/liquid fat ratio at the freezing temperature of ice cream, milk fat undergoes numerous physical changes during ice cream processing that are important for the formation of an optimum product.

Different sources of milk fat are available for use in ice cream such as fresh cream, sweet butter (unsalted butter), anhydrous milk fat (butter oil), plastic cream, milk fat-sugar blends, frozen cream and concentrated milk. Despite the large variety of different milk-fat based ingredients, fresh cream is considered to be the best source of fat to confer premium quality and flavour to the final product; however its perishability and lower quality during some seasons might interfere with the use of this fat source or increase its cost (Marshall et al., 2003; Goff, 2006).

Milk fat has a complex composition of unsaturated and saturated triglycerides with fatty acids ranging in chain length from C4 to C18. Some of these fatty acids are present in large quantities such as palmitic acid (C16:0), that ranges from 26 to 41% of the total fat concentration, and oleic acid (C18:1), which can vary from 18.7 to 33.4%. Most of the other fatty acids are present in low concentration. Volatile fatty acids and lactones are believed to confer the unique flavour characteristics of milk fat (O’Brien,
Due to milk fat complex composition, crystallization can occur in a wide range of temperature.

2.1.1.2. Vegetable Fats

Vegetable fat has been widely used in the formulation of ice cream in places such as United Kingdom, Latin America and some countries of Europe (Goff, 2006). However, it is just becoming a common fat source for ice cream in Canada. With changing regulations, competition between manufacturers and the development of advanced technology, the application of vegetable fats in ice cream has increased, leading to reduced price products and more accessibility to the consumer. Legal definitions may not allow those products to be called “ice cream” in some countries.

The vegetable fats come from a large variety of seeds and fruits such as soybeans, corn, canola, avocado, palm, olive and coconut. Some of them possess a much lower melting point due to their high concentration of mono- and poly-unsaturated fatty acids and position (cis-) of double bonds in the fatty acid molecule. The double bonds of unsaturated fatty acids can assume two distinct configurations cis- and trans-, the cis-configuration being the most common in nature (Gunstone et al., 2007). The differences in physical properties of unsaturated oil and saturated fat, such as melting point, affect significantly the development of ice cream structure. The necessity of a partially crystalline fat for the optimal formation of ice cream structure led to the use of techniques such as hydrogenation to achieve the melting point necessary. Hydrogenation of fat, on the other hand, can synthesize trans-fatty acids, the intake of which has been associated
with cardiovascular disease (Orthoefer, 1996; Gunstone et al., 2007, Marshall et al., 2003).

Blends of vegetable fats and oils are commonly used in the manufacture of ice cream. They are chosen according to flavour, stability and optimal ratio of liquid/solid fat (Marshall et al., 2003). Palm kernel oil, palm oil and coconut oil are the most common vegetable fats used in ice cream (Andreasen and Nielsen, 1998). Palm kernel oil and palm oil are vegetable fats extracted from the fruit of palm tree *Elaeis guineensis*. Palm kernel oil is obtained from the core nut while palm oil is extracted from the pulp of the fruit. The composition of fatty acids and the physical properties of the two vegetable fats are quite different. Palm oil has a very characteristic fatty acid composition with majority of palmitic (C16:0) (44%) and oleic (C18:1) (39%) fatty acids. Palm kernel oil is rich in lauric (C12:0) (48%) fatty acid and possesses a fatty acid composition and concentration very similar to coconut oil. Despite their high concentration of saturated fat, fractionation and hydrogenation of the fats are usually performed in order to increase the melting point profile (Goff and Hartel, 2004).

The health concerns with saturated fat and trans-fat intake and its influence in cardiovascular diseases has led to an increase in interest for the application of vegetable oils in the formulation of ice cream. However its application is limited since partially crystalline fat is required to promote fat destabilization and structure formation. Therefore an increase in the application of vegetable oil in ice cream would demand changes in formulation, adequacy in the process and the use of fat replacers to achieve good quality parameters.
The use of canola oil in ice cream has been reported as an appropriate vegetable oil source because of its high concentration of oleic fatty acid, that confers high oxidation stability, and the health benefits associated with its consumption. Canola oil has a low concentration of saturated fat (7% of saturated fat, against 11% in sunflower oil and 10% in safflower oil) and its consumption has shown to decrease the LDL cholesterol (low density lipoprotein) levels (Marshall et al., 2003).

High-oleic sunflower oil (HOSO) and high-oleic safflower oil are other examples of vegetable oils with high content of oleic fatty acid that promotes high oxidation stability and increases product shelf-life. They have been developed by cross-breeding of selected plants in order to achieve varieties with higher levels of oleic acid. These have also been a typical fat source in the formulation of frozen desserts (O’Brien, 2004; Grompone, 2005).

HOSO is rich in oleic fatty acid (C18:1), in which the concentration ranges from 75 to 85% of its total fatty acid composition. The typical composition and physical characteristics of HOSO are described in Table 2.1. The amount of oleic acid in HOSO represents around 4 times more oleic acid than that contained in normal sunflower oil. Due to its high stability against oxidation, HOSO has been one of the first chosen by producers (O’Brien, 2004; Grompone, 2005). HOSO production is concentrated in North America and France but its consumption is still limited. Production does not exceed 5% of the total world production of sunflower oil (O’Brien, 2004).

The use of vegetable oil in ice cream has been associated with deficiency in texture and creaminess. That is certainly due to the lower concentration of saturated fat that hinders the development of ice cream structure.
Table 2.1. Composition and some physical properties of high-oleic sunflower oil.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acid Composition (%)</td>
<td></td>
</tr>
<tr>
<td><em>Myristic (C14:0)</em></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><em>Palmitic (C16:0)</em></td>
<td>3.0 – 4.8</td>
</tr>
<tr>
<td><em>Palmitoleic (C16:1)</em></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td><em>Stearic (C18:0)</em></td>
<td>2.7 – 6.5</td>
</tr>
<tr>
<td><em>Oleic (C18:1)</em></td>
<td>75.0 – 85.0</td>
</tr>
<tr>
<td><em>Linoleic (C18:2)</em></td>
<td>8.0 – 10.0</td>
</tr>
<tr>
<td><em>Linolenic (C18:3)</em></td>
<td>&lt;0.3</td>
</tr>
<tr>
<td><em>Arachidic (C20:0)</em></td>
<td>0.2 – 0.5</td>
</tr>
<tr>
<td><em>Gadoleic (C20:1)</em></td>
<td>0.1 – 0.5</td>
</tr>
<tr>
<td><em>Behenic (C22:0)</em></td>
<td>0.5 – 1.1</td>
</tr>
<tr>
<td><em>Erucic (C22:1)</em></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Refractive Index, 25ºC</td>
<td>1.468</td>
</tr>
<tr>
<td>Iodine Value</td>
<td>78.0 – 88.0</td>
</tr>
<tr>
<td>Saponification Number (mg KOH/g)</td>
<td>188 - 194</td>
</tr>
<tr>
<td>Melting Point (ºC)</td>
<td>4.4 – 7.2</td>
</tr>
<tr>
<td>α – Tocopherol (ppm)</td>
<td>94 - 430</td>
</tr>
<tr>
<td>β – Tocopherol (ppm)</td>
<td>2</td>
</tr>
<tr>
<td>γ - Tocopherol (ppm)</td>
<td>1</td>
</tr>
</tbody>
</table>

2.1.2. Ice Cream Processing - Structure Development

The manufacture of ice cream involves diverse steps including mix preparation, homogenization, ageing, freezing and hardening. Combined, these steps are responsible for the increase in viscosity of the mix, destabilization of fat droplets, and incorporation and stabilization of air, which confers the optimal structure and physical properties of ice cream.

2.1.2.1. Mix Preparation

The manufacture of ice cream mix can be summarized in the following steps: formulating and combining ingredients, pasteurization, homogenization, and cooling. Some dry ingredients such as milk solids not-fat and cocoa powder should be incorporated into the mix at low temperature to avoid the formation of lumps. Adding dry ingredients while the liquid is being agitated also facilitates incorporation. The stabilizers are the most difficult constituents to dissolve. They should be mixed with sugar before incorporation into the mix, followed by intense agitation (Marshall et al., 2003; Goff, 2006). Table 2.2 describes a typical formulation of an ice cream mix.

The mix is preheated in the combination step. However pasteurization is necessary for eliminating the pathogenic microorganisms existing in the mix. Pasteurization of the mix is achieved by heating the mix to a specific temperature, keeping at that temperature for a certain period of time and finalising by cooling it to 5ºC, which acclimates the mix to the aging step (Marshall et al., 2003; Goff, 1997a). Cooling the sample will avoid exposing the mix to a warm temperature for a long period of time, which will encourage the growth of microorganisms. Some examples of minimal time
and temperature for pasteurization are 69°C / 30 min in a batch process and 80°C / 25 s in a continuous process (Marshall et al., 2003).

Table 2.2. Typical formulation of an ice cream mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (weight in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10 - 16</td>
</tr>
<tr>
<td>Milk solids-not-fat</td>
<td>9-12</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9-12</td>
</tr>
<tr>
<td>Corn Syrup</td>
<td>4-6</td>
</tr>
<tr>
<td>Stabilizers and emulsifiers</td>
<td>0-0.5</td>
</tr>
<tr>
<td>Total solids</td>
<td>36-45</td>
</tr>
<tr>
<td>Water</td>
<td>55-64</td>
</tr>
</tbody>
</table>

Source: Goff, 1997a.

During ice cream processing, a stable emulsion with small particle size is required. Therefore, a homogenization step is necessary to create an ice cream emulsion with a normal distribution of fat globule sizes with diameters lower than 2 µm. The homogenizer operates by forcing a large amount of mix through a small orifice, at a given pressure, causing the large fat droplets to break into smaller ones. The temperature of the mix needs to be above the melting point of the fat. Often used in ice cream processing is a two-stage homogenization with pressures of 14-18 MPa for the first stage and 3-4 MPa to the second stage. For ice cream mix prepared with milk fat, the minimum temperature of homogenization is 60°C. However, the use of temperatures around 80°C increases the efficiency of the homogenization (Berger, 1976; Marshall et al., 2003; Goff, 1997a).
Since an emulsion is a mix of non-miscible liquids (oil in water), the instability of the system is high. The stability of the fat droplets in ice cream mix during homogenization is caused by surfactant adhesion (milk protein and emulsifiers) to the oil/water interface (Ruger et al., 2002; Goff, 1997b). After homogenization, the new oil surface formed is rapidly covered by milk protein. With time, milk proteins are displaced through emulsifier activity. Emulsifiers have the ability to lower the surface tension of the fat/serum interface more than milk protein. This leads to their preferable adsorption at the fat droplet interface after homogenization thus displacing casein and whey proteins (Goff and Jordan, 1989; Adleman and Hartel, 2001). Emulsifier is not required for the stabilization of the ice cream emulsion. However, the adsorption of small molecule surfactants at the oil/water interface creates a weaker membrane that becomes more susceptible to fat destabilization during the freezing process (Goff and Jordan, 1989; Goff, 1997a; Marshall et al., 2003).

Since homogenization conditions affect fat droplet size and the association of emulsifier in the droplet surface, homogenization affects the meltdown stability of ice cream (Koxholt et al., 2001). Homogenization is the first step in fat structure formation (Goff, 1997a).

2.1.2.2. Aging

The aging step is defined as the time in the process where the mix will be cooled to temperatures of 4-5°C and kept at that temperature for a period of 4-24 h (Marshall et al., 2003). Aging conditions promote some significant changes in the ice cream mix. The low temperature leads to the formation of partially crystalline fat droplets and raises the
interfacial activity of emulsifiers. The long period of aging gives enough time for full hydration of the milk proteins and stabilizers (Barfod et al., 1991; Gelin et al., 1994; Berger, 1997).

The complete hydration of protein and stabilizer confers an increase in the viscosity of the ice cream mix. A higher viscosity of the mix is associated with better whipping and aeration, with an increase in meltdown stability of the frozen ice cream and with the reduction in ice crystal growth during storage (Cottrell et al., 1979; Berger, 1997).

At lower temperatures, the dissociation of casein micelles from the oil droplet interface by emulsifier molecules is more evident. Using transmission electron microscopy (TEM) and Kjeldahl analysis, Goff et al. (1987) studied the displacement of casein micelles from the oil-water interface using Polysorbate 80 (Tween 80) as an emulsifier. Overall, emulsifiers reduce the surface tension at the oil/water interface when cooled to a low positive temperature (Barfod et al., 1991; Gelin et al., 1994). Lutton et al. (1969) investigated the physical properties and the effect of temperature on the emulsifier layer formed around the fat droplet in an emulsion system. The reduction in surface tension was associated with the crystallization of the emulsifier monolayer at the fat droplet surface. The interfacial activity of milk proteins is not strongly affected by changes in temperature (Krog, 1991).

Partial crystallization of fat droplets in ice cream mix is important to promote partial coalescence, which leads to formation of a stable ice cream structure (Goff, 1997a). Although there is not much information about the kinetics of fat crystallization during aging, Berger (1997) stated that fat droplets in ice cream mix require a period of
4.5 to 6 h of ageing at 5°C to be fully crystallized. Several studies assert that aging for 4-24 h is fundamental for the crystallization of the fat in the mix (Goff, 1997a; Goff, 2006; Berger, 1997; Adleman and Hartel, 2001). The kinetics of crystallization during aging can be affected by time or temperature of ageing but also by the size of the fat droplets, the melting point of the fat, the rate of cooling and the type and concentration of emulsifier used in the emulsion (Adleman and Hartel, 2001).

Crystallization of fat occurs when the cooling condition is reached. By lowering the temperature of the fat below its melting point, the molecules organize themselves in a unique ordered arrangement, leading to nucleation and crystal formation (Hartel, 2001). Nucleation can occur by two different processes: homogeneous and heterogeneous nucleation. Homogeneous nucleation can be described as the organization of molecules that come together to form a nucleus. From that point, the nucleus can convert to a crystal lattice and easily grow to promote further crystallization. For homogeneous nucleation to occur, intense supercooling condition is required to form the nucleus. Homogeneous nucleation is rare in most of crystallizing species. On the other hand, heterogeneous nucleation is much more likely to occur and can easily be exemplified by the crystallization of bulk liquids. In a bulk system, impurities can act as nucleation sites, in other words, they can be starting points for crystallization to occur. Because of that, a lower supercooling condition is sufficient to start crystallization (Hartel, 2001; Coupland, 2002).

In an emulsion fat crystals do not form as fast as in a bulk fat system due to the formation of an enormous number of fat droplets. According to Adleman and Hartel (2001), 1 g of ice cream contains more than one trillion fat globules. For crystals to form
in the fat droplets, nucleation has to occur inside each droplet. The presence of impurities inside each droplet is necessary for heterogeneous nucleation to occur. However, the number of droplet may exceed the number of impurities that acts as nucleation sites. Therefore, crystallization in emulsions requires, to some extent, homogeneous nucleation. In other words, for crystallization to occur, intense supercooling conditions will be required in order to start nucleation. Coupland (2002) listed some factors that can influence and accelerate the nucleation rate in emulsions such as the presence of surfactant. Also, he mentioned that solid fat droplets can influence the crystallization of liquid fat droplets when in contact after collision.

2.1.2.3. Freezing

The freezing process of ice cream involves the formation of two new dispersed phases: air cells and ice crystals. Fat destabilization of partially crystalline globules creates a network that stabilizes air cells incorporated during agitation. The freezing process is carried out in a barrel shaped scraped-surface heat exchanger. The cold temperature of the barrel wall (-23 to -29°C for a batch freezer) promotes the crystallization of the water that is scraped off by the blades and incorporated into the mix (Berger, 1976; Marshall et al., 2003). As ice crystals are formed and water is removed from the mix, a concentrated serum phase with milk protein, sugars and salts is created. The concentration of the serum phase reduces its freezing point, creating an unfrozen phase in the ice cream that will always exist (Goff, 1997a; Marshall et al., 2003). The presence and shape of ice crystals formed during freezing process is believed to accelerate fat destabilization by enhancing shear forces (Goff, 1997b).
2.1.2.3.1. Destabilization of Fat

Fat destabilization is one of the most critical stages in the development of ice cream structure. It is responsible for the stabilization of the air incorporated in the mix, which results in one of the most important attributes in the final product: the texture.

Fat destabilization is based on two important factors: the intense shear conferred in the freezing process and the weak membrane of the fat droplet caused by the displacement of the protein by emulsifiers. The aggregation of fat droplets occurs by a mechanism called partial coalescence. The freezing process creates intense shearing in the mix by the rotation of the blade and the formation of ice crystals, which is fundamental to cause fat destabilization in ice cream (Marshall et al., 2003). Van Boekel and Walstra (1981) have shown that crystals in partially crystalline fat droplets have the tendency to orient themselves to the interface of the droplet. The weaker membrane around the fat droplets, caused by the replacement of milk proteins by emulsifiers, allows the crystals to easily protrude from the membrane of the fat droplet. If a fat droplet collides with another the crystal can easily puncture the membrane of the second fat droplet. Since the affinity between fat crystals and oil is higher than between fat crystals and water, oil flows out of the droplet and connects the two droplets. Because of the network created by the extensive amount of crystalline fat inside the droplet it has enough structure to keep it rounded in shape (Coupland, 2002; Goff, 1997b). Droplets that undergo partial coalescence are stable because of the presence of fat crystals. The melting of the fat can destabilize and cause complete coalescence of the fat droplets (Vanapalli and Coupland, 2001). Figure 2.1 shows the schematic difference between partially coalesced droplets containing fat crystals and coalesced liquid droplets.
Partial coalescence is a fat destabilization mechanism that can happen only in the presence of partially crystalline fat droplets. The crystals arrange themselves to reach an equilibrium position with a low free energy. As a result of the different forms of arrangement of the fat crystals five types of crystallization in fat droplets have been reported (Table 2.3). L and M types are formed due to melting and re-crystallization of the fat. The tangential orientation of the larger crystalline layer to the oil/water interface reduces the tendency of the crystals to puncture the lamella and undergo partial coalescence. N-type globules are characterized by the formation of crystals inside the droplets not tangentially oriented to the interface. Those crystals have a higher tendency to partial coalescence (Fredrick et al., 2010). Boode and Walstra (1993) have also described a similar effect of the orientation of the crystals inside the droplets and the extent of partial coalescence.

The stability of an emulsion against coalescence, or more specifically to partial coalescence, is dependent on its wetting properties. The contact angle $\theta_w$, measured in the
continuous phase between crystal and interface, can be described as the equilibrium position of the fat crystal; the contact angle is related to the wetting behaviour of the crystals at the interface. A large $\theta_w$ angle describes crystals more likely wetted by the oil phase while smaller angles describe crystals more likely wetted by the aqueous phase. Partial coalescence will be more favourable at $\theta_w \approx 90^\circ$ where the crystal is equally wetted by the aqueous phase and the oil phase (Rousseau, 2000; Boode and Walstra, 1993).

Table 2.3. Types of fat crystallization in oil droplets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Contact angle $\theta_w$</th>
<th>Crystal's size</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>$\theta_w \approx 90^\circ$</td>
<td>large</td>
<td>A large, centered crystal</td>
</tr>
<tr>
<td>N2</td>
<td>$\theta_w \approx 90^\circ$</td>
<td>Small</td>
<td>Small crystals randomly distributed inside the droplet</td>
</tr>
<tr>
<td>L</td>
<td>$\theta_w &gt;&gt; 90^\circ$</td>
<td>large</td>
<td>Large crystals formed after small crystals being melted. Located tangentially to the fat droplet surface.</td>
</tr>
<tr>
<td>M</td>
<td>$\theta_w &gt;&gt; 90^\circ$</td>
<td>Large and small</td>
<td>Large crystals located tangentially to the edge of the fat droplet and small crystals concentrated in the interior of the droplet.</td>
</tr>
<tr>
<td>K</td>
<td>$\theta_w \leq 90^\circ$</td>
<td>large</td>
<td>A large and long droplet allocated tangentially to the exterior edge of the droplet. Crystals are too thick to follow the curvature, so they grow outside of the droplet</td>
</tr>
</tbody>
</table>

Description of the schematic representation of the types of oil droplets observed by *Fredrick et al., 2010.*
2.1.2.3.2. Air Incorporation

During the freezing of ice cream, in addition to the formation of ice crystals, there is a concentration of the serum phase, destabilization and aggregation of fat droplets, and air is incorporated into the mix in the form of air cells.

Air can be incorporated in different ways depending on the type of freezing process applied. In a continuous freezer, small air bubbles are injected into the mix while the mix is freezing. In a batch freezer, air cells are incorporated by whipping the frozen mix (Marshall et al., 2003). Caldwell et al. (1992b) revealed a difference in the microstructure of ice cream processed in a continuous freezer compared to in a batch freezer. The first process differs from the second by the presence of smaller and more spherical air cells, plus the larger number of air cells due to a higher overrun.

As has been already mentioned, the source of the fat is crucial in the incorporation and stabilization of air cells in ice cream. It influences the extent of partial coalescence that forms the network responsible for air incorporation. Shear stress is important in partial coalescence of fat droplets and so is the presence of air bubbles and ice crystals (Goff, 1997a).

2.1.2.4. Hardening

The aim of the hardening step during ice cream manufacture is to quickly reduce the temperature of the ice cream after freezing to about -18ºC or lower. Fast freezing avoids formation of large crystals and large air cells that can compromise the quality of ice cream. The use of cold air that circulates and blows over the sample to reduce the
temperature is a very common method used in hardening of ice cream (Adleman and Hartel, 2001).

2.1.3. Some Ice Cream Structural Components

2.1.3.1. Air Cells

Air bubbles play an important role in the development of the ice cream structure that is directly associated to the quality of the product. The mechanism of air cell formation in frozen ice cream is promoted by air incorporation. During whipping, air is incorporated to the mix and broken down into smaller cells that are stabilized by the presence of fat. Air bubbles start as large units and end up in sizes ranging from 20 to 50 μm.

In ice cream, foam is created by the dispersion of gas into the aqueous phase, which generates an unstable system in the absence of a fat droplets network. The three most common mechanisms related to the destabilization of foam are coalescence, disproportionation (Ostwald ripening) and liquid drainage. Coalescence of air cells is characterized by the formation of a large air cell from the combination of two or more small cells. Ostwald ripening arises from the difference in gas pressure between different sizes of bubbles. Based on the Laplace pressure, a smaller bubble will have a higher pressure than a bigger air bubble. Therefore, because of the concentration gradient difference, gas tends to diffuse from the smaller cell to the bigger cell. Large cells are formed and small cells tend to disappear. Liquid drainage is caused due to gravity which forces the serum phase around the air cells to flow down and the air cells to rise to the top (Chang and Hartel, 2002b).
During whipping, the air cells are stabilized by a fat network. The surface tension of air cells also seems to be important in its stability. Laplace pressure establishes the influence of surface tension on the instability of air bubbles. It can be defined as the difference in pressure between the outside and inside of a bubble. In other words, it is the driving force that leads to the spherical shape of the bubbles. Laplace pressure equation can be described by:

\[ \Delta p = \frac{2\gamma}{r} \]

where \( \gamma \) is the surface tension and \( r \) is the radius of the air bubble (Prins, 1988). Since a spherical shape of bubbles minimizes its surface area and therefore minimizes surface tension, bubbles have the tendency to assume a spherical shape for more stability. Moreover, the adsorption of proteins at the air-water interface also helps to reduce the surface tension and give stability to the air cells. The stabilization of air cells can resist gas diffusion and shrinkage of the ice cream (Turan and Bee, 1999). In ice cream, however, the growth of ice crystals can also interfere with the spherical shape of air bubbles.

2.1.3.2. Fat Droplets

Fat is the constituent most related to the development of texture and flavour of ice cream. According to Marshall et al. (2003), texture can be defined as the mouth-feel sensation associated with the structure of the ice cream. The development of structure seems to start in the homogenization step, where fat droplets are dispersed into an aqueous mix of proteins, sugar and stabilizers. However, the texture and the formation of the final structure of ice cream occur in the freezing step. As the ice cream mix is frozen
a dispersed phase of ice crystals is formed; the shear blades of a scraped surface freezer cause the incorporation of air, fat droplet destabilization and the fat network formation that is responsible for the entrapment of the air cells (Goff, 2006). The extent of fat destabilization and network formation is directly associated to some important properties of ice cream such as shape retention, dryness, meltdown stability and a smooth texture (Goff, 2002).

The destabilization of the fat is due to the partial coalescence of the fat droplets. The agglomeration of fat creates a network that stabilizes air cells, offering resistance against meltdown. Figure 2 shows the network formation around an air cell and the spherical form retention of the fat droplets, resulting from the mechanical strength offered by the crystalline fat inside the droplet.

Figure 2.2. Scanning electron micrograph of a frozen ice cream sample showing the network of spherical milk fat droplets (B) involving the air cell (A).
As has been discussed in the above review, the presence of a partially crystalline fat structure seems to be essential for the partial coalescence of fat droplets and the development of the structure of ice cream. The wide melting profile of milk fat is responsible not only for structure development but also for the mouth-feel sensation conferred by the structure of ice cream. With the worldwide concern related to the consumption of high levels of saturated fat and \textit{trans}-fatty acid, industries have met a challenge that includes the replacement of traditional saturated fat while keeping the texture and flavour usually conferred by the presence of saturated fat. However, finding alternatives to saturated triacylglycerols is not as easy as the manufacturers would like it to be. The following review will introduce a novel structuring method that can be used to structure edible oils and potentially substitute the traditional triglyceride network structuring.

2.2. Organogels

Organogels can be defined as viscoelastic materials formed from the immobilization of an organic fluid by the gelling network of a material. In other words, organogels are solid-like materials with features and rheological properties of a solid but with a greater part of its composition being a liquid (~98%). Edible oil is an example of an organic solvent that can be gelled with relatively low concentration of organogelators. Low molecular weight organogelators have been extensively studied due to their great ability to gel at low concentration by self-assembling in a 3 dimensional network (Weiss and Terech, 2006). Abdallah and Weiss (2000) consider low molecular weight
organogelators, molecules that are in the group of organic compounds and with a molecular weight usually $\leq 3000$ Da.

Several definitions have been used to explain or simply to define the state of gelation of materials. Thomas Graham (1861) was the first to classify a gel as a colloidal system. Dorothy Jordan Lloyd (1926) stated that a “gel is easier to recognize than to define”. Bungenberg de Jong (1949) presented the definition of a gel as a solid-like system that is composed by colloidal particle materials that form a cohesive structure responsible to entrap the continuous phase (usually liquids). Hermans (1949), however, simply defined a gel as the dry material formed by allowing some substances, such as gelatin and cellulose, to swell and adsorb the liquid phase. He also presented the term gel as a “coherent colloidal system formed by at least two components”, with “mechanical properties of a solid state” and with both components “extended continuously throughout the whole system”. A more complex definition was stated by Flory (1974) where he discusses the theory of gelation in terms of the molecular distribution of the components as well as its molecular arrangement and interlinkage. Presently, gel is understood to be formed by the combination of a gelator substance with its appropriate solvent which self-assembles in a network that immobilizes the liquid, due to surface tension, and forms a solid-like material but with most of its composition being a liquid (Vintiloiu and Jean-Christophe, 2007).

Several reviews have described the different types of organogelators to structure lipid materials that are suitable for food application. Among them are fatty acids, fatty alcohols, wax esters, monoacylglycerols, phospholipids, sorbitan esters, phytosterols (Pernetti et al., 2007) and more recently ceramides (Rogers et al., 2011).
Organogels not only differ in the type of structurant but also in the general structuring concept that will control the solvent gelation. According to Marangoni and Garti (2011) five different building blocks can be identified in the structuring process of edible oils. They can be crystalline particles, self-assembled fibrillar network, polymers, solid particles and liquid crystalline mesophases. These are the structuring elements necessary for the formation of the three dimensional network in structured oils. The characteristics of each building block are described in Table 2.4.

Table 2.4. Building blocks for the formation of the three dimensional network in structured oils.

<table>
<thead>
<tr>
<th>Building blocks</th>
<th>Structuring agents</th>
<th>Description of the network</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline Particles</td>
<td>Fatty acids, wax esters, ceramides</td>
<td>Colloidal crystalline triacylglycerol particles that trap oil inside</td>
</tr>
<tr>
<td>Self-assembled Fibrillar Network (SAFiN)</td>
<td>Phytosterols and orizanol, ricinoleic acid, 12-hydroxystearic acid</td>
<td>Crystalline fibres of one dimensional growth that interact with each other forming a three dimensional network</td>
</tr>
<tr>
<td>Polymers</td>
<td>Ethylcellulose</td>
<td>Cross-linked network stabilized by hydrogen bonds and hydrophobic interactions</td>
</tr>
<tr>
<td>Particle-filled Network</td>
<td>Solid or liquid non-fat particles</td>
<td>A high concentration of particles closely package to form a network</td>
</tr>
<tr>
<td>Liquid Crystalline Mesophases</td>
<td>Monoacylglycerol</td>
<td>Orientation of molecules occurs in mesophases. The reversed bilayers of monoacylglycerol are able to laminate oil</td>
</tr>
</tbody>
</table>

Source: Marangoni and Garti, 2011.
2.2.1. Applications

Organogelators have been of great interest to food, drug, cosmetics and petrochemical industries because of their particular ability to structure oils. At the same time, most of the organogelators described in the literature cannot be applied in food, for safety reasons. However, due to the promising trend in this area, different food grade organogelators have been identified and studied lately. The focus has been not only the structuring characteristics of this new promising system, but also to the potential to develop some other important attributes in a wide variety of products. Rogers (2008) has suggested the potential use of organogel to prevent oil migration in fat based foods. In the pharmaceutical industry, organogels have been of considerable importance to drug and vaccine delivery formulations. Some aspects of lecithin organogel have been studied with great interest for topical drug delivery application. In this case, thermoreversibility, viscoelasticity and biocompatibility are some of the aspects provided by the system that improves aspects of the final product (Kumar and Katare, 2005).

2.2.2. Some Classes of Organogelators Suitable for Food Application

2.2.2.1. Fatty Acids

The gelling ability of fatty acids has been used as thickener agents in the production of lubricating grease for decades (Boner, 1954). Ricinelaicd acid (REA) was found to gel edible oils at a minimal concentration of 0.5%. The gel was characterized by the difference in turbidity due to concentration of ricinelaidic acid and the formation of a hard solid-like organogel at 5% fatty acid concentration at 30°C. Polarized light microscopy analysis of the same gel revealed an irregular network characterized by long
fibres, formed by the intermolecular linkage of the fatty acid. The use of sesame oil and diacetylglycerol oil as the organic solvent did not lead to gelation for similar concentrations of fatty acid, which suggested that hydrogen bonding is necessary for the fatty acid gelation to occur. The ricinelaic acid organogel showed very good storage stability with no changes in microstructure after one month-storage period (Wright and Marangoni, 2006; Wright and Marangoni, 2007).

Daniel and Rajasekharan (2003) found a direct relationship between the chain length of saturated fatty acid and its ability to gel. Fatty acids with larger chain length were found to promote gelation faster and in lower concentrations than fatty acids of a shorter chain. They also noticed similar efficacy in gelling for saturated wax esters compared to the fatty acids. According to them, the gelation mechanism can be explained by an alignment of the molecules, head-to-tail, because of its amphiphilic properties. The acyl chain is stabilized by intermolecular hydrogen bonds, which creates a linear structure that has the ability to arrange itself and entrap the oil forming a solid-gel structure.

Terech and Weiss (1997) and Daniel and Rajasekharan (2003) suggested that the chirality of the molecule, as in the case of 12-Hydroxyoctadecanoic acid, is related to an increase in the gelation ability of the fatty acid. However the asymmetry of the molecule does not fully explain the mechanism of gelation in fatty acids.

2.2.2.2. Fatty Alcohols

A synergistic effect of fatty acid and fatty alcohol mixtures, compared to the pure components, in the gelation of vegetable oils was observed by Gandolfo et al. (2004).
The same study showed the ability of fatty acids (palmitic, stearic, eicosanoic and behenic acids) and fatty alcohols (1-hexadecanol, 1-octadecanol, 1-eicosanol and 1-docosanol) to structure edible oils at concentrations of 2% and a temperature of 5ºC. They also presented a better efficiency for the n-alcohols in terms of gelation ability compared to the respective fatty acids for similar concentrations.

On the other hand, Daniel and Rajasekharan (2003) have found that fatty acids had a higher ability to structure oil than the fatty alcohol with the same chain length.

2.2.2.3. Monoglyceride

Monoglycerides are well-known emulsifiers widely used in food products (O’Brien, 2004). They have the ability of gelling and structuring aqueous and oil systems (Pernetti et al., 2007). The organization of the monoglyceride molecules occurs in mesophases where the metastable α-crystalline gel phase is formed. Since the α-gel state is not thermodynamically stable it is converted in a more stable phase characterized by a network of plate-like β-crystals called coagel (Ojijo et al., 2004, Heertje et al., 1998, Sein et al. 2002, van Duynhoven et al., 2005). This unique molecular orientation assigns good structuring and a similar mouth feel to some products high in saturated fat. With the ability to also change the physical properties of oil, a great interest in monoacylglycerol has led to several studies on the development of organogels with the same characteristics of the monoglyceride hydrogels (Ojijo et al., 2004).

In lipids, the gel structure formation is not completely understood but it is believed to occur by an arrangement of the monoacylglycerol molecules in a reversed bilayer. Gel formation is noticed at concentration as low as 1.3%. Long storage time and
cooling rates were associated with changes in the hardness and crystallinity of monoglyceride gels. The cooling rate is associated with the difference in the polymorphic forms of the crystals. In a low cooling rate β-crystals can also be formed, while in high cooling rates only α-crystal can be formed. The presence of β-crystals seems to increase the hardness of the organogel (Ojijo et al., 2004, Marangoni and Garti, 2011).

2.2.2.4. Wax Esters

Fatty acids, fatty alcohols, their salts with alkali metals and esters are known to have the ability to gel organic solvents at lower concentrations (Weiss and Terech, 2006). Candelilla wax (CDW) has been shown to have the ability to structure safflower oil at 1% concentration at 25°C. Its composition is formed by n-alkanes (~50%), esters (20 - 29%), alcohols and sterols (12-14%) and free fatty acids (7-9%). Despite the high concentration of n-alkanes, the crystallization and the arrangement of the three dimensional network revealed to be less intense than the pure n-alkane with a lower structural order and lower rheological properties (Toro-Vazquez et al., 2007; Moralez-Rueda et al., 2009b).

The physical properties of rice bran wax (RBW) organogels were studied and compared to CDW and carnauba wax (CBW) organogels. With morphology characterized by the formation of long needle crystals, RBW showed a higher ability to structure olive oil (minimum of ~0.5%) compared to CDW (~1%) and CBW (~4%). The hardness results from the penetration method were higher for RBW organogel than for CDW and CBW organogels. That is an indication that the very long needle structures are favourable to the formation of the gel and immobilization of the triacylglycerols (Dassanayake et al., 2009).
2.2.3. **Waxes**

Waxes are composed of a mix of several compounds that forms a very complex mixture. Among them are hydrocarbons, fatty acids and fatty alcohols, esters of fatty acids and fatty alcohols, sterol esters, ketones, diol, etc. Most of the waxes tend to contain ester as the major component (Wolfmeier *et al.*, 2002; Kolattukudy, 1976). However, no general definition seems to be adopted to specify the meaning of wax. Some suggest that wax refers to an ester of fatty acid and fatty alcohol (Gunstone *et al.*, 1986; Wolfmeier *et al.*, 2002 and Vali *et al.*, 2005). Bennett (1963) and Wolfmeier *et al.* (2002) give a technical description of waxes as a material composed by materials of different chemical classification. For the purpose of this thesis, waxes will be considered as a mixture of a great number of organic compounds with major concentration of esters of fatty acid and fatty alcohol (Figure 2.3).

![Lignoceric acid (C24:0)](image1.png)

![Tricontanol (C30)](image2.png)

![Ester of Lignoceric acid and tricontanol](image3.png)

Figure 2.3. Schematic representation of a wax ester from RBW (*Dassanayake et al.*, 2011)

Waxes can be classified in two main groups; synthetic and natural waxes (Wolfmeier *et al.*, 2002).

Natural waxes are materials that occur in nature and do not require any chemical treatment to be formed. Natural waxes can be subdivided in five more specific groups:
insect waxes, animal waxes, plant waxes, microorganism waxes and mineral waxes. Among them we can find mineral waxes like paraffin wax, animal waxes like beeswax and whale spermaceti and vegetable waxes, like CDW and RBW (Warth, 1956; Wolfmeier et al., 2002; Bennett, 1963).

On the other hand, synthetic waxes are formed by some chemical reaction. The formation of compounds from wax-like material is considered to be a partial synthesis of wax. Partially synthetic waxes can be formed by reactions to the esterification of fatty acids and fatty alcohol or the cleavage of esters. Fully synthetic waxes are the waxes formed by the aggregation of low molecular compounds to form materials with similar physical properties of natural waxes. Poly-olefin is an example of fully synthetic wax formed by the combination of organic molecules as ethylene, propane and butane (Wolfmeier et al., 2002; Warth, 1956).

Waxes have been extensively used in many industrial sectors due to distinct properties such as hygroscopicity, slipperiness, polishing properties, transparency, adhesiveness, gelling and many others (Bennett, 1963). In the food industry, waxes have been used as release agents in bakery and confectionery, coating for fruits, vegetables and cheese and in the formulation of chewing gums, defoaming agents and microcapsule for flavours (Wolfmeier et al., 2002 and US FDA, 2010). Regarding safety issues relating to the consumption of wax, especially paraffin wax, recommendations and regulations have been created in order to control the use of this compound by the industry. The waxes listed in the Food Additive Status list by Food and Drug administration are: beeswax, borax, CDW, CBW, petroleum wax, paraffin wax and RBW. These are substances generally recognized as safe and their use can be restricted in the case of paraffin wax, or
not, as beeswax. However, without restrictions their use must conform to good manufacturing practices (US FDA, 2010).

2.2.3.1. Rice Bran Wax

Rice oil, better known as rice bran oil, is the oil extracted from internal layers of rice, *Oriза Satива L*. The layers with larger concentration of oil are germ, aleurone and subaleurone layers, which represent only 10% of the grain. Rice bran oil is largely produced in East Asia due to the high consumption of rice. Lately the interest in rice bran oil has increased as a healthier option of edible oil. Its healthier characteristic is attributed to the presence of γ-oryzanol and tocotrienols that have been associated with the ability to control plasma cholesterol levels (Orthoefer, 2005; Gunstone *et al.*, 2007). Rice bran is composed by lipids (18-24%), fibre, protein, carbohydrate, vitamins and minerals, with rice bran being the main source of rice oil. The fatty acid composition consists mainly of palmitic (C16:0) ranging from 12 to 28%, oleic (C18:1) from 35 to 50%, and linoleic acid (C18:2) from 29 to 45% (typically 32%). In lower concentrations, rice bran oil also possesses stearic acid (C18:0) ranging from 2 to 4% and linolenic acid (C18:3), which varies from 0.5 to 1.8% (Gunstone *et al.*, 2007).

RBW is a natural wax found in crude rice oil (Dassanayake, 2009). With the increased consumption of rice bran oil due to its health benefits there is also an increase in interest in production of RBW as a by-product from rice oil. The wax is found both in the bran or bran coat of rice, which is obtained in the milling process that separates hulls from rice (Warth, 1956). The concentration of wax in rice oil can vary (2-5%) depending on the oil extraction conditions as well as the variety of rice (Orthoefer, 1996)
RBW is obtained from the dewaxing process of the crude oil. The process includes cooling the mix to 20-25°C (winterization) and removing the sludge by filtration, centrifugation or solvents that give a more efficient separation (Warth, 1956). The crude wax sludge can be purified by washing it with methyl alcohol, acetone, ether and finally chloroform (Bennett, 1963). Vali et al. (2005) have suggested the use of hexane to remove the oil excess in the crude sludge wax followed by dissolving it in isopropanol and then filtration at room temperature. In their process the RBW was also bleached, which was intended to remove resinous matter that confers a black colour to the crude wax. NaBH₄ (sodium tetrahydridoborate) was used as the bleaching agent. Hydrogen peroxide (H₂O₂), chromium trioxide (CrO₃) and sulphuric acid (H₂SO₄) have also been used as bleaching agents as reported by Warth (1956). An intense bleaching was observed with the combination of peroxide and chromium trioxide, resulting in a wax with a white color.

Some studies have been controversial in terms of the composition of RBW and it seems that the exact composition is not completely defined. Bennett (1963) defined it as a composition of esters of lignoceric acid (C24:0) and myricil alcohol (C30). Warth (1956) defined it as a composition of esters of waxy acids (C22, C24, C26) and alcohols (C26, C28, C30). Later on, Yoon and Rhee (1982) have separated two portions of the wax; a hard and a soft wax. Both waxes showed composition based on hydrocarbons, esters of fatty alcohols and fatty acids where the soft wax was mainly composed by n-Henicosane (C21), n-Nonacosane (C29), Palmitic acid (C16:0), lignoceric acid (C24:0), Cerotic acid (C26:0), lignoceryl alcohol (C24), myricyl alcohol (C30) and the hard wax composed by n-Nonacosane (C29), n-Hentriacontane (C31), lignoceric acid (C24:0),
Cerotic acid (C26:0), behenyl alcohol (C22), lignoceryl alcohol (C24), ceryl alcohol (C26), myricyl alcohol (C30). A more complex composition is presented by Orthoefer (2005) where fatty acids of sterol and alkyl esters, alcohols of longer alkyl esters, alkanes and alkenes have been identified in the RBW material. The main composition was described as palmitic acid (C16:0), behenic acid (C22:0) and lignoceric acid (C24:0). Dassanayake (2009) presented the chemical composition of RBW as fatty acids (C22:0 and C24:0) and fatty alcohol (C28, C30, C32, C34). Vali et al. (2005) relates the differences in composition with different methods of preparation and purification used by the different studies. RBW fatty acid composition and physical characteristics are summarized in Table 2.5.

RBW has the potential to substitute popular waxes as CBW and CDW. The application of RBW in the formulation of cosmetics and pharmaceutical products has already been documented (Vali et al., 2005). But not only that, RBW has been shown to have a potential for application in the food industry as an oil-structuring material. Recently RBW and its ability to structure edible oils have been studied. Dassanayake et al. (2009) have investigated the thermal behaviour and the crystal morphology of RBW organogels. Compared to CDW and CBW, RBW showed a better ability to structure oils at lower concentration. A minimum of 0.5% of RBW was necessary for gelation to occur, compared to 2% for CDW or 4% for CBW. In terms of crystal morphology, RBW formed very long needles that were associated with better gel formation. Moreover, RBW is considered also as a food additive but currently has restrictions on its use and can only be applied as coating fruits, cheese and vegetables and in the formulation of chewing gum (21 CFR 172, 2011).
Table 2.5. Physical characteristics, fatty acid, fatty alcohol and hydrocarbon composition of RBW compared to CDW and CBW.

<table>
<thead>
<tr>
<th>Chain length</th>
<th>Fatty Acid (%)</th>
<th>Fatty Alcohol (%)</th>
<th>Hydrocarbon (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBW&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Candelilla&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Carnauba&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>3.6</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>2.3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>5.3</td>
<td>12</td>
<td>11.5</td>
</tr>
<tr>
<td>22</td>
<td>26.1</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>24</td>
<td>40.5</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>26</td>
<td>11.5</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>28</td>
<td>6.6</td>
<td>7</td>
<td>16.5</td>
</tr>
<tr>
<td>30</td>
<td>3.1</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>32</td>
<td>1.0</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>RBW&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Candelilla&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Carnauba&lt;sup&gt;d,e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting Point (ºC)</td>
<td>78 - 82</td>
<td>60 - 73</td>
<td>80 - 85</td>
</tr>
<tr>
<td>Iodine value</td>
<td>≤ 20</td>
<td>13 - 23</td>
<td>7 - 14</td>
</tr>
<tr>
<td>Saponification value (mg KOH/g)</td>
<td>75 - 120</td>
<td>47 - 61</td>
<td>78 - 95</td>
</tr>
<tr>
<td>Acid Value (mg KOH/g)</td>
<td>≤13</td>
<td>9 - 20</td>
<td>2.9 - 9.7</td>
</tr>
<tr>
<td>Ester Content (%)</td>
<td>92 - 97</td>
<td>28 - 29</td>
<td>84 - 85</td>
</tr>
<tr>
<td>Free Fatty Acid (%)</td>
<td>0 - 2</td>
<td>7 - 9</td>
<td>3 - 3.5</td>
</tr>
<tr>
<td>Free Fatty Alcohol (%)</td>
<td>-</td>
<td>12 – 14*</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Hydrocarbons (%)</td>
<td>-</td>
<td>50 - 51</td>
<td>1.5 - 3</td>
</tr>
<tr>
<td>Resins (Others) (%)</td>
<td>3 - 8</td>
<td>-</td>
<td>6.5 - 10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples included defatted and bleached samples (Dassanayake <i>et al.</i>, 2009)
<sup>b</sup> <i>(Vali et al., 2005)</i>
<sup>c</sup> <i>(Orthoefer, 1996)</i>
<sup>d</sup> CDW vary according to species <i>(Warth, 1956)</i>
<sup>e</sup> Koster Keunen sample’s datasheet
<sup>f</sup> <i>(Tulloch, 1973)</i>
* Includes percentage of sterols and resins
Recently, Ghosh and Bandyopadhyay (2005) studied the crystallization of RBW in hexane. The study was performed to improve the separation of wax from rice bran oil using membrane technology. In this case the formation and growth of large crystals would facilitate adherence to the membrane and consequently wax separation. They have noticed that the crystallization of RBW in hexane is affected by a couple of factors. First is the temperature at which the solutions were allowed to crystallize. Solutions at lower temperature (5-10°C) presented a high viscosity and a delay in the starting point of crystallization, probably due to difficulty in the mobility of the molecules. The second factor that affected the crystallization of RBW was the time of incubation. After 60 min incubation, crystal growth was not increased. The addition of other components such as rice bran gum and rice bran oil had a significant effect on the initiation of crystallization of RBW.

2.2.3.2. Candelilla Wax

CDW is a natural wax originating in north-western Mexico and south-western Texas derived from the leaves of *Euphorbia cerifera* and *Euphorbia antisypilitica*, from the family *Euphorbiaceae*. CDW can also be obtained from *Pedilanthus Pavonis Boissier* and the *Pedilanthus Aphyllus Boissier* plants, however, those plants offer a much lower wax yield (Instituto de la Candelilla, 2004; Bennett, 1963; Morales-Rueda et al., 2009b; Warth, 1956).

The CDW can be extracted from all the external parts of the plant, with exception of the roots. The extraction can be done in boiling water, with sulphuric acid or by solvent, the first being the more traditional method and still the most common (Warth,
1956). The approximate composition of refined CDW is of 49-51% hydrocarbons, 28-29% esters of fatty acids and fatty alcohols, 12-14% alcohols and sterols and 7-9% of free acids (Toro-Vazquez et al., 2007; Morales-Rueda et al., 2009a). Another important characteristic of CDW is its low melting point (70.5°C) compared with the majority of the waxes. Based on that, it has already been used as a hardening agent of other waxes without increasing the melting point (Warth, 1956).

The ability of CDW to structure oil has also been investigated. Despite its high quantity of n-alkanes, polarized light micrographs of 1 and 3% wax organogel have shown a crystal morphology characterized by small crystals compared to the long needle crystals formed by a pure n-alkane (C32) (Morales-Rueda et al., 2009a).

CDW is recognized as a food additive by the Food and Drug administration and can be used with no restriction other than following good manufacturing practices (US FDA, 2010).

2.2.3.3. Carnauba Wax

CBW is a plant wax obtained from the leaves of Copernicia cerifera Martius, a palm tree that mainly occurs in the dry climate of north east Brazil. Because of the arid climate, the carnauba palm releases wax to protect the leaves against intense evaporation of water, providing about 150 g of CBW per plant/year (Wolfmeier et al., 2002; Warth, 1956).

The extraction starts by drying the leaves in the sun, which facilitates the removal of the wax by beating the dried and shrinkage leaves or simply removing the wax off the surface. The process is followed by melting the wax in boiling water, which separates a
crude wax. That can be purified by several methods such as centrifugation and solvent extraction (Wolfmeier et al., 2002; Bennett, 1963). Four different types of wax can be obtained according to the maturity of the leaves. Younger leaves provide type I and type II waxes, while mature leaves generate type III and IV waxes (Warth, 1956). The composition of the CBW can vary for each type, however it can be summarised by the composition described in Table 2.5.

CBW has one of the highest melting points if compared with other natural waxes. Because of this property, CBW is known, between other plant waxes, as a “melting point booster” and can be combined with other waxes to increase their melting point. Other valuable characteristics are its hardness, lustre ability and discrete and pleasant odour (Wolfmeier et al., 2002; Bennett, 1963).

CBW can be used in the fabrication of candles, cosmetics, lubricants, polishes, varnishes, inks, finishes for leather etc. With the aim of application in food, CBW is considered safe under consumption and is usually used as glazing agent in the production of gums, soft candies, gravy and sauces as well as coating for foods (21 CFR 184, 2011).

2.3. Ice Cream Structure Characterization

2.3.1. Standard Methods

2.3.1.1. Overrun

Overrun is the measurement, commonly used by the industry, of the amount of air incorporated in the frozen ice cream. It is expressed by the percentage increase in volume
that the initial ice cream mix undergoes during whipping (batch process) or injection of air (continuous process). In other words, an ice cream with a 100% overrun has had its volume doubled by the incorporation of air (Marshall et al., 2003).

The light and soft texture of ice cream is directly related to its ability to incorporate and stabilize air cells. As has been already mentioned, the destabilization of fat droplets is responsible for the stabilization of air cells and consequently to obtaining a high overrun. Therefore, overrun measurements become an easy way to measure the development of the structure of ice cream. Parameters such as meltdown resistance of ice cream, among others, have been associated with the overrun obtained during freezing (Muse and Hartel, 2004). In addition, a study has shown the relationship between overrun and air bubble size distribution, where an increase in overrun would lead to the formation of smaller air cells in the final ice cream (Rosalina and Hartel, 2004). As noticed, overrun has been widely used to characterize physical properties of frozen ice cream. However, according to Rosalina and Hartel (2004), the use of overrun as a tool to study the structure formation of ice cream has been shown to have conflicting results due to some secondary effects that are usually not evaluated.

2.3.1.2. Meltdown rate

The meltdown rate of ice cream can be determined by placing a known amount of ice cream over a mesh grid, at room temperature, and allowing it to melt. The meltdown rate of the ice cream is defined by the percentage of serum melted over time (Marshall et al., 2003).
The ability of an ice cream to resist meltdown is one of the most obvious attributes related to the structure of ice cream. As already discussed, the destabilization of fat and the formation of a fat network that wraps the air cells is believed to be one of the most important factors affecting meltdown stability. However, some other factors seem to affect the meltdown rate of ice cream such as the presence of a high volume of air in samples with higher overrun. The insulating effect caused by the presence of air seems to affect the heat transfer and consequently the meltdown rate of ice cream (Muse and Hartel, 2004). Muse and Hartel (2004) have also found in their study that ice crystal size and the viscosity of the mix also have an influence in the melting rate of frozen ice cream.

The meltdown stability test includes evaluation of other factors besides the meltdown rate. The shape retention also characterizes the fat network formation around the air cells that gives it structure and support to overcome melting, and roughly, keep the shape of the ice cream. Visual and physical analyses of the retained and dripped phases provide important information on the extent of fat destabilization and structure formation (Bolliger et al., 2000a; Muse and Hartel, 2004).

2.3.1.3. Light Scattering

As the emulsion is formed, controlling and monitoring its stability against aggregation and separation of the fat is important. It is also of interest to characterize the mix in terms of fat droplet size distribution to verify the level of dispersion. A stable emulsion, with small particle size, will lead to a satisfactory destabilization. Light scattering is one of the most common methods used to characterize the particle size of an
emulsion (Dalgleish, 2004). Two different light scattering techniques, dynamic and integrated light scattering, are widely used to measure particle size. In the framework of this thesis, only integrated light scattering (ILS) will be taken into consideration.

The ILS method consists of the application of a laser beam that traverses a clear cell containing a highly diluted solution of the emulsion. The particles in the solution scatter the light in different angles that are detected by the equipment. Software collects the information, and in conjunction with the optical properties of the particle, transforms it into particle size distribution data (Dalgleish, 2004; Aguilera and Stanley, 1999; Murphy, 1997).

ILS has the ability to measure a large range of scattering angles, which facilitate the analysis of a broader range of particle sizes. Additionally, new equipment has brought some advantages, which include backscatter and large angle detectors and a blue light source with a different wave length. These elements also help to improve resolution of the analysis by offering a wider detection range of particle sizes (Malvern Instruments, 2010).

The accuracy of the analyses depends on distinctive factors such as the optical properties of the particles being measured, the coherence of the spherical shape assumption of the particles, and the intensity of dilution of the emulsion being analyzed. In addition, it is important to have in mind that light scattering analysis is used to measure fat destabilization, but it is not capable of differentiating the types of fat destabilization that fat droplets can undergo. In other words, light scattering analysis might give similar results for an emulsion destabilized under partial coalescence or complete coalescence of their fat droplets (Dalgleish, 2004). A fairly well stabilized ice
cream mix should possess a monomodal globule size distribution with the size of the globule not greater than 2 μm and average size around 0.5 to 1 μm. After destabilization and formation of aggregates, due to partial coalescence, a bimodal distribution is expected with less than 50% of the particle sizes smaller than 2 μm. Values such as the volume mean diameter, \( d_{4,3} \), and the surface mean diameter, \( d_{3,2} \), are numbers more likely to be used to report the characteristics of the mix than are the normal mean or median (Marshall et al., 2003).

2.3.1.4. Differential Scanning Calorimetry

Another approach to analyse crystallization of fat in emulsions is by the use of the differential scanning calorimetry (DSC) technique. The technique consists of comparing the energy required (or liberated) to increase (or decrease) the temperature of a DSC pan that contains a small amount of sample, against an empty pan. The energy is exchanged, between the equipment and the pan, in the form of heat. Therefore, temperature and heat power are used to analyse the phase transition of the food system such as crystallization and melting of the fat phase. The data provides onset, middle and final temperatures, as well as the enthalpy of the phase transitions, that allows obtaining information on the extent of crystallization (Hartel, 2001; Aguilera and Stanley, 1999).

DSC has been widely used to investigate the thermal behaviour of edible oils and fats (Tan and Che Man, 2000). The use of DSC to study the crystallization and structural properties of fat droplets in ice cream emulsions has been reported by different studies (Granger et al. 2005; Bazmi and Relkin, 2006; Rosnani et al., 2007). The DSC curves of ice cream mixes have been shown to be affected by formulation, including triacylglycerol
composition and presence of surfactants, and formation of an emulsion, which interferes with the rate of nucleation and consequently with the rate and temperature of crystallization.

In the study of crystallization and melting of fat using DSC, the temperature scanning rate needs to be taken into consideration. The temperature scanning rate affects significantly the onset temperature and the enthalpy of the phase transitions. A low scanning rate will provide larger peaks, reaching higher levels of crystallization, and higher onset temperatures of crystallization, against a high scanning rate that will give a smaller peak with lower crystallization temperature. Because of the influence of the scanning rate, results shown by the DSC have to be carefully interpreted (Hartel, 2001).

2.3.1.5. Protein Analysis

In ice cream emulsions, proteins play an important role in stabilizing the emulsion. By aggregating at the oil droplet interface after homogenization, proteins decrease the interfacial tension of the fat droplets and form a viscoelastic and thick film at the interface that contributes to the stabilization of the fat droplets (Damodaran, 1997).

Emulsifiers have the ability to displace protein from the oil/water interface due to their lower surface tension at the membrane layer. Despite their higher affinity with the oil/water layer, emulsifiers form a much thinner layer that does not confer the same stability as a protein layer. The thin membrane around the fat droplets allows crystals to easily puncture the lamella and undergo partial coalescence (Damodaran, 1997; Goff, 1997a). Therefore, the study and quantification of proteins around the fat droplets is
important to achieve an appropriate level of partial coalescence and consequently desirable aeration in ice cream.

Hunt and Dalgleish (1994) have used a gel electrophoresis (SDS-PAGE) technique to determine the amount of protein adsorbed at the fat interface. By centrifugation, the fat phase was separated from the aqueous phase and analysed. Later on, Segall and Goff (1999) investigated the effect of temperature, centrifuge speed and centrifuging time, on the protein adsorbed at the interface. They suggested that crystallization of the fat phase would lead to destabilization of the fat during centrifugation. Therefore, better results were obtained when centrifugation was performed at temperatures in which the fat phase would be melted.

2.3.2. Microstructure Characterization

In order to achieve a quality ice cream product, an optimal formation of the fat structure as well as good aeration needs to be obtained. Optical and electron microscopy techniques have been applied to better understand the fat structure and the air cell distribution in the ice cream. Those techniques provide good resolution to enhance the visualization of ice crystals, air cells and fat droplets in conjunction with the possibility of studying their influence in the formation of ice cream structure. Cryo-electron microscopy goes even further since it allows the visualization of the ice cream structure in the frozen state.

Electron microscopy differs from light microscopy because electron microscopy uses electron beams instead of light for a greater resolving power due to its shorter
wavelength. A magnification of 30 to 1000 times higher than light microscopy resolution can be achieved (Aguilera and Stanley, 1999).

2.3.2.1. Cryo-Scanning Electron Microscopy

When an electron beam hits a thin section of a sample, electrons can be transmitted through the samples or they can be scattered by different factors. These electrons contain information of the sample that will be captured, and electron intensity will be displayed in the form of light intensity to obtain images. The two most common types of electron microscopy are scanning electron microscopy (SEM), in which the scattered electrons are collected to give the necessary information about the sample, and TEM in which the transmitted electrons are of interest (Williams and Carter, 2009; Aguilera and Stanley, 1999).

In a SEM microscope, an electron beam is created when an electrical current is applied to the tungsten filament. An accelerating voltage between the cathode (tungsten filament) and the anode pulls electrons through an aperture to create the electron beam. Two magnetic lenses, condenser and objective lenses, are used to narrow and give the right shape to the beam. When the beam hits the sample, secondary electrons are ejected out of the sample. Those electrons provide information on the topography of the sample (Aguilera and Stanley, 1999).

In electron microscopy, electrons need a high vacuum to be able to travel at the necessary distance and velocity. Because of that, the reduction of vapour pressure is critical and can be achieved by drying or freezing the samples. Kalab (1985) reviewed different techniques for fat-based dairy products including ice cream. He suggested the
use of cryo-SEM and TEM as the techniques best suited to the study of microstructure. As described by Kalab, conventional SEM is not applicable for most of the fat-based dairy products since fixation and dehydration of the liquid phase are necessary. That requires the removal of fat, water and other volatile components that can lead to loss of structure and changes to dimension. Furthermore, in the case of frozen ice cream samples, the increase in temperatures has a destructive effect on the sample (Caldwell et al., 1992a).

The use of cryo-SEM makes possible the study of the general microstructure of the frozen ice cream. The samples are immersed in liquid nitrogen, which immobilizes the sample and provides an analysis of the complete and undamaged structure. Therefore, a reduction in the temperature makes the vapour pressure of the water and other liquids a minor problem. The use of low temperature can also prevent the collapse of the structure by melting and prevent the creation of chemical artefacts by fixation (Aguilera and Stanley, 1999; Caldwell et al., 1992a; Caldwell et al., 1992b).

In addition to analysing fat structure, the cryo-SEM technique has been used in ice cream to evaluate the stability of air cells during storage conditions. Similar results have been achieved between cryo-SEM and optical microscopy techniques. However, cryo-SEM images can provide more details in terms of distortions and channelling caused in long-term storage ice cream (Chang and Hartel, 2001a).

2.3.2.2. Transmission Electron Microscopy

Concisely, a TEM microscope has a similar structure as a SEM microscope because it uses an electron beam in a vacuum system. A high voltage is applied to a
tungsten filament or lanthanum hexaboride (LaB$_6$) crystals. A narrow electron beam that travels at high velocity is created. Magnetic lenses and magnetic fields are used to focus the electron beam. The electron signal is captured and converted to an image to be visualized (Williams and Carter, 2009; Aguilera and Stanley, 1999). TEM technique has been successfully used to evaluate fat droplets and its crystal morphology in dairy emulsions (Goff et al., 1987; Allan-Wojtas & Kalab, 1984). Sample preparation of ice cream emulsions is very challenging and involves different steps, including encapsulation of the emulsion and fixation of the proteins and the fat droplets. The samples also have to be embedded in resin, for further sectioning. The process is done at temperatures close to ambient temperature, which impedes the application of this technique for frozen ice cream (Goff et al., 1987).

Another approach is the use of freeze substitution and low-temperature embedding for frozen materials. This technique has been applied in ice cream to study fat and air structures, but the study of crystal morphology in fat droplets in frozen ice cream or ice cream mix, using this technique has not been reported in the literature yet (Goff et al., 1999; Smith et al., 2004).

2.3.2.3. Image Analysis

Image analysis can be plainly defined as the use of software to analyse and obtain information from an image. The use of a computerized system instead of a human visual system avoids biased results or errors due to fatigue. It also accelerates the analysis and is more accurate in terms of measurement of quantitative results. However, the ability of the
scientist to correctly interpret the picture is the first and crucial step for good results in image analysis (Glasbey and Horgan, 1995; Aguilera and Stanley, 1999).

The use of image analysis for counting air cells has been reported in several studies (Chang and Hartel, 2002a,b; Caillet et al., 2003). Image analysis can be summarized by the following steps:

- Acquisition of the images, in which digital images are collected from the microscope.

- Image processing: in this step, coloured and monochrome images are converted to gray scale images. This conversion is extremely important as the basic principle of image analysis is to convert gray pixels into numerical values. The application of filters can improve the quality and remove noise of images. Binarization and segmentation can also be applied.

- Measurement analysis: numerical values will be obtained from the images. In the end, the quantitative data is subjected to statistical analysis for more successful and accurate interpretation of the data (Aguilera and Stanley, 1999).
3. MATERIALS AND METHODS

3.1. Organogel

3.1.1. Wax Organogel Preparation

Rice bran wax (RBW) organogel was obtained by combining 10% (wt) of RBW (Koster Keunen LLC, Watertown, CT, USA) and 90% (wt) of high oleic sunflower oil (HOSO) (Trisun 80, Nealanders International Inc., Mississauga, ON, Canada). The temperature of the blend was increased up to 80ºC to melt the wax. The hot blend was vigorously stirred to guarantee complete and homogeneous dissemination of the wax in the oil. Samples were then ready to be analyzed or incorporated in the ice cream mix. Candelilla wax (CDW) and carnauba wax (CBW) organogels were prepared as described for the RBW organogel.

3.1.2. Polarized Microscopy of Wax Organogels

The crystallization of RBW, CDW and CBW (Koster Keunen LLC, Watertown, CT, USA) was observed using polarized optical microscopy. Organogels of the different waxes were prepared as described in Section 3.1.1. The blends were cooled to room temperature until sample hardened or the wax crystallized. A small amount of sample, about 3 to 5 mm³, was placed between two glass slides on a temperature controlled microscope stage (CSS450 Linkam Optical Shearing System, Linkam Scientific Instruments Ltd, Surrey, UK). The temperature of the sample to be observed was increased to 95ºC to assure complete melting of wax crystals. The sample was then
crystallized by cooling the gel from 95°C to 30°C using two different cooling rates: 1°C, and 30°C/min. Samples were observed by polarized optical microscopy using a Leica DM RXA2/CTR MIC optical microscope (Leica Microsystems, Richmond Hill, ON, Canada). A 20x magnification objective lens with a 0.4 numerical aperture was used (Leica NPLAN L 20x/0.40, Leica Microsystems Inc., IL, USA). Images were acquired using a CCD camera (Q-imaging Retiga, Burnaby, BC, Canada).

3.1.3. Separation of Organogel Phase from Emulsions

In this process, 10% (wt) RBW organogel (prepared as described in Section 3.1.1.) was combined with 87% (wt) water and 3% (wt) of polysorbate 80 (Germantown™ Emulsifier 76, Danisco Inc., ON, Canada). The temperature of the mix was increased to 80°C for complete melting of the RBW crystals. The mix was homogenized at 20.4/6.9 MPa using a two-stage homogenizer (31MR Laboratory Homogenizer, APV Gaulin Inc., MA, USA). After emulsion formation, the fat particle size distribution was determined using light scattering (Section 3.4.2.). Emulsions made using HOSO or butter as the fat source were also prepared following the same formulation and process described above. The emulsions were stored at 5°C overnight.

Sodium Chloride (Aurora Fine Sea Salt, Aurora Importing & Distributing LTD, ON, Canada) was added to the emulsion until saturation. The emulsion was poured into centrifugation tubes and placed in a water bath at 40°C until destabilization of the emulsion. Centrifugation was applied using a low-speed clinical centrifuge (IEC Model CL – Clinical Centrifuge, International Equipment Company, MA, USA) at about 3000
rpm to completely separate the fat and water phase. Pictures of the separated fat phase were taken using a Sony 10-Megapixel Camera (Sony of Canada Ltd., ON, Canada).

3.2. Ice Cream

3.2.1. Formulation and Ingredients

Ice cream was prepared following the formulation described in Table 3.1. Types and concentrations of fat and emulsifier were varied according to the characteristic to be studied and will be separately indicated for each test performed.

Table 3.1. Description of the ingredients and their concentrations used in the formulation of all ice cream mixes

<table>
<thead>
<tr>
<th>Mix Component</th>
<th>Content by weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10 or 15</td>
</tr>
<tr>
<td>Milk solid non-fat</td>
<td>10</td>
</tr>
<tr>
<td>Sugar</td>
<td>12</td>
</tr>
<tr>
<td>Corn Syrup</td>
<td>4</td>
</tr>
<tr>
<td>Emulsifier/stabilizer</td>
<td>0.1% guar gum</td>
</tr>
<tr>
<td></td>
<td>0.015% carrageenan</td>
</tr>
<tr>
<td></td>
<td>From 0 to 0.2% polmo or GMO (glycerol monooleate)</td>
</tr>
<tr>
<td>Water</td>
<td>≈ 63.6</td>
</tr>
</tbody>
</table>
Butter fat (Unsalted Butter, Gay Lea Foods Co-operative Ltd, Mississauga, ON, Canada), palm kernel oil (PKO) (Fractionated Palm Kernel Oil SP-91, ACH Food Companies Inc., Memphis, TN, USA), HOSO (Trisun 80, Nealanders International Inc., Mississauga, ON, Canada) and organogels made with RBW, CDW and CBW (Koster Keunen LLC, Watertown, CT, USA) were the fats used in the formulation of the ice cream mixes. Skim milk powder (SMP) (Instant Skim Milk Powder Fortified, Parmalat Canada Inc., Toronto, ON, Canada), sucrose (Fine White Sugar, Lantic Inc., Montreal QC, Canada), corn syrup solids (42DE, Casco Inc., Etobicoke, ON, Canada), guar gum (Grindsted® Guar 80, Danisco Inc., ON, Canada) and carrageenan (Grindsted® Carrageenan CL 900, Danisco Inc., ON, Canada) were the sweeteners and stabilizers incorporated into the mixes. Two types of emulsifiers were used in the preparation of the mixes. The first was a commercial blend, polmo (Germantown™Polmo, Danisco Inc., ON, Canada), which contain 80% mono- and diglycerides and 20% polysorbate 80. The second emulsifier was Glycerol monooleate (DIMODAN® SO/D K-A, Danisco Inc., ON, Canada), an unsaturated monoglyceride extracted from soybean oil.

3.2.2. Ice Cream Preparation

Ice cream was made by two different processes: batch and continuous. In the batch process, formulations were performed in triplicate while in the continuous process formulations were carried out only once. All ice creams were prepared in the pilot plant of Guelph Food Technology Centre, located at the University of Guelph, ON, Canada.
3.2.2.1. Ice Cream Mix Preparation

3.2.2.1.1. Batch Process

Each batch was formulated to produce 3 Kg of ice cream mix. In a stainless steel double boiler pot, SMP was premixed with cool water. The mix was heated. Sugar, carrageenan and guar gum that had been previously mixed together were slowly incorporated into the mix at 35ºC, followed by the addition of corn syrup solids. Emulsifier and fat source were incorporated at higher temperatures (60ºC and 70ºC respectively) and allowed to melt completely before pasteurization. The mix was constantly heated and stirred until pasteurization. Due to the high melting point of RBW organogels, pasteurization was performed at 85ºC for 30 s. After pasteurization, mixes were pre-homogenized using a high speed mixer (Silverson L4RT, Silverson Machines, Inc., Chesham, Bucks, UK) at 7000 rpm for 2-3 min to ensure complete dispersion of the ingredients and to facilitate homogenization. Mixes were homogenized using a two-stage homogenizer (31MR Laboratory Homogenizer, APV Gaulin Inc., Wilmington, MA, USA) at 20.4/6.9 MPa. After homogenization, the batches were cooled by immersing the container with the mix in ice water. Ice cream mixes were aged overnight at 5ºC. All the formulations studied had been prepared using batch pasteurization and batch freezing (Section 3.2.2.2.1.).

3.2.2.1.2. Continuous Process

Due to the high melting point of wax organogels, the continuous process had to be adapted. In this process, 30 kg of mix was prepared and batch pasteurized at 80ºC for 30 s in a 67 L tank attached to a mixer (Lightnin mixer model ND-1, Lightnin, Rochester, NY,
USA). The mix was subsequently homogenized (GEA homogenizer, Niro Inc., Hudson, WI, USA) in 2 stages (20.4 MPa – first stage and 6.9 MPa – second stage) and cooled to 4°C using a tubular heat exchange (Microthermics Model 25, Microthermics, Inc., Wellington, Raleigh, NC) with a 4 L/min flow rate. The mix was aged at 5°C for 24 h.

Six different formulations were prepared following the described process. Two 10% fat ice cream mixes were made with RBW organogel but differing in the type of emulsifier used (polmo or GMO). Two other RBW organogel samples were made with 15% fat and with 0.2% GMO as the emulsifier. In this case the concentration of wax in the organogels was different, being 10% for one sample and 6.67% for the other. Oil and butter ice cream mixes containing 10% fat and 0.2% GMO were also evaluated. Table 3.2 describes the compositions of the six different ice cream mixes.

Table 3.2. Composition of ice creams prepared using a continuous process.

<table>
<thead>
<tr>
<th>Mix Component</th>
<th>Mix 1 (wt.%</th>
<th>Mix 2 (wt.%</th>
<th>Mix 3 (wt.%</th>
<th>Mix 4 (wt.%</th>
<th>Mix 5 (wt.%</th>
<th>Mix 6 (wt.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Milk solids non-fat</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sugar</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Corn Syrup</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Emulsifier/stabilizer</td>
<td>0.1% guar gum</td>
<td>0.1% guar gum</td>
<td>0.1% guar gum</td>
<td>0.1% guar gum</td>
<td>0.1% guar gum</td>
<td>0.1% guar gum</td>
</tr>
<tr>
<td></td>
<td>0.015% carrageenan</td>
<td>0.015% carrageenan</td>
<td>0.015% carrageenan</td>
<td>0.015% carrageenan</td>
<td>0.015% carrageenan</td>
<td>0.015% carrageenan</td>
</tr>
<tr>
<td></td>
<td>0.2% polmo</td>
<td>0.2% GMO</td>
<td>0.2% GMO</td>
<td>0.2% GMO</td>
<td>0.2% GMO</td>
<td>0.2% GMO</td>
</tr>
<tr>
<td>Water</td>
<td>63.7</td>
<td>63.7</td>
<td>58.7</td>
<td>58.7</td>
<td>63.7</td>
<td>63.7</td>
</tr>
</tbody>
</table>
3.2.2.2. Freezing and Hardening of Ice Cream

3.2.2.2.1. Batch Freezing

After aging, 1.5 L of mix was poured into a 3 L scraped surface freezer to be frozen (Model 104-27 Batch Ice Cream Freezer, Taylor Company, Rockton, IL, USA). The mix was frozen, while being constantly whipped, for 5.5 min or until the temperature of the mix reached -5ºC. At this temperature, further whipping was applied, resulting in a total process time of 10.5 min. The first portion of drawn ice cream was collected in a fixed volume container for the overrun measurement. Ice cream was continuously collected in 150 and 250 mL containers and hardened in a blast freezer at -30ºC where they were kept for at least 24 h. Samples were transferred from -30ºC to a freezer at -20ºC 24 h before further analysis.

3.2.2.2.2. Continuous Freezing

After ageing, 30 kg of ice cream mix were frozen using a Vogt Freezer (Model VA-80, Cherry-Burrell Corporation, Chicago, IL, USA) to -5ºC. A working back pressure of 20 psi was used. Ice cream was collected in 150 mL containers for meltdown measurements and 2 L containers for other analyses. Samples were immediately placed in a hardening room at -30ºC. Ice creams were transferred to a freezer at -20ºC, 24 h before analysis. Only the six samples described in Table 4.3 were frozen by this process.
3.2.3. *Measuring Ice Cream Mix Properties*

3.2.3.1. Fat Droplet Size Distribution

The fat droplet size distribution was determined using light scattering. A Mastersizer 2000 (Malvern Instruments, Malvern, Worcs, UK) was used to calculate the size of fat droplets by correlating size with the light scattered by the particles. Soon after the homogenization, ice cream mixes were analysed by diluting, usually a single drop of mix, in the sample chamber of the Mastersizer with Mili-Q water in the proportion of 1:1000 (mix/water). A different SOP (standard operating procedure) was created for each fat source used. The refractive index of RBW organogel was determined using a refractometer (J357 Automatic Refractometer, Rudolph Research Analytical, Hackettstown, USA) to be 1.474.

3.2.3.2. Protein Adsorption Analysis

Ice cream mixes were prepared following the formulation presented in Table 3.1. Emulsions prepared by batch and continuous process were analyzed.

Ice cream mixes prepared in a batch process had the concentration of polmo emulsifier varied from 0 to 0.2% (0, 0.05, 0.10, 0.15 and 0.20%). RBW organogel (10% RBW/90% HOSO) was used as the fat source; 10% fat ice creams were prepared. A sample containing 0.2% GMO as the emulsifier was also investigated. A butter sample with 0.2% emulsifier was analyzed as a control.

Six different samples, prepared by a continuous process, were analysed (Section 3.2.2.1.2.). Table 3.3 describes the samples analyzed.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Process</th>
<th>Type of fat</th>
<th>Fat concentration (%)</th>
<th>Type of emulsifier</th>
<th>Emulsifier concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.05</td>
</tr>
<tr>
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<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>batch</td>
<td>Butter</td>
<td>10</td>
<td>polmo</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>batch</td>
<td>RBW Org.</td>
<td>10</td>
<td>GMO</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>continuous</td>
<td>RBW Org.</td>
<td>10</td>
<td>polmo</td>
<td>0.2</td>
</tr>
<tr>
<td>9</td>
<td>continuous</td>
<td>RBW Org.</td>
<td>10</td>
<td>GMO</td>
<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>continuous</td>
<td>RBW Org.</td>
<td>15</td>
<td>GMO</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
<td>continuous</td>
<td>RBW Org.</td>
<td>15</td>
<td>GMO</td>
<td>0.2</td>
</tr>
<tr>
<td>12</td>
<td>continuous</td>
<td>Oil</td>
<td>10</td>
<td>GMO</td>
<td>0.2</td>
</tr>
<tr>
<td>13</td>
<td>continuous</td>
<td>Butter</td>
<td>10</td>
<td>GMO</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The fat phase was separated from the ice cream emulsions by centrifugation (Optima LE-80K Ultracentrifuge, Beckman Coulter, Inc., USA) at 20°C, 12000 rpm for 40 min. The supernatant layer was removed with a spatula and deposited on a filter paper (Qualitative P5, Fisher Scientific Company, Ottawa, ON, Canada) to allow the adsorption of serum phase residue. The filter paper was kept overnight at 4°C to eliminate water from the fat layer. The samples were transferred to a closed container until further analysis. The protein concentration in the supernatant layer was determined using the Dumas method. A FP-528 Protein/Nitrogen Analyzer (LECO Corp., St. Joseph, MI,
USA) was used to determine the nitrogen content of the sample. The adsorbed protein was calculated by dividing the protein concentration of the supernatant layer (mg protein/g fat) by the specific surface area (m$^2$/g fat) determined by light scattering. In this experiment four replications were performed for each formulation.

3.2.3.3. Differential Scanning Calorimetry Analysis

Tests were done to evaluate the crystallization and melting events in the RBW organogel in ice cream. Bulk RBW, HOSO, RBW organogel (10%) and polmo emulsifier were analysed, as well as ten emulsions that differed in composition. The ten different emulsions included five ice cream mixes whose compositions were the same as described in Table 3.1 but differed in emulsifier content (0%, 0.05%, 0.10%, 0.15% and 0.20%); two wax organogel/water emulsions, one emulsified by SMP and the other emulsified by polmo (80% mono and di-glycerides and 20% polysorbate); and three ice cream mixes with 0% fat content whose composition consisted of 10% msnf, 12% sucrose, 4% corn syrup, and, when stabilized, 0.1% guar gum and 0.015% carrageenan, and, when emulsified, 0.20% polmo emulsifier. Table 3.4 describes the 14 different samples analysed by DSC.

Samples were weighed and sealed into aluminum pans and analysed using a differential scanning calorimeter (DSC Q1000, TA Instruments, USA). An empty hermetically sealed aluminum pan was used as a reference. Calibration was carried out using indium metal standard (m.p. 156.6°C, $\Delta H_f = 28.45$ J/g). The samples were heated to 100°C, equilibrated at that temperature for 5 min, cooled to -60°C at the rate of 5°C/min, equilibrated at -60°C, and heated to 100°C at a rate of 5°C/min.

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Table 3.4. Samples analyzed by differential scanning calorimetry.

<table>
<thead>
<tr>
<th>Number</th>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>high oleic sunflower oil</td>
<td>Bulk</td>
</tr>
<tr>
<td>2</td>
<td>RBW</td>
<td>Bulk</td>
</tr>
<tr>
<td>3</td>
<td>RBW organogel 10%</td>
<td>Bulk</td>
</tr>
<tr>
<td>4</td>
<td>Polmo emulsifier</td>
<td>Bulk</td>
</tr>
<tr>
<td>5</td>
<td>Ice cream mixes (formulation</td>
<td>0% polmo emulsifier</td>
</tr>
<tr>
<td></td>
<td>described in Table 3.1.)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.05% polmo emulsifier</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.1% polmo emulsifier</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.15% polmo emulsifier</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.2% polmo emulsifier</td>
</tr>
<tr>
<td>10</td>
<td>Non-fat emulsions</td>
<td>74% water + 10% msnf, 12% sucrose, 4% corn</td>
</tr>
<tr>
<td></td>
<td></td>
<td>syrup</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>73.9% water + 10% msnf, 12% sucrose, 4% corn</td>
</tr>
<tr>
<td></td>
<td></td>
<td>syrup + 0.1% guar gum, 0.015% carrageenan</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>73.7% water + 10% msnf, 12% sucrose, 4% corn</td>
</tr>
<tr>
<td></td>
<td></td>
<td>syrup + 0.1% guar gum, 0.015% carrageenan + 0.2% polmo emulsifier</td>
</tr>
<tr>
<td>13</td>
<td>RBW emulsion</td>
<td>80% water + 10% RBW + 10% SMP</td>
</tr>
<tr>
<td>14</td>
<td>RBW emulsion</td>
<td>85% water + 10% RBW + 5% polmo</td>
</tr>
</tbody>
</table>

The cooling rate (5°C/min) was chosen as the most appropriate to represent the cooling rate of ice cream mixes prepared in the pilot plant by a batch process. Samples with higher content of water (5-13) were heated to 80°C instead of 100°C to avoid complete evaporation of water. The thermograms were analysed with TA Universal
Analysis Software to obtain the crystallization and melting onset ($T_{on}$, °C), offset ($T_{of}$, °C) and peak ($T_m$, °C) temperatures and enthalpy ($\Delta H$, J/g). For the bulk systems (1-4), three replicates per sample were analysed. For each formulation of emulsions (5-14), two separate batches were prepared and three replicates per batch were analysed.

3.2.4. Characterization of Frozen Ice Cream

3.2.4.1. Overrun

The amount of air incorporated in the frozen ice cream samples was measured by filling a fixed volume container with frozen ice cream immediately after finishing the total freezing/whipping time of 10.5 min. Considering that the density ($d$) of ice cream mix is 1.1 kg/L and the container volume ($v$) and weight being 227 mL and 40.5 g respectively, the overrun percentage was determined by the following formula:

$$ov\% = \left(\frac{gIC_{mix} - gIC_{frozen}}{gIC_{frozen}}\right) \times 100$$

Where:

$gIC_{mix} = d \times v$. The weight of ice cream mix in the fixed volume container

$gIC_{frozen}$. The weight of frozen ice cream in the fixed volume container

3.2.4.2. Meltdown Stability

The stability of the ice cream against melt was analyzed for all formulations. In the ice cream preparation, samples were collected into a 150 mL container to facilitate the meltdown test. Prior to the test, the ice cream samples were kept at -20°C for at least 24 h. Samples were first weighed and then removed from the container by quickly
immersing it in warm water. The samples, in their original shape, were placed in a stainless steel mesh grid placed over a tripod stand at room temperature and allowed to melt. The melted serum that passed through the mesh was collected and weighed every 10 min for a total of 90 min. The meltdown rate was calculated from the percentage of ice cream melted per min. Each sample was measured in triplicate. Pictures of the melted samples were taken using a Sony Ciber-Shot 10-Megapixel Camera (Sony of Canada Ltd., ON, Canada) at different times.

3.2.4.3. Fat Particle Size Distribution

The fat particle size distribution was determined by integrated light scattering using a Mastersizer 2000. Frozen ice cream samples (~100-200 g) were allowed to melt at 5ºC for a period of 24 h. The melted ice cream was analysed at room temperature by diluting it in the sample chamber with Mili-Q water in the proportion of 1:1000 (mix/water). A different SOP was used for each fat source used. Parameters such as volume moment mean (d$_{4,3}$, μm), specific surface area (m$^2$.g/fat) and the surface area moment mean (d$_{3,2}$, μm) were recorded.

3.2.4.4. Differential Scanning Calorimetry Analysis

Differential scanning calorimetry was performed in frozen ice cream samples. Two different samples were evaluated. The first frozen ice cream sample was a 10% fat ice cream made with RBW organogel (10%) as the fat source. The second sample was also a 10% fat ice cream made with 10% lipid that was composed of 10% wax and 90% HOSO. However, in the second sample, wax and oil were added separately to the rest of the mix and not in the form of organogel. Therefore, during the mix preparation of the
second sample, two other samples were prepared (samples were named A and B). The sample A was an ice cream mix formulated with 100% RBW as the fat source. The sample B was an ice cream mix formulated with 100% HOSO as the fat source. The samples A and B were prepared separately. After reaching 85°C, the three mixes (first, A and B) were homogenized at 20.4/6.9 MPa using a two-stage homogenizer. After homogenization, the second sample was prepared blending 10% of the sample A with 90% of the sample B. The first and second samples were aged overnight at 5°C. The freezing process was performed as described in Section 3.3.3.1.

Calibration was carried out using an indium metal standard (m.p. 156.6°C, ΔHf = 28.45 J/g). An empty hermetically sealed aluminum pan was used as a reference. Samples were prepared in a walk-in freezer (-18°C). Frozen ice cream pieces of 2 to 3mm³ were placed and sealed into aluminum pans, whose weight was known, and rapidly transferred to a differential scanning calorimeter with the sample chamber previously cooled to -25°C. The samples were heated from -25°C to 80°C, equilibrated at that temperature for 5 min and cooled to -50°C at the rate of 5°C/min. After analysis, the pan containing the sample was removed from the equipment and weighed. The thermograms, which were normalized by sample weight, were analysed with TA Universal Analysis Software to obtain the crystallization and melting onset (Ton, °C), offset (Tof, °C) and peak (Tm, °C) temperatures and enthalpy (ΔH, J/g). For each formulation of emulsions, two separate batches were prepared and three replicates per batch were analysed.
3.2.5. *Microstructure Characterization*

3.2.5.1. Cryo-Scanning Electron Microscopy

The cryo-scanning electron microscopy (cryo-SEM) technique was used to evaluate the fat aggregation on the surface of the air cell, to characterize the shape of the air cells, and to study the overall structure developed in ice cream samples made with RBW organogels. Ice cream frozen in the batch freezer and continuous freezer were analysed. Butter and oil ice creams were used as control samples.

Frozen ice cream at -18°C was scooped from the center of the container and immediately placed into liquid nitrogen (-198°C) for freezing and immobilization of the microstructure of the sample. A small sample was placed into a copper double screw specimen holder (Caldwell *et al.*, 1992a) while still immersed in liquid nitrogen. The holder containing the two specimens was rapidly transferred into the cryogenic preparation unit (Emitech K1250X, Ashford, Kent, UK) under vacuum, where samples at -160°C were fractured with the aid of a blade located in the interior of the chamber. The fracture procedure was responsible for exposing the internal structure of ice cream by creating a fresh surface. After fracturing, when applied, sublimation was carried out at -80°C for 5 min., which facilitates the visualization of the internal structure.

The fractured samples were sputter-coated with a thin layer of gold (30nm) at -160°C and then transferred under vacuum into the cold stage (≤ -140°C) of the scanning electron microscope (Hitachi S-570, Tokyo, Japan). Samples were visualized at 10kV accelerating voltage and pictures were captured using Quartz PCI (Version 7.0, Quartz Imaging Corporation, Rexdale, ON, Canada) at different magnifications.
3.2.5.2. Air Cell Size Distribution and Shape

Air cell distribution was studied using the method described by Chang and Hartel (2002a). Samples were observed using a cryo-scanning electron microscope, at 300 x magnification, following the same technique described in the Section 3.2.4.5. For this test, sublimation was not performed since the presence of ice crystals facilitates the differentiation between ice and air cells. Pictures of batch and continuous process of ice creams were analysed using Adobe Photoshop image analysis software (Photoshop CS5 Extended, version 12.0, Adobe Systems Inc., Sao Jose, CA, USA). The measurements were performed by manually tracing the perimeter of each air cell. Perimeter, equivalent diameter and circularity of air cells were some of the parameters recorded.

3.2.5.3. Transmission Electron Microscopy

The ultrastructure of butter, HOSO, RBW, CDW and CBW organogel droplets in ice cream mixes were analysed using transmission electron microscopy (TEM). Analyses were performed according to the method described by Goff et al., (1987) with some modifications. In covered small containers, mixes at 5ºC were combined with a 2% Ultra-low Gelling Temperature Agarose (Type IX – A, Sigma-Aldrich Co., St Louis, MO, USA) in the proportion of 1:3 (v:v) respectively. The agarose gel was previously prepared and cooled to 25ºC before mixing. The mix was equilibrated at 4ºC overnight.

The hardened agarose gel containing the ice cream mix was cut into pieces of approximately 1mm³ and fixed with 4% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.0) for 24 h, at room temperature. The samples were rinsed at least 3 times with phosphate buffer with an interval of 15 min between each rinse. After rinsing, the samples were post-fixed in 1% osmium solution in 0.1 M phosphate / imidazole buffer.
(1:1 v/v; pH 7.0) for 48 h at room temperature. This solution was prepared by dissolving 0.25 g of crystalline OsO₄ in 12.5 mL of phosphate buffer (pH 7.0) and combining equal volume of this solution with a 0.2 M imidazole buffer solution adjusted to pH 7.0 with 1 N HCl. The samples were rinsed with phosphate/imidazole buffer and dehydrated by aqueous dilutions of ethanol up to 100% ethanol and finally embedded in Spurr’s resin. A typical schedule of dehydration and embedding was followed, as described in Table 3.5.

Samples with resin were inserted in molds and polymerized at 70°C overnight. The blocks were cut in thin sections (90 nm thickness) with an ultramicrotome (Reichert Ultracut S, Leica, Concord, ON, Canada), and immediately transferred to a grid (Formvar/Carbon Coated – Copper). The samples in the grid were double stained with uranyl acetate and lead citrate following the method described in Hayat (2000). Samples were viewed using a Philips EM300 transmission electron microscope (Eindhoven, The Netherlands) operating at 80 kV.

Table 3.5. Dehydration and embedding schedule used in TEM.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>Ethanol (50%)</td>
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</tr>
<tr>
<td>Ethanol (70%)</td>
<td>15min</td>
</tr>
<tr>
<td>Ethanol (80%)</td>
<td>15min</td>
</tr>
<tr>
<td>Ethanol (90%)</td>
<td>15min</td>
</tr>
<tr>
<td>Ethanol (100%)</td>
<td>15min</td>
</tr>
<tr>
<td>Spurr’s (25%) + Ethanol (75%)</td>
<td>30 min</td>
</tr>
<tr>
<td>Spurr’s (50%) + Ethanol (50%)</td>
<td>30 min</td>
</tr>
<tr>
<td>Spurr’s (75%) + Ethanol (25%)</td>
<td>30 min</td>
</tr>
<tr>
<td>Spurr’s (100%)</td>
<td>At least 1h</td>
</tr>
<tr>
<td>Spurr’s (100%)</td>
<td>Overnight</td>
</tr>
<tr>
<td>Change into Spurr’s (100%)</td>
<td>Embed</td>
</tr>
</tbody>
</table>

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4. RESULTS AND DISCUSSION

4.1. Emulsion Formation

The possibility that rice bran wax (RBW) organogel formed droplets in an emulsion was first evaluated by separating the dispersed fat phase of emulsions. RBW organogel, pure high oleic sunflower oil (HOSO) or milk fat (melted) were dispersed in water and stabilized using Tween 80. The aim was to determine, by a simple test, if the dispersed phase of RBW organogel emulsions would be formed into droplets with the characteristics of a gel.

The fat separation was accomplished by three different methods that involved the addition of salt, the use of centrifugation and the use of a temperature gradient. With the use of these three steps, it was possible to avoid high speed centrifugation or high temperatures to achieve the desired separation of the dispersed phase. The use of a lower temperature was intended to avoid melting RBW in the organogel and the use of low speed centrifugation was intended to avoid possible separation of the RBW crystals from the liquid oil. The results suggest strongly that the dispersed phase is in the form of a gel rather than as liquid oil. Figure 4.1 A, D, E and F show the dispersed fat phase from the emulsion formed by the combination of RBW organogel (10% RBW), water and Tween 80. Figure 4.1 B and C are, respectively, the fat phase separated from an emulsion of milk fat and from HOSO in water. As seen in Figure 4.1 E, the consistency of the dispersed gelled phase from the RBW organogel emulsion was firm enough to be easily removed from the centrifuge tube while keeping its original shape. The milk fat was removed from
the centrifuge tube at 40ºC and it could be observed after crystallization as in Figure 4.1B. HOSO sample is seen in its liquid form (Figure 4.1 C).

Figure 4.1. Fatty phase separated from an emulsion made with RBW organogel (A, D, E, F), milk fat (B) and HOSO (C). Samples were at room temperature (~ 25ºC).

The wax crystallization in the emulsion is of interest because the structure of RBW crystals within the liquid oil droplets affects oil structuring. A couple of factors that affect the crystallization of wax within the droplet can be described as follows: 1. crystallization of fat in an emulsion is known to be strongly dependent on the fat droplet size; 2. the formation of a large amount of small droplets makes supercooling necessary to initiate crystallization (Coupland, 2002). At the same time, cooling rates seem to affect significantly the crystallization of RBW (discussed in more details in
Section 4.4.1). Because a large number of small droplets form, RBW may not be disseminated within all the droplets. In other words, some droplets could end up being free of wax and consequently the structuring of the liquid oil won’t occur. Large fat droplets are less likely to be lacking in wax crystals and will require a lower degree of supercooling, which facilitates the oil structuring. However, larger droplets are associated with emulsion instability and are not appropriate for the development of ice cream structure. Based on that fact, the quantification of fat droplet size is important to evaluate the development of organogel in emulsions. The fat droplet size distribution of the RBW organogel emulsion was evaluated to ensure that small particles would have the ability to become organogel droplets when the degree of supercooling is reached. Figure 4.2 shows the fat globule size distribution in the RBW organogel/water emulsion.

![Fat globule size distribution in the RBW organogel/water emulsion.](image)

**Figure 4.2.** The distribution of fat globule sizes of wax organogel/water emulsion using Tween 80 as an emulsifier. The distribution has a $d_{4,3}$ of 0.394.

The distribution of fat globule size was obtained using light scattering. The normal distribution of small particle size with most of the droplets having diameters...
smaller than 2 μm suggests the formation of a stable emulsion. The characteristics described above for the fat globule size distribution of wax organogel/water emulsion are also parameters desired for a well homogenized and stable ice cream emulsion that will lead to satisfactory destabilization during freezing process (Marshall et al., 2003). Believing that droplets dispersed in an ice cream mix can be in the form of organogel, studies were continued with ice cream mixes and the structuring of frozen ice cream.

4.2. Rice Bran Wax Organogel Ice Cream

The properties of ice cream mix and frozen ice cream containing RBW organogel (10% RBW) were evaluated. In this study, polmo, a commercial blend of mono- and diglycerides (80%) and polysorbate 80 (20%), was used as the emulsifier. The ice creams tested at this part of the study contained 10% fat in the formulation.

4.2.1. Ice Cream Mix Characterization

4.2.1.1. Mix Fat Droplet Size Distribution

Light scattering was carried out to evaluate fat globule size distribution of ice cream mixes formulated with RBW organogel. The distribution was compared to three controls: a typical milk fat ice cream mix, a non-dairy fat ice cream mix formulated with palm kernel oil (PKO) (50% wt) and HOSO (50% wt), and an unsaturated oil (HOSO) ice cream mix as shown in Figure 4.3.

Analysis revealed a bimodal distribution for the sample formulated with RBW organogel with particle size lower than 10 μm, compared to monomodal normal
distributions presented by the controls with particle size lower than 2 μm. The presence of a second peak could be related to slight instability of the organogel droplet or simply to limitations in processing.

The batch process described for the preparation of organogel ice cream mix had to be adapted to accommodate the melting profile of RBW organogels. High temperatures (>75ºC) were necessary to conduct the homogenization step. However, temperature of the mix was not maintained constant during the process. During the homogenization step, the temperature can drop significantly until the total volume of mix is homogenized, promoting the crystallization of the wax and hindering the breaking of large fat droplets into smaller ones. That could lead to the appearance of a second peak in the fat size distribution of RBW organogel ice cream mixes.

![Figure 4.3](image-url)

Figure 4.3. The fat globule size distribution of RBW organogel (10% RBW) (---) ice cream mix compared to milk fat (——), HOSO (-----) and PKO (50%) (-----) ice cream mixes. The distribution presented \( d_{4,3} \) of 0.569, 0.403, 0.422 and 0.503 respectively.
Rousseau (2000) discussed the interactions between protein or surfactants and fat crystals and the effect of these interactions on emulsion stability. The same review discussed the effect of fat crystal size and microstructure at the oil droplet interface on food emulsion stabilization. Considering that the amount of milk protein in an ice cream mix is enough to stabilize the oil droplets after homogenization (Goff, 1997b), instability could be caused by lower adsorption of protein at the oil droplet interface due to the nature of RBW organogel (intraglobular crystalline microstructure). Figure 4.4 presents the fat globule size distribution of ice cream mixes formulated with concentrations of emulsifier (polmo) varying from 0 to 0.2%.

![Fat globule size distribution](image)

**Figure 4.4.** The fat globule sizes distribution of RBW organogel (10% RBW) ice cream mix with 0% (—), 0.05% (—), 0.10% (……..), 0.15% (___) and 0.20% (____) polmo emulsifier. Each distribution has $d_{4,3}$ of 1.065, 0.627, 0.787, 0.765 and 0.569 respectively.

It can be observed that the second peak decreases when concentration of emulsifier increases, which emphasizes the possibility of poor protein adsorption at the interface because the addition of emulsifier contributes to emulsion stability.
4.2.1.2. Surface Protein Adsorption

The concentration of protein adsorbed at the organogel droplet interface, and the effect of emulsifier concentration on protein displacement at the organogel droplet interface was investigated. Six different samples were analysed. Five ice cream samples were formulated using RBW organogel as the fat source with different emulsifier concentrations that varied as follows: 0, 0.05, 0.10, 0.15 and 0.20% of polmo. Milk fat ice cream mix containing 0.2% polmo emulsifier was used as a control.

The results presented in Figure 4.5 revealed a common behaviour of protein adsorption at the fat droplet interface represented by the amount of protein adsorbed per surface area of fat. The sample with no emulsifier shows the higher concentration of protein (4.91 ± 0.41 mg/m²) at the stage when it is expected to be fully coated with protein. When emulsifier was added to the mix it displaced protein from the fat droplet interface, and protein concentration was decreased (3.01 ± 0.26 mg/m² at 0.2% polmo).

If the nature of organogel could result in a decrease of protein adsorption at the RBW organogel droplet interface, the emulsifier probably would be stabilizing the oil droplets by filling the interface areas lacking in protein instead of displacing it. Feijter et al. (1987) have stated that the degree of protein displacement is related to surfactant concentration and type. According to them, the type of fat does not have a large effect on protein displacement. However, Sung (2009) found a significant effect of composition of fat droplets on protein surface load.
Figure 4.5. Concentration of protein adsorbed at the oil droplet interface for ice cream mixes formulated with different concentrations of emulsifier: 0% ( ), 0.05% ( ), 0.1% ( ), 0.15% ( ) and 0.2% ( ). The results from milk fat ( ) ice cream mix is presented as control. Means followed by the same letter are not significantly different (p>0.05).

The mechanism of emulsifier action in ice cream formulated with milk fat occurs by displacing protein at the fat droplet interface because it has a preferable adsorption at the surface layer (Goff and Jordan, 1989). The emulsifier adsorption creates a thinner shear-sensitive membrane which increases the instability of the fat droplet. The mechanism leads to fat destabilization during ice cream processing (Bolliger et al., 2000b; Goff and Jordan, 1989; Lin and Leeder, 1974). The same trend is observed for RBW organogel samples. The increase in emulsifier concentration leads to a significant reduction in protein load at the oil droplet surface, which could increase the ability of fat droplets to undergo partial coalescence and develop ice cream structure. Also, protein adsorbed at the RBW organogel droplet interface was significantly (p<0.05) lower than
the milk fat sample, which could suggest more instability of RBW organogel droplets against partial coalescence. However, lower protein adsorption may not necessarily be correlated to a high fat destabilization during freezing according to Povey et al. (2006). Pelan et al. (1997) reported that milk fat ice cream samples containing 0.2% emulsifier had protein coverage at the oil surface around 13 mg/m² when a mix of saturated and unsaturated monoglyceride was used as emulsifier compared to 3.65 mg/m² in the present study. Such a difference could be due to the different emulsifiers used but also the difference in the methods used for measuring the surface protein load in both studies.

4.2.1.3. Differential Scanning Calorimetry of Fat Constituent

Differential scanning calorimetry (DSC) was used to observe crystalization and melting temperatures of RBW organogel. It has also been a useful technique to study the crystalization temperatures of droplets in emulsions as well as defining the crystal structure of the droplets. This information can be correlated to freezing temperatures and freezing point depression. Elwell et al. (2004) have reported the use of DSC to study changes in composition of lipid droplets in emulsion. In the study, crystallization temperatures of two mixed emulsions were observed during the time (0 to 104 h) through changes in crystallization peak temperatures.

The goal of this investigation was to determine the composition of the RBW organogel droplets in emulsion. This would be one more tool to evaluate and validate the formation of organogel droplets in an ice cream mix.

The study was conducted by analysing ice cream mixes formulated with RBW organogel and comparing them with pure materials (bulk RBW, HOSO and RBW
organogel) and some emulsions containing different compounds that are also contained in the ice cream mixes. Fourteen different materials and emulsions were analysed (Table 4.4) because of the complex composition of ice cream. With all the compounds separated, it was possible to assure that the peaks investigated were associated only with the crystallization of RBW and HOSO, the two constituents of RBW organogels. Figure 4.6 shows the melting thermograms of bulk RBW, emulsifier polmo, RBW organogel (10% RBW), and HOSO.

The RBW organogel melting curve revealed two peaks that correspond to the melting point of RBW (70.18°C) and HOSO (-7.28°C). The freezing point depression between RBW (70.18°C) in organogel and bulk RBW (81.30 ºC) is expected because of the dilution of the wax molecules caused by the addition of 90% (wt) of HOSO.

When RBW organogel was emulsified, the crystallization temperature of RBW in organogel droplets was considerably reduced due to difficulties in nucleation. As previously mentioned, crystallization of fat in oil/water emulsions occurs predominantly by homogeneous mechanism, which requires a supercooling condition to start (See Appendix A for cooling thermograms).
Figure 4.6. DSC melting curves of bulk RBW, RBW organogel (10% RBW), HOSO and polmo emulsifier. For transition temperatures see Appendix B. The scale for the RBW melting curve is shown on the right side of the figure.

No melting peak (~ 70°C) is identified in curves a, b and c (Figure 4.7 A) that corresponds to the ice cream emulsion formulated without fat, without fat and emulsifier and without fat, emulsifier and stabilizer respectively. The results suggest that the peak identified around 70°C is associated with the fat portion of the ice cream mix, more precisely, the melting peak of RBW. The crystallization peak of HOSO was identified around -40°C (Figure 4.7 B) in ice cream mixes. The crystallization peak of HOSO was presented instead of the melting peak since the former was masked by the melting peak of water and could not be identified in the melting thermogram of the ice cream emulsion.
Figure 4.7. Melting (A) and cooling (B) curves of ice cream mixes with 0 (g) and 0.2% (h) polmo emulsifier, no fat added (c), no fat and no emulsifier added (b), no fat or emulsifier or stabilizer added (a), 100% RBW (d) as the fat source, and O/W emulsions of RBW organogel stabilized by SMP (e) or polmo (f). For transition temperatures see Appendix A. The scale for h curve is shown on the right side of the figure.
The comparison between the peaks identified in the ice cream emulsion and in the bulk systems reveals similar temperatures (~ 70°C) of the melting peak of RBW in the bulk organogel and in the ice cream emulsion. The effect of freezing point depression was evident for both systems, which suggested that in both bulk and emulsion, RBW is surrounded by HOSO molecules. Evidence was also shown by the curve of the ice cream mix formulated with pure RBW instead of RBW organogel. Freezing point depression was not observed, and the melt of RBW occurred around 80°C, similar to the melting temperature of pure RBW. In summary, the results suggest that RBW remains dispersed in oil after homogenization and therefore organogel droplet formation is possible.

No difference in crystallization temperature was seen between ice cream mixes formulated with different concentrations of emulsifier. (More details are presented in Appendix A.) McClements et al. (1993) reported the influence of the type of emulsifier on the rate of fat droplet crystallization. According to them, induced crystallization can occur when the emulsifier lowers the interfacial tension of fat droplets allowing crystals to protrude through the interface. By collision of the fat droplets, fat crystals can penetrate other oil droplets inducing heterogeneous nucleation. The authors also state that emulsifiers with similar structure of oil can act as a nucleation starting point for crystallization altering the rate of crystallization. In the present study, the effect of emulsifier on crystallization was not observed and that could be related to the high melting point of RBW. By the time the emulsifier starts to crystallize RBW is already in the crystalline form.
4.2.2. Frozen Ice Cream Characterization

4.2.2.1. Overrun, Fat Destabilization and Meltdown Stability

Overrun increased when ice cream was formulated with organogel in comparison with HOSO samples (Figure 4.8). The larger incorporation of air in the RBW organogel samples created a lighter sample with better texture and good appearance. The overrun of RBW organogel ice cream was significantly different to standard samples such as milk fat ice cream and PKO frozen dessert (p<0.05). However it should be considered that the range of overrun between standard samples and organogel samples was narrow, varying from 50 to 70% only. The results suggest that the presence of organogel is, to some extent, improving the structure of ice cream by allowing more incorporation of air.

![Figure 4.8](image)

Figure 4.8. Overrun of ice cream made with HOSO ( ), RBW organogel (10%RBW) ( ), milk fat ( ) and PKO (50%) ( ). Means followed by the same letter are not significantly different from each other (p>0.05).

The increase in overrun, however, was not associated with an increase in fat destabilization. RBW organogel, milk fat and PKO (50%) possess a similar $d_{4,3}$, which is
not significantly different between them or when compared to HOSO ice cream (Figure 4.9).

Figure 4.9. Comparison of mean fat particle diameter ($d_{4,3}$) of ice cream prepared with RBW organogel (10% RBW) and 0% ( ), 0.05% ( ), 0.1% ( ), 0.15% ( ) and 0.2% ( ) polmo emulsifier. The results from milk fat ( ), HOSO ( ) and PKO (50%) ( ) ice creams using 0.2% polmo are presented as controls. Means followed by the same letter are not significantly different from each other ($p>0.05$).

A significant increase in the mean particle diameter is seen when concentration of polmo emulsifier is increased from 0 to 0.2%, which supports the results presented on surface protein displacement. It suggests that with an increase in emulsifier concentration, milk protein is displaced from the surface of the droplet thereby creating a much thinner membrane that increases the instability of the droplet and leads to larger fat destabilization after shear. The type of fat does not seem to influence the degree of
destabilization when polmo is used as an emulsifier since no difference in mean particle diameter was found between samples with different types of fat (p>0.05).

The degree of fat destabilization, however, is much lower than as described by Bolliger et al. (2000b). In their study, for an ice cream formulated with 0.15% mono- and diglycerides and 0.04% polysorbate 80, the mean fat particle diameter (d4,3) of the frozen ice cream was around 40 μm. The presence of polysorbate 80 as the emulsifier has been associated with greater fat destabilization during freezing (Goff et al., 1987; Goff and Jordan, 1989; Bolliger et al., 2000b). The same trend, however, was not observed in the present study.

The ability to resist meltdown is presented in Figure 4.10. No improvement was seen for samples formulated with RBW organogel (10% RBW). Samples seem to melt even faster than samples formulated with pure HOSO. After 1 h 30 min, about 80% wt of the RBW organogel sample was already melted. According to Hartel et al. (2003, cited by Muse and Hartel 2004), the amount of air incorporated can affect the meltdown rate of ice cream. They assert that a large amount of air affects the heat transfer during melting because the serum has a more tortuous path to follow, which slows down the melting. However, this effect was not noticed in this study. RBW organogel ice creams incorporated more air than HOSO ice creams, but the former presented higher rates of melting. This difference could be associated with the source of fat used. In Hartel et al.’s (2003) study, milk fat was used, and this fat probably behaves differently from organogel. Another reason could be that the study referred to much higher overrun than the ones obtained in the present study.
Figure 4.10. Meltdown curves of frozen ice creams formulated with RBW organogel with 0
( ◆ ), 0.05 ( ▲ ), 0.1 ( ★ ), 0.15 ( ▼ ) and 0.2% ( ● ) polmo emulsifier. HOSO ( ■ ), PKO (○ ) and milk fat ( ● ) controls were also presented.

The expected relationship between overrun, fat destabilization and meltdown stability presented in numerous studies and reviews (Muse and Hartel, 2004; Bolliger et al., 2000a, Barfod, 2001; Koxholt et al., 2001, etc.) was not observed in RBW organogel samples formulated with polmo emulsifier.

4.2.2.2. Differential Scanning Calorimetry of Frozen Ice Cream

Frozen ice cream samples were analysed using DSC. The experiment was conducted to evaluate any difference in crystallization or melting profile of the two samples. The study aimed to find evidence that could support the assumption that RBW remains in the dispersed phase (organogel) after the intense shear applied in the freezing process. The first sample was an RBW organogel (10% fat) ice cream formulated with 0.2% polmo emulsifier. The second sample was an ice cream mix that had RBW (10%)
and HOSO (90%) dispersed separately. (For more details of sample preparation, see Section 3.2.4.4.). The samples were first allowed to melt in the differential scanning calorimeter and then they were crystallized again.

The melting curve of the frozen RBW organogel ice cream reveals that the melting temperature (~ 70ºC) of the RBW in the frozen ice cream was similar to the melting temperature of the RBW in the ice cream mix of the same sample (Figure 4.11Ac and d). This temperature is similar to the melting temperature of the RBW in the bulk RBW organogel (70.18ºC) that is presented in 4.2.1.3 when freezing point depression occurred by the presence of HOSO.

The melting curves for the mix and frozen ice cream, prepared by dispersing RBW and HOSO separately, revealed melting temperatures (~ 80ºC) higher than the one from the previous sample (~ 70ºC). Since the melting temperature of the RBW in the organogel still exhibits freezing point depression, probably caused by the dilution of HOSO, the separation of RBW crystals from the RBW organogel does not seem to occur, at least not to a large extent that would be detected by the DSC. This is good evidence that RBW organogel remains in a gel state even after the freezing process.

The cooling curves for the frozen ice cream compared to their ice cream mix are presented in Figure 4.11B. The two frozen samples analysed were melted (Figure 4.11A) and recrystallized (Figure 4.11 B). The cooling curves of ice cream mix and frozen ice cream, formulated with RBW organogel, reveals a large difference in crystallization temperature. The cooling curve for the frozen sample formulated with RBW organogel revealed three main peaks with different crystallization temperatures that were not observed in the ice cream mix of the same sample. The intense shear applied by the
freezing process creates an agglomeration of droplets of different sizes. Larger agglomerates have the ability to easily crystallize, which results in higher temperatures of crystallization. The opposite is also true. Single droplets or small agglomerates will result in lower temperature of crystallization when a larger degree of supercooling is necessary for crystallization to occur. That is possibly the main cause for the features observed in the cooling profile of the frozen RBW organogel ice cream. The presence of different peaks agrees with the wide range of particles sizes in frozen RBW organogel ice cream revealed by light scattering.

On the other hand, the second sample that contained droplets of RBW and HOSO separately dispersed do not show changes in the crystallization temperature of wax before and after the freezing process; RBW droplets in the ice cream do not seem to agglomerate after shear was applied. According to Fredrick et al. (2010) fat particles with high concentration of solid fat do not aggregate or form clumps after collision because of the hardness of the droplets and the absence of oil. When oil is not present for wetting the crystals aggregation does not occur. That is the reason for the similarity in cooling profile between the ice cream mix and frozen samples. In conclusion, it is possible to say that droplets of RBW, in this case, are not participating in the fat destabilization; agglomeration is formed only by the coalescence of the oil droplets.

No major changes were noticed on the HOSO crystallization peak (results not presented).
Figure 4.11. Melting curves (A) and crystallization curve (B) of the ice cream mix (a) and the frozen ice cream (b) of the mix which had HOSO and RBW dispersed separately and a RBW organogel ice cream mix (c) and frozen RBW organogel ice cream (d). The scale for a and c (ice cream mixes) curves is shown on the right side of the figure.
4.2.3. Microstructure of Ice Cream

Until this point, the formulation of ice cream using RBW organogel has not shown a great ability to structure ice cream. This peculiar material has behaved in a distinct way raising many questions about its function in ice cream stability. The study of the microstructure of the ice creams aims to better understand ice cream structuring using RBW organogel.

4.2.3.1. Cryo-Scanning Electron Microscopy

The overall structure development of RBW organogel ice cream was investigated using cryo-scanning electron microscopy (cryo-SEM). The aggregation of fat at the air cell interface in RBW organogel ice cream at different concentrations of emulsifier is presented in Figure 4.12. The micrographs reveal an increase of fat aggregation when emulsifier concentration increases. Samples without emulsifier had almost no aggregation of fat at the air cell interface (Figure 4.12 A). The serum of the same sample, however, presented a large number of single spherical fat droplets. When emulsifier concentration was increased, more fat aggregation at the air interface was observed and the presence of single fat droplets at the serum phase was reduced.

The Cryo-SEM results, as well as light scattering and protein adsorption results, support many studies that described the mechanism of droplet destabilization and aggregation at the air cell surface. However, those results have not been associated with air cell stability for ice cream formulated with RBW organogel (10% RBW) using polmo as an emulsifier. Several reasons to explain the observed behaviour can be suggested. The first is that the internal structure of the organogel droplet may not be sufficient to avoid
oil spreading out of the droplet. The second is that the organogel structure may be broken during the concomitant freezing and whipping process; the third can be simply because partial coalescence is the most important mechanism of fat destabilization in ice cream to promote the formation of a network that will offer stability to air cells. 10% RBW in the composition of the organogel may not be enough to accomplish this mechanism. The mechanism of fat destabilization and microstructure formation in ice cream will be discussed in more details in Section 4.3.3.2.

Figure 4.12. Cryo-scanning electron micrographs of ice cream samples formulated with RBW organogel and different concentrations of emulsifier: 0 (A), 0.05 (B), 0.10 (C), 0.15 (D) and 0.2% (E) (wt). Scale bar = 3 μm.

The aggregation of fat at the air cell interface in RBW organogel ice cream is evident for different sources of fat (Figure 4.13). Milk fat ice cream (A) shows the
presence of a large number of spherical droplets that protrude out of the air bubble interface. Ice cream formulated with HOSO was also investigated as a control sample (Figure 4.13C). The smooth membrane visible around the droplet could be caused by oil spreading at the interface. A fat network is not formed in that case thereby compromising incorporation of air and stabilization of the air cell. RBW organogel seems to aggregate at the interface forming a rough membrane but with no definition of droplet shape. However, the roughness and large aggregation of fat at the air bubble surface does not seem to be enough to stabilize the air cell and protect ice cream against meltdown. RBW organogel sample also shows the presence of crystals that protrude from the fat membrane around the droplet (Figure 4.13B).

Figure 4.13. Cryo-scanning electron micrographs of ice cream formulated with different types of fat: milk fat (A), RBW organogel (10%RBW) (B) and HOSO (C). The micrographs depict the air cell interface covered by fat. Scale bar = 3 μm.

The presence of needle crystals in the RBW organogel samples has been associated with the crystallization of HOSO during the hardening process since similar crystals have been identified in HOSO ice cream samples. The needle crystals observed in RBW organogel and HOSO ice creams seem to occur in larger numbers at the
interface of air cells in RBW organogel samples. One explanation is that the presence of RBW in the crystalline form behaves as a nucleation starting point for heterogeneous crystallization of HOSO, which would facilitate the crystallization of the liquid oil. Another explanation would be that the needle crystals observed are a combination of HOSO and RBW crystals. In both scenarios, HOSO would be separated from fat droplets probably due to intense shearing during the freezing process. If dewetted from the organogel droplets, HOSO is free for formation of crystal and thus exposes the crystal network formed by RBW that could be observed by SEM.

At lower magnifications, the overall microstructure of ice creams was also investigated (Figure 4.14). The micrographs revealed two samples with good air cell distributions formed by a large population of air cells with small diameters, as well as ice crystals with uniform shape and size, a continuous serum phase and visible fat aggregation at the air interface.

Figure 4.14. Cryo-scanning electron micrographs of the overall structure of ice creams formulated with RBW organogel (10%RBW) (A) and milk fat (B). Scale bar = 120 µm. a = air cell, i = ice crystal.
4.2.3.2. Air Cell Distribution and Shape

Air cell distributions were similar for milk fat and RBW organogel ice cream formulated with 0.2% polmo emulsifier (Table 4.1). The small value for median and mean equivalent diameter describes distributions characterized by the presence of a large number of small air cells. Although the results for mean equivalent diameter are statistically different (p<0.05), the difference is minor if compared with HOSO ice cream samples. Air cell distribution of HOSO ice cream is not presented in this part of the study, but the results can be compared with data presented in Section 4.6.3.

### Table 4.1. Mean equivalent diameter of air cell distribution of ice cream formulated with milk fat and RBW organogel measured by cryo-scanning electron microscopy.

<table>
<thead>
<tr>
<th></th>
<th>Milk fat ice cream</th>
<th>RBW Org. ice cream</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>353</td>
<td>314</td>
</tr>
<tr>
<td>Median (μm)</td>
<td>4.68</td>
<td>9.04</td>
</tr>
<tr>
<td>Mean (μm)</td>
<td>8.95</td>
<td>10.61</td>
</tr>
<tr>
<td>Std. Dev (μm)</td>
<td>14.72</td>
<td>11.96</td>
</tr>
<tr>
<td>Min. (μm)</td>
<td>1.39</td>
<td>1.51</td>
</tr>
<tr>
<td>Max. (μm)</td>
<td>98.35</td>
<td>103.0</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.78</td>
<td>0.67</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>7.40</td>
<td>9.28</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>10.49</td>
<td>11.93</td>
</tr>
</tbody>
</table>

CI = confidence interval

Fat, from either source, plays a significant role in the air cell distribution. Eisner et al. (2005) and Bolliger et al. (2000b) stated that the formation of air cells with small diameters created a more stable foam with better resistance against melting, conferring
some attributes important in ice cream such as creaminess. However, the improvement in the distribution of air cells was not associated with stability against meltdown in RBW organogel ice cream. An improvement in creaminess was attributed to RBW organogel ice cream if compared to the oil ice cream sample. Sensory analyses, however, were not carried out.

One of the most visible differences between the milk fat and the RBW organogel ice cream structure presented in Figure 4.14 is the distorted shape of the air cells. The roughness of the interface and the more elongated shape of the air cells in RBW drew attention because of the divergent meltdown results. Because of those features, circularity of air cell in wax organogel ice cream and milk fat ice cream was quantified (Figure 4.15). The use of cryo-SEM enabled the study of distortion of air cells thanks to cryo-preservation of the internal structure, and consequently, the original shape of air cells was preserved.

![Histograms of circularity of air cells in RBW organogel and milk fat ice creams measured from cryo-scanning electron micrographs.](image)

Figure 4.15. Histograms of circularity of air cells in RBW organogel (■) and milk fat (□) ice creams measured from cryo-scanning electron micrographs.
The results of circularity measurements of air cells showed the difference between milk fat and RBW organogel ice cream. The circularity algorithm calculates how close the shape of an object is to a circle. For example, when air cells assume an elongated shape, circularity is reduced. A significant difference in mean circularity was observed between the two samples \((p<0.05)\). It is known that air cells tend to assume a spherical shape that results in the smallest surface area per volume. The surface area is directly related to surface tension, since a larger surface area increases the boundary energy that must be minimized to create stability. Laplace’s equation correlates the increase in pressure difference at the air interface with surface tension.

\[
\Delta p = \frac{2\gamma}{r}
\]

According to the equation, any increase in surface tension will intensify the pressure difference at the air interface. In other words, an enlargement in curvature will increase surface area, which increases the surface tension and intensifies the difference in pressure that consequently increases the instability of the air cells. If the aggregation of organogel droplets at the air cell interface is able to increase surface tension by changing the curvature of the air cell interface, RBW organogel droplets can be one of the causes of air instability in these ice cream samples. Also, if the increase in surface tension is great enough, the fat network formed may not be sufficient for air cell stabilization. As pointed out before, the deformation of air cells could be one of the reasons why RBW organogel ice creams have not achieved resistance against meltdown. Some reviews have associated the pressure difference at the air cell interface with some mechanisms of destabilization such as coalescence and Ostwald ripening (van Vliet, 1999; Sofjan and Hartel, 2004).
4.2.3.3. Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to investigate the ultrastructure of RBW organogel droplets in ice cream. As already mentioned, crystallization of fat droplets in emulsions was affected by numerous factors that can be reflected not only in the crystallization rate but also in the crystal structure (Coupland, 2002).

The TEM images illustrate the presence of wax crystals within the fat droplets (Figure 4.16). Casein micelles can also be clearly identified around the membrane of the fat droplet. The crystallization of RBW is represented by the lighter thread inside the droplet, since penetration of osmium is hindered by the solid state of the wax. The presence of the wax crystals is one more piece of evidence that oil structuring can occur when a mix of liquid oil and wax is emulsified. The existence of crystals in small droplets is a good indication that the RBW was efficiently disseminated.

Figure 4.16 A and B represent an RBW organogel ice cream mix formulated with 0% of emulsifier, and C and D were taken from samples formulated with 0.2% of emulsifier. Not much difference was observed in the surface layer of the fat droplets with and without emulsifier. The number of casein micelles does not seem to differ between the two samples suggesting lower protein adsorption at the fat droplet surface. Also, in the absence of emulsifier, fat droplets remain spherical while the presence of emulsifier seems to deform the droplets.

Polmo is a blend of 80% mono- and diglycerides and 20% polysorbate 80. Polysorbate 80 is a strong emulsifier, known for its ability to promote a large fat destabilization and increase the meltdown stability in milk fat ice cream (Goff et al.,
1987; Goff and Jordan, 1989; Bolliger et al., 2000b). However its effect in RBW organogel is not understood. The effect of different emulsifiers will be discussed in more details in Section 4.3.

The arrow in Figure 4.16C points to RBW crystals that appear to be cutting through the membrane, which could increase the instability of the droplet. Several studies have associated this phenomenon with droplet stability against partial coalescence (Coupland, 2002; Rousseau, 1999; Fredick et al., 2010). At the same time Figure D, from the same sample, shows the presence of crystals at the edge of the droplet and crystals within the droplets. Similar morphology was seen by van Boekel and Walstra (1981). They described different fat crystal structures in milk fat droplets. According to observations, the presence of L and M types of fat droplets was associated with instability and aggregation of fat droplets. L- and M-types were characterized by the presence of crystals tangentially oriented to the surface with M-type having also needle crystals within the droplet. Small crystals were not observed in the RBW organogel droplets; however that does not mean that they are not part of the structure. Overall, the presence of emulsifier seems to affect the ultrastructure of fat droplets, which influences their stability, aggregation at the air interface, and structure formation.
Figure 4.16. Transmission electron micrographs of RBW organogel ice cream mix formulated without emulsifier (A and B) and with polmo emulsifier (C and D) showing the internal structure of the organogel droplets with RBW crystals. Scale bar = 500 nm (A) and 1μm (B, C and D).
4.3. The Use of Different Emulsifiers in Rice Bran Wax Organogel Ice Cream

Ice creams were formulated with RBW organogel and a different type of emulsifier, glycerol monooleate (GMO), in an effort to improve the meltdown resistance. Some studies showed the ability of GMO to develop an ice cream with good structure, high meltdown stability and good shape retention (Zhang and Goff, 2005; Sung, 2009). The results that will be presented in this section are from ice creams formulated with 0.2% emulsifier (GMO or polmo) and 10% fat (RBW organogel, milk fat or HOSO).

4.3.1. Ice Cream Mix Characterization

GMO, as a polmo emulsifier replacement, did not have a considerable influence on the parameters of RBW organogel ice cream mix. The results for homogenization are shown in Figure 4.17. There was not a large difference in fat droplet size distribution between the ice cream mixes prepared using GMO or polmo. In both mixes, emulsions were formed with curves of similar shape. Both distributions were still characterized by the presence of a second hump, whose cause is not understood. According to the conclusion given by Bolliger et al. (2000a), a distribution characterized by a small d_{4,3} is a result of homogenization pressure more than type of surfactant since sufficient protein and emulsifier are available for emulsion stability.

Protein adsorption on the ice cream mix formulated with GMO was measured in order to better understand fat destabilization that takes place in the freezing process of ice cream. The protein displacement at the oil droplet interface caused by GMO was not significantly different from that caused by polmo in ice cream mix, although a slight decrease in protein surface load was observed in GMO samples (Figure 4.18).
Figure 4.17. The distribution of fat globule size in ice cream mixes prepared with RBW organogel (10% RBW) and polmo (—), and RBW organogel (10% RBW) and GMO (>). Each distribution has $d_{4,3}$ of 0.569 and 0.614 respectively.

Polmo is a blend of 80% mono- and diglycerides and 20% polysorbate 80. Bolliger et al. (2000a) reported a large increase in protein displacement when 12 to 30% (wt) of polysorbate 80 was added to mono- and diglycerides and used as the emulsifier. The presence of polysorbate 80 increased fat agglomeration and fat droplet aggregate size in ice cream emulsions. Goff and Jordan (1989) studied the destabilizing power of several emulsifiers, including GMO and polysorbate 80. They report a much higher fat destabilization when polysorbate 80 was used as an emulsifier. Polysorbate 80 was shown to be effective in displacing protein even when the emulsifier was added after homogenization.

The studies described above suggest a large effect in protein displacement when polysorbate 80 is present in the mix. The same effect was not seen in the present study (Figure 4.18). However, the cited studies did not investigate the effect on protein displacement when GMO or a blend of 80% mono- and diglycerides and 20%
polysorbate 80 were used as the emulsifier in ice cream mix under the same conditions. Zhang and Goff (2005) showed a greater ability of GMO to displace casein compared to glycerol monostearate. GMO led to a high degree of fat destabilization and partial coalescence. The same study also suggested that GMO strongly displaces protein at the air cell interface. Barfod et al. (1991) stated that the use of 0.2% GMO resulted in an unstable emulsion and that a large agglomeration of fat droplets was observed, even before ageing of mix.

![Figure 4.18](image-url)

**Figure 4.18.** Concentration of protein adsorbed at the oil droplet interface for ice cream mixes formulated with RBW organogel and polmo (■) or GMO (▲) as the source of the emulsifier. Means are not significantly different (p<0.05).

4.3.2. Frozen Ice Cream Characterization

4.3.2.1. Overrun, Fat Destabilization and Meltdown Stability

No significant improvement in air incorporation (overrun) was seen when the polmo emulsifier was replaced by GMO (Figure 4.19) (p>0.05). Although the difference was not
significant, samples formulated with GMO presented slightly lower overrun values when compared to ice creams formulated with polmo. According to Zhang and Goff (2005), the high displacement of protein from the fat droplet interface, caused by the presence of GMO, can lead to large agglomeration and spreading of fat at the air droplet interface that can cause instability and collapse of air cell.

![Overrun of RBW organogel ice creams made with GMO and polmo.](image-url)

Figure 4.19. Overrun of RBW organogel ice creams made with GMO (■) and polmo (□). Milk fat ice cream with polmo (●) and HOSO ice cream with polmo (□) were used as controls. Means followed by the same letter are not significantly different from each other (p>0.05).

Fat destabilization in milk fat ice cream emulsions is known to occur by partial coalescence of fat droplets when shear is applied. In RBW organogel ice creams the mechanism of aggregation is not understood, and further results cannot be directly associated with partial coalescence. However, as in milk fat emulsions, greater fat destabilization in RBW organogel ice cream seems to be correlated with an improvement in meltdown stability.
Figure 4.20 presents the mean fat particle diameter \( (d_{4,3}) \) of ice cream prepared with RBW organogel and GMO compared to previous results formulated using polmo. A significant increase in particle size was observed. Greater fat destabilization implies development of structure in ice cream. The results of fat agglomeration diameters, however, were unexpected due to the trend seen in protein adsorption.

According to the mechanism of action of emulsifier in fat destabilization, the emulsifier displaces protein, which is aggregated at the oil droplet interface after homogenization. The presence of a thinner layer at the droplet interface increases the instability of the droplet which undergoes destabilization. A small, but not significant \( (p>0.05) \), decrease in the concentration of protein at the droplet interface is observed in Figure 4.18. Perhaps the small difference is enough to lead to higher fat agglomeration in GMO samples \( (d_{4,3} \text{ of 23.02}) \). Interfacial tension could also be investigated as a predictor of fat destabilization. Goff and Jordan (1989) have reported higher interfacial tension for GMO in a solution of anhydrous milk fat and milk solids non-fat, when compared to polysorbate 80 in similar solution. As discussed before, polmo is a blend composed of only 20% polysorbate 80 and this could be the reason for the differences seen in the studies presented above.

Extremely low levels of protein displacement caused by using polmo and GMO emulsifiers could be one more reason for differences in interfacial tension. No significant improvement in protein displacement could be measured. Since that is the case, GMO must have had an extra effect on the droplets to cause greater aggregation. Davies et al. (2000) suggested that GMO was able to influence the growth of crystals, thereby affecting its morphology within the droplet. They also imply that GMO can encourage
the growth of crystals through the interface of the droplet, which would increase destabilization.

The composition of GMO is another factor that could account for the discrepancy in the results presented by the different authors. Although some studies have not reported the source of the monoglyceride, distilled monoglyceride can be animal or vegetable oil based. Distilled monoglycerides are concentrated monoglycerides obtained by molecular distillation. They are composed of high levels of monoglycerides (usually 95%) with the composition of fatty acids being similar to the fat source (Krog and Sparsø, 2004). In a previous study in our laboratory, Mendez-Velasco (2010) quantified the composition of the GMO used in the present study. The GMO (Dimodan® SO/D K-A) is made from soybean oil with a fatty acid composition of palmitic (C16:0) (11%), stearic (C18:0) (5%), oleic (C18:1) (25%), linoleic (C18:2) (50%) and linolenic (C18:3) (9%) fatty acids. Commercial blends of monoacylglycerols usually contain a minimum of 90% monoesters, but the fatty acid composition can vary considerably. Davies et al. (2001) who investigated the effect of GMO on emulsion stability reported the use of a commercial emulsifier, Dimodan, with a composition of 72% monoolein, 11% monopalmitin and 11% monostearin. The same authors investigated the effect of using GMO on the shear-stability of the emulsion. In that study, GMO was composed of 95-98% monoglycerides of which 92% were monoolein, with only 1% monostearin and 1% monopalmitin (Davies et al., 2000). Apparently the composition of the monoglyceride can affect the function of the emulsifier on the displacement of protein, as well as the crystallization of fat and the viscoelasticity of the droplet surface, which increases instability against shear (Krog and Sparsø, 2004).
Figure 4.20. Mean fat particle diameter ($d_{4,3}$) of ice cream prepared with RBW organogel (10% RBW) and polmo (■) or GMO (□). Milk fat ice cream (■) and HOSO ice cream (□) were used as controls. Means followed by the same letter are not significantly different from each other (p<0.05). Each distribution have mean $d_{4,3}$ of 11.283, 23.02, 11.854 and 7.097 respectively.

Meltdown stability of the RBW organogel ice cream formulated with GMO was also evaluated (Figure 4.21). This ice cream showed an improvement in meltdown resistance if compared with RBW organogel or HOSO ice cream formulated with polmo. Although the slope of the curve in Figure 4.21 (empty circle) does not seem to be much smaller than the above curves (filled square and star), a significant difference in meltdown rates between the curves was observed (p<0.05).

According to the results discussed above, the use of GMO seems to confer more instability to the fat droplets in ice cream by facilitating fat agglomeration. The increase in $d_{4,3}$ was also correlated with an increase in meltdown stability. The mechanism of fat
agglomeration and increase in foam stabilization, however, were not associated with protein displacement at the interface. In other words, the shear-sensitivity of the ice cream emulsions was found to be higher for GMO samples, but the increase in shear-sensitivity could not be correlated with higher protein displacement. A possible mechanism for the action of GMO through the increase in shear sensitivity of RBW organogel ice cream emulsion will be presented later.

Figure 4.21. Meltdown curves of frozen ice creams formulated with RBW organogel and polmo (→), RBW organogel and GMO (←), milk fat and polmo (▲) and HOSO and polmo (❖). Meltdown rates were 1.02 (± 0.04), 0.70 (± 0.05), 0.21 (± 0.01) and 0.97 %wt melt/min (± 0.05) respectively.
4.3.3. Microstructure of Ice Cream

4.3.3.1. Cryo-Scanning Electron Microscopy

Cryo-SEM was used to better investigate the formation of structure in ice creams formulated with RBW organogel and different emulsifiers. Differences in the interfaces of air cells in ice creams formulated with several types of fat are presented in Figure 4.22. Ice cream formulated with milk fat present a small air cell covered by spherical droplets and aggregation of fat droplets by partial coalescence is indicated by the arrow (Figure 4.22A). The liquid oil spread around the air cell of an ice cream formulated with pure HOSO is evident (Figure 4.22B). Oil seems to be spread also around the air cells of RBW organogel ice creams. Air bubbles in RBW organogel ice cream with GMO present a rough air interfaces covered by fat (Figure 4.22D). Aggregation, however, is not as smooth as in air cell covered by pure HOSO, neither are fat droplets spherical as in ice cream formulated with milk fat. In polmo samples, air cells seem to have a large number of needle crystal projections already identified (Figure 4.22C). The formation of crystals in a needle-like form was previously associated with the crystallization of HOSO that occurs during the hardening process. As observed before, the presence of needle-like crystals at the air cell interface is more prominent in polmo than GMO samples. That suggests that GMO can possibly change the ultrastructure of organogel droplets, creating droplets that reduce oil spreading because they are less shear-sensitive. It appears, in this case, that HOSO molecules are not as detached from the network as they are in polmo samples. HOSO spreading out from the wax structure facilitates it crystallization. That could be the reason why needle crystals were not so evident in the GMO samples.
It was also observed that spherical droplets were more evident in the serum phase of samples prepared with GMO (Figure 4.22C). This observation reveals not only the formation of spherical droplets but also a possible resistance of the droplets to spreading after shear applied by the freezing process. That, however, does not mean resistance against aggregation since GMO samples have shown larger aggregation diameters.

Figure 4.22. Cryo-scanning electron micrographs of ice cream samples formulated with milk fat and polmo (A), HOSO and polmo (B), RBW organogel and polmo (C) and RBW organogel and GMO (D). Arrow indicates partial coalescence of fat droplets. Scale bar = 6 µm (A) and 3 µm (B, C and D).
One of the most interesting characteristics revealed by cryo-SEM is the difference in the shape and distribution of air cells of GMO ice cream compared to polmo ice creams as shown in Figure 4.23. Despite the presence of small air cells in both samples (Section 4.2.2.4), samples formulated with GMO show the presence of much more uniform and spherical air cells, a characteristic associated with ice cream meltdown stability (Caldwell et al., 1992a). What previously had been attributed to a possible effect of the presence of organogel at the air cell interface seems now to also be related to the type of emulsifier. GMO seems to play a critical role in the stabilization of air incorporated into the ice cream mix during freezing.

Another important observation is the presence of large channels in the polmo sample. Smith et al. (1999) defined channels as black areas inside the air cells that can be a result of coalescence of other air cells. In their study, the structure of whipped cream was evaluated. They associated the appearance of channels with instability of the samples against gravity. Channels could be one more indication of instability of the polmo sample against meltdown.

According to Caldwell et al. (1992b), the formation of large ice crystals was associated with low structure stability. In the description by Muse and Hartel (2004), when ice cream melts, the liquid water has to diffuse through the unfrozen serum, passing through other structural elements in ice cream of which ice is one. If large ice crystals are present, when they melt, the large space left by the ice crystals creates a less tortuous pathway which facilitates diffusion of water and consequently the melting of ice cream. The presence of relatively larger ice crystals with irregular shapes is observed in polmo
samples (Figure 4.23B). This can be one more reason for the fast meltdown for samples formulated with polmo as the emulsifier.

Figure 4.23. Cryo-scanning electron micrographs showing the overall structure developed in ice creams formulated with RBW organogel and GMO (A) or polmo (B). a = air cell, i = ice crystal. Scale bar = 60μm.

4.3.3.2. Transmission Electron Microscopy

It is evident that the presence of GMO causes a different effect in the structure stability of RBW ice cream. Changing the composition of the interfacial layer of fat droplets or modifying the crystallization of the wax within the oil droplets could be a mechanism of action for this emulsifier. TEM was performed to investigate in more detail the influence of GMO in the RBW organogel droplets. Figure 4.24 (C and D) reveals that the addition of GMO to the mix creates RBW organogel droplets with a more uniform spherical shape with much less distortion if compared to polmo ice cream mix (Figure 4.24A and B). While crystallization of RBW in oil droplets of polmo samples has been
identified by the presence of a single crystal in the outer edge or middle of the droplets, GMO samples show crystals with more branching. That could help in oil structuring, creating a droplet with higher shear resistance.

Another important structural aspect of GMO samples is the presence of large crystals protruding out of the organogel droplet (Figure 4.25). When intense shear is applied and collision of droplets is inevitable, the crystal at one droplet interface can pierce the membrane of another droplet, enhancing fat agglomeration. This observation explains the larger particle size seen in GMO ice creams and revealed by light scattering. According to Fredrick et al.’s (2010) classifications of semi-crystalline oil droplets, the organogel droplets in GMO samples are similar to the K-type fat droplets. The authors explained that in K-type globules, crystals are too thick to follow the curvature of the droplet. Therefore, they grow tangentially to the outer edge of the fat droplet. This effect is intensified by the addition of water-soluble surfactants. It was suggested that partial coalescence is certain to occur in this case.

GMO showed the ability to encourage crystal formation outside of the fat droplet. Davies et al. (2000) suggested that the surfactant is capable of causing an alignment of the oil molecules with its hydrocarbon chain. This alignment is believed to cause the orientation of the crystals to the outer interface of the droplet. The author’s observations also described the effect of GMO on the crystallization of RBW fat in the present study.

Partial coalescence is believed to be the most prevalent form of fat aggregation in ice cream. It has been the main type of aggregation discussed until this point. However it is important to remember that most of the studies presented were conducted using milk fat or any other blend of saturated and unsaturated fat, while in this study RBW organogel has been analysed.
Figure 4.24. Transmission electron micrographs of RBW organogel ice cream mix formulated with polmo emulsifier (A and B) and GMO emulsifier (C and D) showing the internal ultrastructure of the RBW organogel droplets. Arrow = branching, circle = shape of droplets. Scale bar = 1µm.
Figure 4.25. Transmission electron micrographs of RBW organogel ice cream mix formulated with GMO. The RBW crystals can be seen projecting out of the fat droplet membrane (pointed by arrows). Scale bar = 500 nm (D), 1 µm (A and C) and 2 µm (B).
In milk fat (or butter), as well as any other blend of saturated and unsaturated triacylglycerides (TAGs), the saturated TAGs crystallize into small crystals that interact by non-covalent forces forming clusters. With a high ratio of solid/liquid fat, the three-dimensional network formed by the aggregation of clusters can structure liquid oil to have the physical properties of butter. On the other hand, RBW has the ability to form a continuous network using much lower ratios of wax/liquid oil. Higher interactions and the presence of microcrystals are factors that lead to the ability of wax to structure oil at low concentrations (Rogers, 2009). So, it is hard to believe that interactions and mechanisms of action would be similar between two very different forms of structured fat.

Coalescence and flocculation are some mechanisms of fat aggregation that may occur in ice cream. Flocculation is a type of aggregation where attractive forces bring droplets together to form a cluster. However, different from coalescence, during flocculation droplets keep their integrity, and fusion of droplets does not occur (Fredrick et al., 2010). Recently, fat flocculation was suggested by Goh et al. (2006) to be the main type of aggregation in milk fat ice cream. They studied ice cream with different ratios of milk fat and flaxseed oil. Samples were either treated or not with a solution of sodium dodecyl sulfate (SDS) and ethylene diamine tetraacetic acid (EDTA) to dissociate any fat flocculated. Milk fat ice cream not treated with SDS/EDTA showed large \( d_{4,3} \) that is drastically reduced when treated with SDS/EDTA. Flaxseed oil ice cream not treated with SDS/EDTA has shown similar \( d_{4,3} \) to the sample treated with SDS/EDTA. Goh et al. (2006) associated the form of structure stabilization with flocculation instead of partial coalescence.
The analyses that evaluate the behaviour of milk protein adsorption at RBW organogel interfaces have shown peculiar results. The divergence in the results presented by the present study (protein adsorption, TEM, different emulsifiers) seems to suggest a complex composition of the organogel droplet interface that could be affecting protein binding. The nature of organogel and specifically the composition of organogel droplet interface could interfere in the repulsive or attractive forces between two droplets and could increase instability of droplets against flocculation. How the composition of RBW may affect droplet interface composition and its possible effects in emulsion stability will be discussed in Section 4.4.3.1.

4.4. The Use of Different Wax Organogels in Ice Cream

4.4.1. Wax Crystallization

Dassanayake *et al.* (2009) have shown the ability of RBW to form long crystals in liquid oil, and this ability has been related to better gel formation. Considering the size of fat droplets in an emulsion, the ability of long crystals to form a network to structure oil within the droplets has been questioned. Because of that, the crystallization of other waxes was evaluated. The crystal morphology of RBW, CDW (Candelilla wax) and CBW (Carnauba wax) dispersed in HOSO is presented in Figure 4.26. CDW and CBW formed microcrystalline particles at high cooling rates (30°C/min) (Figure 4.26C and E). These crystals were much smaller than those formed during RBW crystallization in HOSO under the same conditions (Figure 4.27).
In ice cream, crystallization rate is affected by the formation of a large number of small droplets. Supercooling is necessary for crystallization to occur (Coupland, 2002). In the batch processing adapted for ice cream production in this study, the cooling rate of the emulsion was relatively low, which certainly affected the crystallization of the wax within the oil droplets. Because of that, the wax crystallization was evaluated after a lower cooling rate (1°C/min) that was more comparable to the cooling rate of ice cream mix.

The lower cooling rate seems to disturb the crystal formation of the RBW by creating agglomeration of crystals instead of a branched network, which certainly affects the structural characteristics of the organogel (Figure 4.27B). CBW crystallization is also largely affected by cooling rate when the formation of spherulitic agglomeration of crystal is seen (Figure 4.26F). As seen for RBW, the crystallization of the wax does not cover the whole volume of sample, therefore affecting the organogel formation.

An emulsion is formed by an enormous amount of droplets of very small volume. If spherulitic crystallization occurs inside each droplet and is able to cover its entire volume, a network could be established resulting in oil structuring. CDW is not greatly affected by cooling rate (in a micro scale) which also shows a great potential for the formation of organogel droplets in an emulsion.

Based on the results discussed above, tests were performed using CDW organogel (10% wax) and CBW organogels (10% wax) in ice cream. Because of the potential seen in the use of CDW, ice creams tested were formulated with CDW and GMO or polmo as an emulsifier. However, with CBW organogel, only GMO was added to the mix because better results were achieved with this emulsifier.
Figure 4.26. Polarized optical micrographs of different wax crystals in HOSO. The crystallization of RBW (A and B), CDW (C and D) and CBW (E and F) was observed when the wax organogel was cooled at two different cooling rates (30°C/min and 1°C/min). Scale bar = 100 μm.
4.4.2. *Ice Cream Mix Characterization*

The particle size distribution of CDW and CBW organogel ice creams using GMO as the emulsifier is displayed in Figure 4.27. Both samples showed a second peak less prominent and more diffused than the first large peak. The use of polmo in CDW organogel ice cream did not cause a reduction in the second hump. The presence of a second hump was evident in all the samples analysed. However, a possible effect from the adapted batch process has not been discarded.

![Graph showing particle size distribution of ice cream mixes](image)

Figure 4.27. The distribution of fat globules sizes of ice cream mix prepared with RBW Organogel (10% wax) with GMO ( -•-) or polmo ( -•-), CDW Organogel (10% wax) with GMO ( -•-) or polmo ( -•-) and CBW Organogel (10% wax) with GMO ( -•-). Each distribution have d4,3 of 0.732, 0.569, 0.814, 0.708 and 0.698 respectively.

4.4.3. *Frozen Ice Cream Parameters*

4.4.3.1. *Overrun, Particle Size and Meltdown Stability*

The incorporation of CDW and CBW organogels in ice cream did not result in any increase in overrun; the mixes were able to incorporate similar volume of air. Figure
4.28 displays the distribution of particle size diameter of the frozen ice cream formulated with CDW and CBW organogels. This figure also presents the results of RBW organogel ice creams for comparison. Interesting results were found for the use of these waxes.

Figure 4.28. The distribution of fat globules sizes of frozen ice cream prepared with RBW Organogel (10% wax) and GMO (———) or polmo (———), CDW Organogel (10% wax) and GMO (---) or polmo (-----) and with CBW Organogel (10% wax) and GMO (·-·-·). Each distribution have d4,3 of 23.02, 11.283, 7.746, 5.713 and 2.721 respectively.

The use of CDW and CBW organogel in ice cream surprisingly did not lead to large fat destabilization. The mean d4,3 was low: 2.721 for CBW organogel ice cream compared to a d4,3 of 23.02 for RBW organogel ice cream, both formulated with GMO.

A large decrease in meltdown stability was observed for these samples (Figure 4.29). Meltdown rates were even greater than for pure HOSO ice cream. Again, the higher overrun of the wax ice cream did not have any impact on meltdown stability. Possible reasons for the effects seen for these waxes will be discussed next.
Another important point that should be introduced is the composition of the waxes. Dassanayake et al. (2009) had investigated the thermal properties of RBW, CDW and CBW in the bulk state. They associated broader DSC peaks with the complex composition of the waxes. RBW showed a narrow single peak as a result of being composed mainly by wax esters. CDW and CBW showed the presence of two peaks within a much wider range of temperature, which indicates a more complex composition of these waxes. The different components in the waxes can be one of the main causes for the different crystal habit shown in Figure 4.26. The composition of waxes used in this study is summarized in Table 4.2.

![Figure 4.29. Meltdown curves of frozen ice creams formulated with RBW organogel and GMO ( ), CDW organogel and GMO ( ) or polmo ( ) and CBW organogel and GMO ( ) Meltdown rates were 0.70 (± 0.05), 1.021 (± 0.04), 1.33 (± 0.03), 1.29 (± 0.04) and 1.31% wt melt/min (± 0.042) respectively. Meltdown rate of RBW organogel and GMO is significantly different from RBW organogel and polmo that is significantly different from the other waxes (p<0.05).](image-url)
Table 4.2. Composition of RBW, CDW and CBW from the datasheet of Koster Keunen sample.

<table>
<thead>
<tr>
<th>Composition</th>
<th>RBW&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Candelilla&lt;sup&gt;d,e&lt;/sup&gt;</th>
<th>Carnauba&lt;sup&gt;d,e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ester Content (%)</td>
<td>92 – 97</td>
<td>27 – 35</td>
<td>84 – 85</td>
</tr>
<tr>
<td>Free Fatty Acid (%)</td>
<td>0 – 2</td>
<td>7 – 10</td>
<td>3 – 3.5</td>
</tr>
<tr>
<td>Free Fatty Alcohol (%)</td>
<td>-</td>
<td>10 – 15*</td>
<td>2 – 3</td>
</tr>
<tr>
<td>Hydrocarbons (%)</td>
<td>-</td>
<td>50 – 65</td>
<td>1.5 – 3</td>
</tr>
<tr>
<td>Resins (Others) (%)</td>
<td>3 – 8</td>
<td>-</td>
<td>6.5 – 10</td>
</tr>
</tbody>
</table>

*Includes percentage of sterols and resins

Free fatty acid and fatty alcohol behave as surface active impurities. When present in an emulsion, they will accumulate at the oil/water interface where they can decrease surface tension and affect the stability of the oil droplet (McClements, 2005; Lyklema, 2000). Fatty alcohols are well known antifoam agents. Antifoams are characterized by the ability of prevent foam formation and enhance air cell coalescence (Joshi et al., 2005). The presence of a high concentration of free fatty acid and free fatty alcohol in the composition of CDW and CBW can be one reason why meltdown rates were higher. It would also be expected that ice creams prepared with CDW and CBW would result in large destabilization because of these components. However, a very small amount of fat agglomeration was observed. TEM pictures revealed an important mechanism for stability through the CDW and CBW organogel droplets.

4.4.4. *Transmission Electron Microscopy*

The crystallization of CDW and CBW in the oil droplet was also investigated. The transmission electron micrographs are presented in Figure 4.30. The crystallization of CBW in oil droplets was characterized by the presence of a long thin crystal around the interface (Figure 4.30B arrow indicating the crystal). It could be also a thin layer of
submicrometer crystals as described by Rousseau (2002). The author investigated the stabilization of emulsions achieved when fat droplets are covered by fat crystals.

The crystal monolayer formed by CBW was strongly correlated with the stability of CBW organogel droplets against aggregation as is evident from the particle size measured in frozen ice cream. CBW organogel ice cream have shown the lowest mean particle diameter ($d_{4,3}$ of 2.721) of all the samples tested, which shows the ability of the CBW crystals in stabilize the emulsion against fat aggregation. The ability to protect the interface is evident by the droplet, indicated by the arrow, in Figure 4.30A. The penetration of the fixative was probably blocked by a crystal shell around the droplet interface.

CDW crystallization was observed to form a pronounced crystal in the middle of the droplet or at the surface (Figure 4.30C and D). According to the description of Rousseau (2002) and Fredrick et al. (2010), the crystallization of fat in oil droplets can be observed as submicrometer crystals concentrated at the interface and crystals dispersed within the droplet. So, as mentioned before, the observation of large crystals positioned at the droplet interface is not necessarily an indication that crystals are not present in the rest of the volume of the droplet. The method may not be enough to observe the formation of small crystals. The effect of polmo and GMO emulsifiers in the crystallization of CBW and CDW within the droplets was not investigated.
Figure 4.30. Transmission electron micrographs of CDW organogel ice cream mix (A and B) and CBW organogel ice cream mix (C and D) showing the internal structure of the droplets of wax organogels. Ice cream mixes were formulated without emulsifier. Scale bar = 500 nm (B, C and D) and 1 µm (A).
4.5. The Effect of Fat Concentration on Ice Cream

According to Marshall et al. (2003), the fat content of ice cream is directly related to quality and value of the product. Premium ice cream has a fat content varying from 14 – 18%; the increase is associated with an increase in creaminess and buttery flavour. However, our interest is on the efficiency of the fat in developing the structure of ice cream. An increase in fat content may lead to higher levels of fat destabilization that could promote better meltdown stability in RBW organogel ice creams.

In this part of the study, ice creams were formulated with 15% fat and were compared to previous results of RBW organogel, HOSO (15% fat) and milk fat (15% fat) ice creams. The effect of the total concentration of RBW in ice cream stability was also investigated. Since the addition of GMO has improved meltdown stability, this emulsifier was chosen to be used in the following study.

Overrun measurements showed similar results for all the samples formulated with 15% fat (10% RBW organogel). However, it was observed that in these ice creams the ability to incorporate air was limited. Also, no difference was seen in the fat globule size distribution of the ice cream mixes with 15% fat (results not shown). Figure 4.31 displays the mean fat particle diameter (d₄₃) of frozen ice creams.

The fat particle size in ice cream composed of 15% fat of RBW (10% wax) organogel and GMO emulsifier appeared to be larger than any other sample tested. As discussed in Section 4.3.3.2., the formation of RBW crystals at the outer edge of the fat droplet, caused by the addition of GMO, is believed to be the cause of greater destabilization of this sample.
Figure 4.31. Mean fat particle diameter ($d_{4,3}$) of 15% fat ice creams prepared with GMO and RBW organogel (10% RBW) ( ), RBW organogel (7% RBW) ( ), or RBW organogel (5% RBW) ( ). Butter ( ) and HOSO ( ) 15% fat ice creams prepared with GMO and RBW organogel (10% RBW) ( ) 15% fat ice cream prepared with polmo were used as controls. Previous results from 10% fat ice creams formulated with RBW organogel (10% RBW) and polmo ( ) or GMO ( ) were presented for comparison. Means followed by the same letter are not significantly different from each other ($p > 0.05$).

The formation of larger particles indicates higher levels of fat aggregation and fat network formation. GMO seems to be causing greater destabilization of fat and the concentration of fat also seems to be important for the formation of large fat agglomerates. The level of fat destabilization also increased when RBW concentration in the organogel phase was increased. This suggests that the increase in wax concentration promotes more resistance against coalescence. Ice cream formulated with 10% RBW
(10% wax) organogel and polmo emulsifier had the lowest level of destabilized fat, which has also been correlated with the highest meltdown rate.

The presence of large fat droplet aggregates in RBW organogel ice cream was also associated with an increase in melting resistance (Figure 4.32). According to Muse and Hartel (2004), destabilized fat is one of the most critical element that affect meltdown resistance. Ice cream formulated with 15% fat (10% RBW organogel) as the fat phase and GMO as the emulsifier have shown a significant (p<0.05) reduction in melt rate when compared to an ice cream with 15% fat (10% RBW organogel + polmo) or 15% HOSO. Although the meltdown rate achieved by the samples was relatively low, it was significantly (p<0.05) different from the meltdown rate for milk fat ice creams.

The concentration of RBW did not show a significant (p>0.05) effect on the meltdown stability of RBW organogel samples. Similar meltdown curves for samples formulated with 15% fat (10% RBW)/ (7%RBW)/ (5%RBW) organogels are displayed in Figure 4.32 (dashed lines).

As described by Bolliger et al. (2000), the melting rate describes the physical change in the state of the ice, but it does not indicate relevant properties such as shape retention or collapse of structure during melting. Shape retention is also an indicator of fat destabilization and network formation. During ice cream melting, pictures were taken every 10 minutes for a period of 1 h and 30 min and final shapes are presented in Figure 4.33.
Figure 4.32. Meltdown curves of 15% fat ice creams formulated with GMO and RBW organogel (10% RBW) ( - ■ - ) (0.22 ± 0.02), RBW organogel (7% RBW) ( - - - - ) (0.24 ± 0.02), or RBW organogel (5% RBW) ( - ◊ - ) (0.29 ± 0.05). Butter ( - ▼ - ) (0.08 ± 0.01) and HOSO ( - ● - ) (1.07 ± 0.03) 15% fat ice creams prepared with GMO were used as controls. RBW organogel (10% RBW) 15% fat ice cream with polmo ( - ◊ - ) (1.19 ± 0.04) and previous results for 10% ice creams formulated with RBW organogel (10% RBW) and polmo ( - ▼ - ) (1.02 ± 0.05) or GMO ( - ▽ - ) (0.71 ± 0.05) were presented for comparison. Meltdown rates are indicated in parentheses.

Samples formulated with 15% fat, RBW organogel and GMO showed better shape retention in comparison to samples made with 10% fat, RBW organogel and GMO (Figure 4.33A and F). In the latter, nearly the entire mass of ice cream dripped through the mesh, while in the former, the network formed by the highly destabilized fat helped sustain the foam on top of the mesh. The dripped fluid from samples with 10% fat was white, with the presence of air bubbles which indicated that the foam was passing the mesh and the fat network was not able to hold the structure. The dripped fluid from
samples with 15% fat was watery, transparent and did not contain air bubbles. For comparison, results for milk fat and HOSO 15% fat ice cream formulated with GMO are also presented (Figure 4.33). HOSO did not show any meltdown stability while milk fat samples had the best shape retention of all samples.

Shape retention of samples formulated with RBW organogel containing different concentrations of wax can also be seen in Figure 4.33B and C. Ice cream formulated with firmer gels (RBW (10% RBW) organogel) showed a slightly lower meltdown rate and somewhat better shape retention. Higher levels of RBW increased oil structuring, forming harder droplets. Conversely, lower concentration of wax in the organogel can give a greater ability for the oil to spread at the interface. The spreading can result in the disruption of the foam, reducing the stability of the sample against meltdown.

The use of 15% fat ice cream formulated with RBW organogel and polmo did not show any improvement in meltdown rate or shape retention when compared to 10% fat ice creams. This lack of improvement could be related to the effect of polmo emulsifier on the crystallization of the wax within the droplet. The crystals concentrated in the edge of the droplets could have caused droplet stability. This idea is supported by the light scattering results that have shown smaller particle diameters for ice creams formulated with polmo.

Overall, RBW organogel ice creams with larger fat destabilization showed lower meltdown rates and better shape retention. The use of high concentration of fat (15%) and using GMO as the emulsifier seems to be necessary to achieve better ice cream structure when RBW organogel is used as the fat source. The melting resistance of these ice
creams was also correlated with an optimal development of microstructure as will be presented next.

Figure 4.33. Shape retention of ice creams at 90 min of meltdown test for ice creams formulated with 15% fat RBW organogel (10% RBW) and GMO (A), 15% fat RBW organogel (7% RBW) and GMO (B), 15% fat RBW organogel (5% RBW) and GMO (C), 15% fat milk fat and GMO (D), 15% fat HOSO and GMO (E) and 15% fat RBW organogel (10% RBW) and polmo. Previous results for 10% fat ice creams formulated with RBW organogel (10% RBW) and polmo (F) are presented for comparison.

4.5.1. Microstructure Analyses

4.5.1.1. Cryo-Scanning Electron Microscopy

The improvement in the meltdown stability in 15% fat RBW organogel ice cream using GMO, was investigated further by evaluating the microstructure using cryo-SEM (Figure 4.34).
Ice cream formulated with 15% MF and GMO (Figure 4.34A) was used as the standard for comparison with other formulations. Ice cream formulated with 15% RBW (10% RBW) organogel and GMO (Figure 4.34C) presented spherical air cells containing a large number of smaller air cells and small, well defined, ice crystals. This formulation was also correlated with high meltdown stability. The microstructure of RBW organogel ice cream was similar to milk fat ice cream (Figure 4.34A and C). The microstructure of 15% HOSO ice cream (Figure 4.34B) was characterized by larger ice crystals and air cells of irregular shape. A 15% RBW (10% RBW) organogel ice cream formulated with polmo (Figure 4.34D) had large ice crystals of less defined shape and smaller air cells of irregular shape. Both samples were correlated with poor meltdown stability. In summary, the microstructure of ice creams based on the different formulations was correlated with the results for meltdown stability.

It was possible to observe, at higher magnification images, the fat aggregation at the air cell interface. However, no differences were seen in the characteristics of fat aggregation between 15% and 10% fat ice creams.

The melting behaviour of the ice cream formulated with RBW organogel seems to be explained by fat aggregation and fat network formation. The shape and size of air cells also proved to have an influence in meltdown resistance. Because of these influences, air cell distribution in the 15% fat RBW (10%RBW) organogel ice creams formulated with GMO was also evaluated and compared with milk fat ice creams of similar composition.
4.5.1.2. Air Cell Distribution and Shape

The results of air cell distribution confirmed the visual analyses of cryo-SEM micrographs of the samples. The air cell distribution of ice creams formulated with 15% fat and GMO showed the presence of a large number of small cells with mean values of 11.49 μm for milk fat ice cream and 10.49 μm for RBW (10% RBW) organogel. There was no significant difference in mean air cell diameters between the milk fat and the RBW organogel samples (Table 4.3).

As presented in Section 4.2.3.2, ice creams formulated with 10% fat, RBW organogel and polmo emulsifier showed air cells with distorted shapes. It was thought that the shape of the air cells could have an effect in structure stability. When the concentration of fat was increased (15% fat) and the emulsifier was replaced by GMO, air cells assumed a more spherical and uniform shape (Table 4.3). Unlike the results presented for polmo ice creams, there was no significant difference in the circularity of...
the above samples. In other words, the improvement in circularity of air cells was significantly correlated with better meltdown resistance.

According to Chang and Hartel (2002b), distortion of air cells can also be associated with some destabilizing mechanisms of foams, such as disproportionation, coalescence and channelling. Because of the connection between distortion and destabilization, an improvement in circularity of air cells can also be associated with a lower predisposition of cells to initiate these mechanisms of destabilization.

Table 4.3. Mean equivalent diameter and circularity of air cell distribution of ice cream formulated with 15% milk fat or RBW (10% RBW) organogel measured by cryo-scanning electron microscopy. GMO was the emulsifier used.

<table>
<thead>
<tr>
<th></th>
<th>15% Butter Ice Cream + GMO</th>
<th>15% RBW(10%Wax) Organogel Ice Cream + GMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>264</td>
<td>253</td>
</tr>
<tr>
<td>Median (μm)</td>
<td>7.46</td>
<td>6.34</td>
</tr>
<tr>
<td>Mean (μm)</td>
<td>11.49</td>
<td>10.49</td>
</tr>
<tr>
<td>Std. Dev (μm)</td>
<td>12.23</td>
<td>11.19</td>
</tr>
<tr>
<td>Min. (μm)</td>
<td>1.76</td>
<td>1.18</td>
</tr>
<tr>
<td>Max. (μm)</td>
<td>99.58</td>
<td>71.0</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.75</td>
<td>0.70</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>10.0</td>
<td>9.11</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>12.97</td>
<td>11.88</td>
</tr>
</tbody>
</table>

CI = confidence interval
4.6. Replication in a Continuous Freezer

In order to validate the results on an industrial level, the development of structure in ice cream was investigated using a continuous freezer. The freezing technique used is known to produce different effects on the formation of air cells and the fat aggregation of fat droplets (Goff et al., 1999).

Six ice cream formulations were chosen for the study (Table 4.3) to evaluate each criteria (fat concentration, type of emulsifier, fat source) separately. Although polmo did not show any ability to promote fat destabilization or increase meltdown resistance, the samples formulated with 10% fat and polmo (mix 1) showed the highest levels of air incorporation (overrun), and therefore were tested. GMO was used as the emulsifier in most of the samples; the 15% fat ice creams were also evaluated. RBW was the only wax organogel tested.

Table 4.4. Composition of mixes prepared using continuous process.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat concentration</th>
<th>Emulsifier</th>
<th>Fat source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix 1</td>
<td>10%</td>
<td>Polmo</td>
<td>RBW (10% RBW)</td>
</tr>
<tr>
<td>Mix 2</td>
<td>10%</td>
<td>GMO</td>
<td>RBW (10% RBW)</td>
</tr>
<tr>
<td>Mix 3</td>
<td>15%</td>
<td>GMO</td>
<td>RBW (6.7% RBW)</td>
</tr>
<tr>
<td>Mix 4</td>
<td>15%</td>
<td>GMO</td>
<td>RBW (10% RBW)</td>
</tr>
<tr>
<td>Mix 5</td>
<td>10%</td>
<td>GMO</td>
<td>Milk fat</td>
</tr>
<tr>
<td>Mix 6</td>
<td>10%</td>
<td>GMO</td>
<td>HOSO</td>
</tr>
</tbody>
</table>

Continuous freezing of the mixes was performed only once. Since not enough replications were performed, statistical inference cannot be done for these samples.
4.6.1. Ice Cream Mix Characterization

Figure 4.35 presents the fat globule size distribution of the mixes prepared for continuous freezing. Stable emulsions were formed. They were characterized by monomodal distributions with a high percentage of the distribution lower than 2μm. One unexpected characteristic was the absence of the second peak which had been observed in samples prepared for batch freezing (Section 4.2.1.1). The possibility that temperature control during the homogenization process (temperature control versus no control of temperature) influenced the fat droplet distribution is now confirmed.

After homogenization, milk protein immediately adheres to the fat droplet interface. With ageing time and temperature, the emulsifier displaces protein at the interface creating a thinner membrane on the droplet. In this study, the ice cream mix formulated with polmo was the only sample that had to be homogenized twice, but after that still presented a mean particle size (1.871μm) larger than GMO ice cream mixes. The fact that emulsifier type affects homogenization may suggest poor protein adsorption or possibly the dispersion of the emulsifier in the organogel phase. GMO could be reducing the melting temperature of the organogel phase thus facilitating the homogenization process.

Protein adsorption at the fat droplet interface was evaluated (Figure 4.36). As expected, the protein adsorption is slightly higher for samples with higher concentration of fat. With the same concentration of emulsifier but an increase in oil droplet surface area, the amount of emulsifier able to displace protein by surface area is reduced, which results in higher protein adsorption at the oil droplet interface (Figure 4.36 mix 3 and 4).
Figure 4.35. The distribution of fat globule sizes of ice cream mix 1 (--), mix 2 (---), mix 3 (----), mix 4 (-----), mix 5 (-----) and mix 6 (-----) prepared for continuous freezing. Each distribution has d_{4,3} of 1.871, 0.686, 0.994, 1.033, 0.797 and 0.757 respectively.

Figure 4.36. Concentration of protein adsorbed at the oil droplet interface for ice cream mix 1 ( ), mix 2 ( ), mix 3 ( ), mix 4 ( ), mix 5 ( ) and mix 6 ( ). Means followed by the same letter are not significantly different (p>0.05).
4.6.2. *Frozen Ice Cream Characterization*

The mean equivalent diameter of the destabilized fat was taken as a measure of the extent of fat destabilization caused during the freezing process (Figure 4.37). An increase in fat destabilization was observed when polmo was replaced by GMO. This same trend was seen in samples prepared by the continuous freezing process. Large fat aggregations were observed for samples with higher concentration of fat with GMO as the emulsifier. Mix 1, when polmo was used as the emulsifier, gave unexpected results. This ice cream (mix 1) did not show much destabilization of fat.

Mix 1 and 2 presented higher levels of fat destabilization when batch freezing was performed. On the other hand, larger fat agglomeration was observed in mix 3, 4, 5 and 6 when continuous freezing process was used. Goff et al. (1999) found that batch freezing produced greater fat destabilization if compared to continuous freezing when mono and diglycerides and polysorbate 80 are used as the emulsifier. On the contrary, Marshall *et al.* (2003) stated that continuous freezer imposes greater shearing than batch freezer and therefore causes greater fat destabilization.

The larger fat destabilization observed in mix 3, 4 and 5 (RBW (6.7% RBW), RBW (10% RBW) and milk fat respectively) was associated with an enhancement of meltdown resistance. After hardening, ice creams were allowed to melt and images were taken of the shape of the ice creams after 1 h and 30 min melting (Figure 4.38). A significant improvement in shape retention and meltdown rate is seen for samples with higher amount of fat (mix 3 and 4), which are comparable with ice cream formulated with milk fat. Shape retention is an indication of the development of fat structure.
Figure 4.37. Malvern Matersizer 2000 data showing the distinction between mean fat particle diameter ($d_{4,3}$) of ice cream prepared by a continuous freezer (CF) and a batch freezer (BF). Frozen ice cream prepared from mix 1 ( ), mix 2 ( ), mix 3 ( ), mix 4 ( ), mix 5 ( ) and mix 6 ( ) were analysed.

Samples formulated with lower concentrations of wax (mix 3) showed similar meltdown resistance to samples formulated with 10% wax organogel (mix 4). In other words, the increase in wax concentration from 0.7 to 1% (wt) wax in the total ice cream was not as critical as the presence of greater concentration of organogel. The development of physical structure of ice cream using RBW organogel turned out to be feasible.
Figure 4.38. Picture of the shape retention of ice creams at 90 min of meltdown test for ice creams prepared from mix 1 (A), mix 2 (B), mix 3 (C), mix 4 (D), mix 5 (E) and mix 6 (F).

Figure 4.39 displays the meltdown rate of ice creams prepared in a continuous freezer and ice creams of similar formulation prepared in a batch freezer. For mix 3 and 4, both formulated with RBW organogel, meltdown rate dropped considerably from 0.36 to 0.21 % melt/min and 0.28 to 0.19 %melted/min (Figure 4.39). The increase in meltdown stability of these samples must be related to larger fat particle sizes in the frozen ice cream. That reinforces the ability of the continuous freezer to apply intense shear during the freezing process. Meltdown rates were reduced for most samples when the continuous freezing process was used instead of the batch freezing process.
Figure 4.39. Comparison of melt rate for ice creams processed by a continuous freezer (CF) and a batch freezer (BF). Frozen ice cream prepared from mix 1 ( ), mix 2 ( ), mix 3 ( ), mix 4 ( ), mix 5 ( ) and mix 6 ( ) were analysed.

4.6.3. Ice Cream Microstructure

The aggregation of fat at the air interface for the different ice creams can be seen in Figure 4.40. There appears to be a large difference in the form of fat aggregation for the 6 samples. As expected, milk fat destabilization occurs by partial coalescence forming agglomeration of well defined spherical droplets (Figure 4.40F mix 5). Mix 1, formulated with RBW organogel and polmo emulsifier, did not show large aggregates as was expected by the results revealed for the fat particle size distribution in the frozen ice cream. Only a few single droplets are seen at the interface. Not much explanation can be given for the atypical behaviour of mix 1. It has to be considered that only one test was performed using the continuous freezing process. Therefore, mistakes in process or formulations cannot be dismissed. In mix 2 and 6 few aggregates were observed. Crystallization of the oil, represented by the presence of needle crystals, was observed in
both samples. However, when concentration of fat was increased (mix 3 and 4) the aggregation at the air cell interface also increased as represented by a rough surface with a greater number of needle long crystals. A much larger number of crystals was seen in mix 3, which was formulated with a smaller concentration of wax (6.7%). A softer gel could allow the oil to easily spread out of the structured network being feasible for crystallization.

![Image](image.png)

**Figure 4.40.** Cryo-scanning electron micrographs of ice cream prepared from mix 1 (A), mix 2 (B), mix 3 (C), mix 4 (D), mix 5 (E) and mix 6 (F). The air cell interface, covered by fat, can be observed. Scale bar = 30 µm (A, C, D and E), 15 µm (B) and 10 µm (E).

Unique fat aggregation was observed in air cells from mix 4 (Figure 4.41) where two completely different forms of aggregation were observed for two air cells that were only 10 µm apart. Wax concentration and wax composition could be some of the causes.
for that observation. However, no further investigation was done to evaluate the form of aggregation of fat occurring in these samples.

![Image](A.png)

Figure 4.41. Two different forms of fat aggregation at the interface of two different air cells in ice cream (from mix 4) revealed by cryo-SEM. Scale bar = 10μm.

The results of shape retention and melting rate properties in the extruded samples were also validated by cryo-SEM images. Air cell size distribution of the different mixes can be observed in Figure 4.42. The difference in the size of the air cells in each ice cream formulation, and their shape is evident. Samples prepared with RBW and GMO (mix 2, 3 and 4) showed more improvement towards spherically shaped air cells. If compared to a HOSO ice cream (mix 6), the application of RBW led to a large decrease in air cell sizes. Mix 1 showed the presence of large agglomeration of air, but with no consistent shape.

Deformed ice crystals shape can be observed particularly for mix 3 and 5 (Figure 4.42C and E). Five min sublimation of the ice was performed before sample coating because it facilitates differentiation of ice crystals from the other structural components.
But sublimation has a great influence on the overall appearance of the ice cream microstructure (Caldwell et al., 1992a). The surface of ice crystals appear to have been deformed, as if detached from the side, adopting an aspect of folded sheet what could be a result of intense sublimation.

![Image](image1.png)

Figure 4.42. Cryo-scanning electron micrographs of the overall structure of ice cream prepared from mix 1 (A), mix 2 (B), mix 3 (C), mix 4 (D), mix 5 (E) and mix 6 (F). The different sizes of air cells are highlighted by the dashed lines. Scale bar = 100μm.

To confirm the discrepancy in air cell distribution between the six ice cream formulations frozen in a continuous freezer, air cell size distribution was measured using cryo-SEM images (Table 4.5). It appears that in mix 1 the incorporation of air was observed in the form of holes instead of defined air cell. Because of that the air cell size distribution for mix 1 was not evaluated.
Larger air cells are usually observed in ice creams frozen in continuous freezer (Marshall et al., 2003). However, that trend was not observed in these ice cream samples. Mix 5, formulated with butter, had similar air cell size distribution from samples prepared in a batch freezer (Table 4.1). Also presented is the lower mean equivalent diameter of all samples. Mix 2 and 4 had a remarkable increase in mean equivalent diameter in comparison to results from the batch freezing ice creams with similar formulation (Table 4.1, Table 4.3). Mix 4, which was formulated with the higher concentration of wax had a marked decrease in mean equivalent diameter if compared with mix 3, which had a lower concentration of wax for 15% fat ice creams. Median of mix 3 is considerably larger than mix 4, which is characterized by a greater number of small air cells. Despite convincing evidence of the difference in air cell distribution in mix 3 and 4 this could not be correlated with fat destabilization and meltdown stability.

Table 4.5. Mean equivalent diameter of air cell of ice cream formulated from mix 2, mix 3, mix 4, mix 5 and mix 6.

<table>
<thead>
<tr>
<th></th>
<th>Mix 2</th>
<th>Mix 3</th>
<th>Mix 4</th>
<th>Mix 5</th>
<th>Mix 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>187</td>
<td>242</td>
<td>269</td>
<td>302</td>
<td>389</td>
</tr>
<tr>
<td>Median (μm)</td>
<td>27.41</td>
<td>44.85</td>
<td>15.45</td>
<td>7.56</td>
<td>36.09</td>
</tr>
<tr>
<td>Mean (μm)</td>
<td>58.55</td>
<td>60.52</td>
<td>33.91</td>
<td>10.29</td>
<td>73.60</td>
</tr>
<tr>
<td>Std. Dev (μm)</td>
<td>71.18</td>
<td>62.89</td>
<td>48.11</td>
<td>12.58</td>
<td>91.81</td>
</tr>
<tr>
<td>Min. (μm)</td>
<td>1.22</td>
<td>1.38</td>
<td>1.17</td>
<td>1.39</td>
<td>2.54</td>
</tr>
<tr>
<td>Max. (μm)</td>
<td>297.64</td>
<td>320.86</td>
<td>310.90</td>
<td>155.69</td>
<td>579.85</td>
</tr>
<tr>
<td>Std. Error</td>
<td>5.2</td>
<td>4.04</td>
<td>2.93</td>
<td>0.72</td>
<td>4.65</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>48.28</td>
<td>52.564</td>
<td>28.13</td>
<td>8.86</td>
<td>64.44</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>68.82</td>
<td>68.49</td>
<td>39.6871</td>
<td>11.71</td>
<td>82.74</td>
</tr>
</tbody>
</table>

CI = confidence interval
5. CONCLUSION

Rice bran wax (RBW) organogels showed to have potential as a fat substitute in ice cream.

Fat phase separation, differential scanning calorimetry (DSC) analyses and transmission electron microscopy (TEM) images revealed that RBW is dispersed in the oil droplets after emulsion formation and the dispersed RBW organogel droplet is in the form of a gel rather than liquid oil.

The application of RBW organogel was correlated to the formation of a stable ice cream emulsion characterized by small particle sizes and unimodal distribution (revealed by light scattering of ice cream samples prepared from continuous freezing). The frozen ice cream, formulated with polmo emulsifier, revealed higher levels of overrun that was not associated with higher meltdown resistance. Similar levels of fat destabilization were observed among RBW organogel ice cream and controls, when polmo was used as the emulsifier. The addition of emulsifier increased fat aggregation at the air cell interface. However, greater fat aggregation, when a higher concentration of emulsifier was applied, seemed to result in the deformation of air cells. High meltdown stability was not achieved by samples using polmo as the emulsifier. As revealed by TEM images, the use of polmo showed also an effect in the shape of fat droplets in ice cream emulsion causing them to be more elongated.

The use of polmo or glycerol monooleate (GMO) as the emulsifier was found to influence differently the stability of the RBW organogels droplets. Light scattering results showed that the addition of GMO formed organogel droplets that were more likely to
undergo destabilization. The instability of the organogel droplets caused by the presence of the emulsifier can be a consequence of the wax crystallization, which occurs at the outer edge of the droplet according to the results revealed by TEM images. The large aggregation of the destabilized droplets resulted in an increase in meltdown resistance. Also, a significant difference was observed in the spherical shape of the organogel droplets in the ice cream mix containing GMO when compared to organogel droplets in the polmo ice cream mix. The latter showed droplets with a more elongated shape and possibly higher instability. The replacement of polmo by GMO emulsifier was also observed to have an effect on the overall structure of ice cream. Structural elements such as ice crystals and air cells appeared with uniform shape and much more spherical air cells were observed.

The application of candelilla wax (CDW) and carnauba wax (CBW) organogels in ice cream did not cause any impact on the meltdown behaviour of the ice creams. Initially, CDW showed great potential for the formation of organogel droplets in an emulsion. That was because CDW crystallizes in high oleic sunflower oil (HOSO), forming a fat network of small crystals that are not greatly affected by cooling rate. However, TEM images revealed that the crystallization of CDW occurred on the edge of the organogel droplet covering the droplet with a crystalline shell of CDW. Because of this coverage, it is possible to conclude that a micro crystallization is not enough to predict the ultrastructure of the organogel droplet. CDW and CBW organogel droplets also showed lower fat destabilization. The greater stability of the droplets against aggregation could be an effect of crystal formation. With a hard solid wax at the edge of the droplet, instability upon collision is reduced and large aggregates are not formed. This
effect is evident in the light scattering results of the frozen ice cream. Lower fat destabilization was correlated with poor meltdown stability. The complex composition of CDW and CBW could have had an influence on the instability of the ice creams against meltdown.

The increase in fat content, accompanied by the use of GMO, was shown to be responsible for a large improvement in the structure of ice cream. Numerous factors were found to influence the development of ice cream structure: large fat destabilization, large aggregation of fat at the air interface, the formation of spherical air cells, the presence of well defined ice crystals, and the ultrastructure of the droplet.

Successful replication of the batch freezer samples was obtained when samples of similar composition were prepared using a continuous freezer. When compared to a batch freezing process, the use of a continuous freeing process promoted a greater destabilization of the organogel droplet containing GMO emulsifier. The higher fat content and the formation of larger fat destabilization generated samples with impressive meltdown stability, as revealed by the significant improvement in shape retention of the ice creams. The more intense shear applied by a continuous freezing process is likely to be the reason for the larger fat destabilization. An improvement in meltdown stability was also correlated with the shape and size of air cells, as observed in cryo-scanning electron microscopy (cryo-SEM) analyses.

The melting resistance of the organogel ice creams, which is correlated with fat network formation, seems to be explained by large fat destabilization and the small spherical shape of air cells. These factors were accomplished with an increase in fat concentration and the use of GMO emulsifier.
The application of wax organogel in ice cream showed great improvements in the quality of ice cream when compared to a pure liquid oil ice cream. If better quality parameters can be achieved by optimization of the formulation, RBW organogel has the potential to be used as a saturated fat substitute in ice cream. That would allow an increase in the concentration of unsaturated oils, creating some health benefits associated with the consumption of the product.

5.1. Future work

The mechanism of organogel droplet aggregation is not completely understood. Light scattering analyses revealed large fat destabilization in samples with good meltdown stability and shape retention. However, the differentiation of partial coalescence, coalescence and flocculation was not explained by these results. A more detailed investigation of the flocculation mechanism would be possible with the use of of sodium dodecyl sulfate (SDS) and ethylene diamine tetraacetic acid (EDTA) to dissociate any fat flocculated, as described by Goh et al. (2006).

Better understanding the mechanism of fat destabilization and structure formation in ice creams formulated with RBW organogel can facilitate the application and study of different forms of food grade organogels.

The optimization study of the formulation of the RBW organogel ice creams is necessary to evaluate the structure formation in ice cream with lower concentrations of fat (< 15%). Formulations with lower concentration of fat, about 10%, are of greater commercial interest than formulations with higher concentration of fat.
More elaborate investigation of the wax crystallization within the organogel droplets and the effect of different emulsifiers on the wax crystals may help improve the effect of fat concentration in the formation of ice cream structure. The presence of crystals protruding out of the organogel droplet was associated with large fat destabilization and structure formation. Using emulsifier that has the ability to largely encourage the crystal formation at the outer edge of the droplets may lead to greater fat destabilization and possibly the formation of ice cream structure in ice creams with lower concentrations of fat (< 15%).

The effect of different emulsifiers on the crystallization of CDW and CBW within the organogel droplets should be studied to be able to apply CDW and CBW organogels effectively in ice cream. If the crystallization of the CDW and CBW inside the droplet could be manipulated by the use of a certain type of emulsifier, the use of CDW and CBW organogels could be effective in the application of these wax organogels in ice cream. Since CDW organogel is firmer than CBW and RBW organogels, a lower concentration of wax is enough to form a firm gel structure. With a successful application of this wax organogel in ice cream, the use of lower concentrations of wax in ice cream would be possible, which would improve flavour.
6. REFERENCES


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7. APPENDIX A

Cooling Thermograms

A) Name of samples indicated in the figure.
B) RBW organogel ice cream mixes with 0 (a), 0.05 (b), 0.1 (c), 0.15 (d) and 0.2% (e) polmo emulsifier. Ice cream mix made with 100% RBW as the fat source (f).
8. APPENDIX B

Transition temperatures revealed by DSC analyses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Onset Temperature (°C)</th>
<th>Peak Temperature (°C)</th>
<th>Offset Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-13.75 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-7.36 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.57 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>76.99 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.63 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.66 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>-13.69 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.10 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-7.63 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>-16.2 ± 0.92</td>
<td>-5.51 ± 0.76</td>
<td>5.32 ± 0.86</td>
</tr>
<tr>
<td>4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>31.85 ± 0.69</td>
<td>35.53 ± 0.66</td>
<td>38.05 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>50.47 ± 0.18</td>
<td>52.63 ± 0.006</td>
<td>52.89 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>56.04 ± 0.59</td>
<td>58.77 ± 0.04</td>
<td>64.56 ± 0.30</td>
</tr>
<tr>
<td>5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-34.99 ± 0.47&lt;sup&gt;★&lt;/sup&gt;</td>
<td>62.21 ± 1.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-40.09 ± 0.48&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-35.48 ± 0.43&lt;sup&gt;★&lt;/sup&gt;</td>
<td>62.27 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-40.10 ± 0.51&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-35.23 ± 0.52&lt;sup&gt;★&lt;/sup&gt;</td>
<td>62.24 ± 0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-40.00 ± 0.63&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-35.11 ± 0.40&lt;sup&gt;★&lt;/sup&gt;</td>
<td>61.92 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-39.98 ± 0.51&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-32.87 ± 1.24&lt;sup&gt;★&lt;/sup&gt;</td>
<td>60.52 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-37.05 ± 1.83&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
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</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12&lt;sup&gt;★&lt;/sup&gt;</td>
<td>50.68 ± 0.48</td>
<td>57.57 ± 0.43</td>
<td>51.83 ± 0.15</td>
</tr>
<tr>
<td>13&lt;sup&gt;★&lt;/sup&gt;</td>
<td>-33.95 ± 0.59&lt;sup&gt;★&lt;/sup&gt;</td>
<td>61.40 ± 0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-38.94 ± 0.43&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
<tr>
<td>14&lt;sup&gt;★&lt;/sup&gt;</td>
<td>-32.86 ± 1.24&lt;sup&gt;★&lt;/sup&gt;</td>
<td>61.25 ± 1.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-37.04 ± 1.82&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(-) No peak was identified

(*) Samples with two or more peaks. Temperatures are presented from the lowest temperature peak to the highest temperature peak.

<sup>a</sup> HOSO melting temperatures

<sup>b</sup> RBW melting temperatures

<sup>★</sup> crystallization peak of HOSO. Melting peak couldn’t be identified because of water melting peak.

(sample 4) Pure polmo emulsifier. Four peaks were identified and are represented in crescent order of temperature.