Properties of oil-in-water emulsions and ice creams made from coconut milk

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Abstract

Coconut milk (CM) containing coconut oil extracted from the endosperm (meat) of coconut fruit is not stable and undergoes a rapid separation into a creaming layer at the top and a serum phase at the bottom. In this study, coconut milk was separated into coconut cream (CC) and coconut skim milk (CSM). The ability of proteins in CM, CC and CSM to form and stabilise oil-in-water (O/W) emulsions containing coconut oil was investigated. The unstable nature of coconut oil droplets in CM was found to be due to types of proteins adsorbed on the surface of oil droplets, which were predominantly 11S globulins, known to be hydrophobic and salt soluble proteins. The oil droplets stabilised by 11S globulins in CM and CC were larger in size and highly flocculated, probably due to hydrophobic interaction, thus resulting in rapid creaming and phase separation, compared to those stabilised by proteins (e.g. water-soluble albumins) in CSM. Smaller droplet size with less droplet flocculation and slower phase separation was obtained when emulsions were prepared with a predominance of proteins present in CSM. The CSM-based emulsions were relatively more stable but they were only able to provide short-term stability against phase separation. The results suggest that the ability of proteins (both globulins and albumins) in CM to stabilise the emulsion oil droplets was not high because these proteins did not seem to posses the ability to provide steric and electrostatic stabilisation to the emulsion droplets stabilised by them. An addition of small molecule surfactants, particularly a water-soluble surfactant of Tween 80, induced the formation of smaller droplets in the CM- and CSM-based emulsions, thereby improving their emulsion stability to a certain extent. However, the addition of oil-soluble small molecule surfactants (e.g. mono- and diglycerides and/or partially unsaturated mono- and diglycerides) in the absence of Tween 80 caused a significant increase in droplet size of emulsions prepared from CSM. In contrast, this phenomenon was not observed in emulsions made from CM. The formation and properties of coconut milk ice creams differing in the concentration of CSM proteins, as well as ratios of solid fat-to-liquid oil (blends of coconut oil and sunflower oil) were also investigated. The differences in those variables were found to have a significant influence on the properties and stability (particle size, flow properties, droplet flocculation) of ice cream mixes as well as the characteristics of ice creams, such as overrun, melt resistance and shape retention. Several instrumental analyses, including

size measurement, flow behaviour and small-deformation oscillatory tests, showed the presence of an agglomerated structural network in ice creams based on CSM containing oil blends as well as in ice creams based on dairy milk. From the findings, the agglomerated fat structural network in the CSM-based ice creams containing the suitable solid fat content at 68% could change ice creams with a slow melting rate and the more ability to retain their shape during melting compared with those of the dairy milk-based ice cream and ice creams made directly from CM. Overall the results suggest that coconut milk proteins do not possess the properties of proteins suitable for making very small droplets as well as stable emulsions against phase separation, particularly 11S globulins that are one of the major constituent proteins in coconut milk. However, albumins, which are the predominant proteins in coconut skim milk, may be suitable for use as the surface-active proteins for making smaller emulsion droplets in coconut oil-in-water emulsions, but their concentration needs to be increased for use probably by membrane filtration or freeze drying after removal of some carbohydrates from coconut skim milk.

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Chapter 1
Introduction

Coconut milk is a natural oil-in-water (O/W) emulsion extracted from fresh mature coconut meat (endosperm) (Hagenmaier et al., 1972, Monera and del Rosario, 1982, Seow and Gwee, 1997). Coconut milk is unstable, thereby readily separating into two distinct phases, cream and serum, by gravitational force soon after its extraction (Monera and del Rosario, 1982, Seow and Gwee, 1997). This could be due to the particle size of coconut oil droplets in freshly extracted coconut milk that is rather too big and the nature of the interfacial layers that may not have the high ability to protect the oil droplets against flocculation and coalescence (Monera and del Rosario, 1982).

Since this study was aimed to use coconut milk as the main ingredient for making ice cream, the instability of the coconut milk emulsion was hypothesised to interfere with the production of the ice cream with desired properties when it was made from coconut milk. However, a study of characterising the properties of ice creams solely based on coconut milk without added dairy milk or dairy ingredients has not been shown in the literature. There has been little research conducted on the stability and properties of natural coconut milk emulsions although some studies have shown that a creaming behaviour of flocculated oil droplets is a main cause of the instability of coconut milk emulsions (Onsaard et al., 2005, Onsaard et al., 2006, Tangsuphoom and Coupland, 2005, 2008b). The properties of different proteins in coconut milk that cause the emulsion instability (e.g. droplet flocculation and coalescence) are not well understood and needs to be investigated.

The stability of emulsions stabilised by proteins can be adversely or favourably affected by the addition of some other emulsifiers, such as small molecule surfactants, during the formation of emulsion droplets by homogenisation. However, this has not been well studied despite the fact that coconut milk is highly unstable against droplet flocculation and phase separation. This is very important to understand the functionalities of added surfactants in the structural formation of ice cream made from coconut milk. Ice creams

are mostly made from dairy milk and cream ingredients containing milk fat. Vegetable oils as well as plant proteins are becoming more popular and are accepted as alternative ingredients that can replace dairy milk fat and dairy proteins (Marshall et al., 2003, Méndez-Velasco and Goff, 2011, 2012a). Vegetable oils, such as coconut oil, sunflower oil and palm oil, have gained their use as ingredients for ice creams (Granger et al., 2005b, Granger et al., 2005c, Méndez-Velasco and Goff, 2011, 2012a). In recent years, several studies showed that the properties of ice creams, such as viscosity, meltdown rate and hardness, were shown to be affected by the incorporation of coconut oil (Choo et al., 2010, Kurultay et al., 2010). However, no studies have been shown concerning about a structural network of fat droplets in ice cream based on coconut oil or coconut milk.

It is essential to study the stability and properties of emulsions based on coconut milk in order to understand the properties of ice cream made from coconut milk. This is because the main quality and sensorial attributes of ice creams are dependent on the physicochemical properties and behaviours of emulsion droplets in the ice cream mix when subjected to the various stages of ice cream making processes, including homogenisation, ageing and freezing/whipping. Coconut milk contain two types of proteins, that is, albumins and globulins that are water and salt soluble proteins, respectively (Tangsuphoom and Coupland, 2009). The ability of these two proteins to emulsify and stabilise oil droplets in emulsions has also not been well understood.

The objectives of this project were to: 1) gain the understanding about the formation, properties and stability of coconut oil-in-water emulsions, 2) understand the influence of small molecule surfactants on the coconut proteins-stabilised oil-in-water emulsions and 3) investigate the properties of ice cream mix emulsions and the corresponding ice creams made from coconut milk.

Chapter 2
Literature review

2.1 Coconut milk

Coconut milk is one of the main traditional ingredients commonly used in foods and desserts in many Southeast Asian countries. It is normally called "santan" in Malaysia, "gata' in the Philippines (Seow and Gwee, 1997) and "kati" in Thailand. In Thailand, coconut milk is used as an important raw material for making a frozen dessert, normally known as coconut milk ice cream, one of the traditional frozen desserts (Surapat and Rugthavon, 2003, Vatanasuchart et al., 2000).

Coconut milk is a milky fluid extracted from the endosperm (kernel, meat) of mature coconut (*Cocos nucifera* L.) fruits (Hagenmaier et al., 1973, Hagenmaier et al., 1974, Seow and Gwee, 1997, Tangsuphoom and Coupland, 2005). To extract coconut milk, the mature fresh coconut meat (edible white flesh of coconut fruit) is separated from the shell of coconut nut, grated and pressed either with or without added water which is then separated from the meat fibres (Hagenmaier et al., 1973, Monera and del Rosario, 1982, Seow and Gwee, 1997). The structure of coconut nut is comprised of three main parts, such as outer shell, solid white endosperm and hollow space filled with a liquid (coconut water), as shown in Figure 2.1. Coconut water is a clear liquid naturally present in coconut nut, which is different from coconut milk that is extracted from the coconut endosperm.

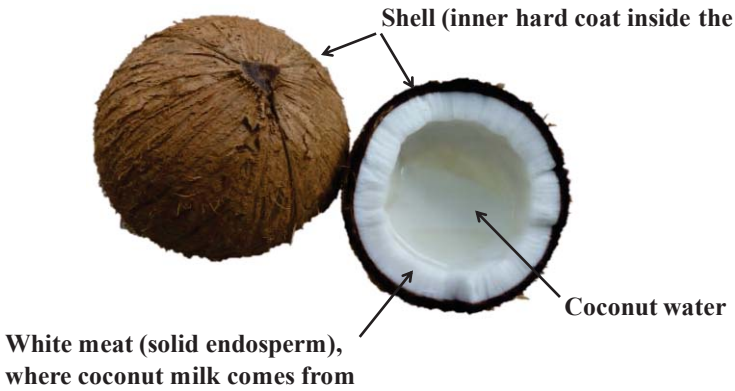

Figure 2.1 Structure of coconut nut

2.2 Coconut milk products

For products of coconut milk, the term "coconut milk" has been interchangeably used with "coconut cream" (Seow and Gwee, 1997). The Codex Alimentarius Commission (2003) defines coconut milk and coconut cream into four categories based mainly on their composition, such as total solids, non-fat solids, fat content and moisture content. The categories are 1) light coconut milk; 2) coconut milk; 3) coconut cream and 4) coconut cream concentrate. The standard composition of each type is shown in Table 2.1. Coconut milk is distinguished from coconut cream by fat and solids non-fat contents. Commercial coconut milk and coconut cream products may have other ingredients added, such as coconut water, maltodextrin and sodium caseinate (Codex Alimentarius Commission, 2003). Coconut milk and coconut cream products are allowed to be added with additives such as bleaching agents, emulsifiers, preservatives, stabilisers or thickeners (Table 2.2).

Commercial coconut milk and coconut cream products normally have a long shelf life. The good quality products should have the colour, flavour and odour similar to the characteristics of fresh raw coconut milk. In order to extend the shelf life, the products can be heat treated, spray dried or frozen. Currently, there are three main forms of products available in markets, including 1) liquid products in hermetically sealed packages such as can or aseptic packaging, for instance, ultra-high temperature (UHT)-treated or pasteurised; 2) powdery products and 3) frozen products. A typical product composition is given in Table 2.3. The main form of consumer packaging for coconut milk and cream is aseptic packaging in 200-250 ml Tetra-Brik® or Combibloc® containers with a shelf life of 24 months (Seow and Gwee, 1997). A problem associated with these products is coagulation of coconut milk proteins that occurs once the temperature reaches 80°C. This leads to curdled products, mainly in more concentrated products. Separation of the oil and water phases usually occurs when coconut milk is processed without using appropriate surface active agents and stabilisers and a homogenisation treatment is not applied adequately (Seow and Gwee, 1997).

Table 2.1 Standard composition (wt%) of different types of coconut milk and coconut cream products

Product	Total Solids (Min-Max)	Solids non-fat (Min)	Fat (Min)	Moisture (Max)	pH (Min)
Light coconut milk	6.6-12.6	1.6	5.0	93.4	5.9
Coconut milk	12.7-25.3	2.7	10.0	87.3	5.9
Coconut cream	25.4-37.3	5.4	20.0	74.6	5.9
Coconut cream concentrate	37.4 min.	8.4	29.0	62.6	5.9

Source: Codex Alimentarius Commission (2003)

Table 2.2 Types and amounts of food additives allowed in coconut milk and coconut cream

Additive type	Food additive	INS No.	Maximum level
Bleaching agents	Sodium metabisulphite	223	30 mg/kg
	Potassium metabisulphite	224	
Small molecule surfactantrs	Polyoxyethylene (20) sorbitan monolaurate	432	1000 mg/kg
	Polyoxyethylene (20) sorbitan monooleate	433	
	Polyoxyethylene (20) sorbitan monopalmitate	434	
	Polyoxyethylene (20) sorbitan monostearate	435	
	Polyoxyethylene (20) sorbitan tristearate	436	
	Mono- and diglycerides*	471	*Limited by GMP[a]
	Sucrose esters of fatty acid	473	1500 mg/kg
Preservatives	Sodium benzoate (only for pasteurised coconut milk)	211	1000 mg/kg
Stabilisers/ Thickeners	Guar gum	412	Limited by GMP
	Xanthan gum	415	
	Gellan gum	418	
	Sodium carboxymethyl cellulose	466	

[a] = good manufacturing practice. Source: Codex Alimentarius Commission (2003)

The stability of processed coconut milk emulsions is affected by various factors, such as coconut oil concentration, type and amount of stabilising agents added and homogenisation pressure and thermal process conditions (Seow and Gwee, 1997), other ingredients added, such as sucrose and salt (Kim et al., 2003, Kulmyrzaev et al., 2000, Onsaard et al., 2005) and pH and ionic strength (Tangsuphoom and Coupland, 2008a). For instance, coconut milk at its isoelectric pH 3.5-4 becomes unstable and tends to form lumps with oil droplet flocculation but exhibits no lump formation at pH 6 (Peters, 1960 cited in Hagenmaier et al. 1972). A number of stabilising agents, such as carboxymethyl cellulose (CMC), gum Acacia and maltodextrin, small molecule surfactants and protein and polysaccharide emulsifiers, such as polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monolaurate (Tween 20), sodium caseinate, and whey protein isolate, have been used to stabilise coconut milk emulsions (Chiewchan et al., 2006, Jena and Das, 2006, Jirapeangtong et al., 2008, Peamprasart and Chiewchan, 2006, Tangsuphoom and Coupland, 2005).

Coconut milk can be dehydrated by spray drying to yield a creamy white coconut milk powder. Spray-dried powder is easily dissolved in water at ambient temperature, and about 60-100 g powder is equivalent to one coconut (Seow and Gwee, 1997). Often prior to the spray drying process, ingredients such as maltodextrin, casein, skim milk and corn syrup are added. A typical composition of coconut milk powder is also shown in Table 2.3. Frozen coconut milk products are made by freezing the pasteurised coconut milk and cream. The frozen products have the shelf life up to one year at -23.3°C storage condition (cited in Cancel, 1979, Seow and Gwee, 1997).

2.3 Chemical composition and physical properties of coconut milk

Coconut milk is a dispersion of coconut oil in an aqueous phase comprising of natural phospholipids and proteins (Monera and del Rosario, 1982). The natural composition of coconut milk varies depending on variety, geographical location, cultural practice, maturity of nut and method of extraction including amount of water (if added). The composition of undiluted coconut milk reported in the literature is shown in Table 2.4.

Table 2.3 Composition and pH of commercial coconut milk and coconut milk powder

Constituent (wt%)	Canned products				Spray-dried products	
	Thailand[a]	Malaysia[b]	Singapore[b]	West Somalia[a]	Seow & Leong (1988)[c]	Gonzalez (1986)[c]
Moisture	76.2	78.8	84.4	75.8	2.2	0.8-2.0
Fat	15.0	14.0	11.0	18.4	63.6	60.5
Solids non-fat	9.0	7.2	ND	ND	ND	ND
- Protein (N x 6.25)	0.3	0.3	0.5	0.9	4.5	6.9
- Ash	ND	ND	ND	ND	1.0	1.8
- Carbohydrates*	8.1	6.2	3.5	4.2	28.7	27.3
- Minerals	0.4	0.7	0.6	0.7	ND	ND
- Free fatty acid	0.2	ND	ND	0.5	ND	ND
- Crude fibre	ND	ND	ND	ND	ND	0.02
pH	6.2	ND	ND	6.3	ND	ND

ND = not reported, * = by difference calculated from [100-water-fat-protein-ash]
Source: [a] Seow and Gwee (1997), [b] Arumughan et al. (1993), and [c] cited in Seow and Gwee (1997)

Table 2.4 Composition of fresh undiluted coconut milk

Constituent (% wt)	Nathaneal (1954)[a]	Popper et al. (1966)[a]	Jeganathan (1970)[a]	Simuang et al. (2004)
Moisture	50.0	54.1	50.0	54.0
Fat	39.8	32.2	40.0	34.0
Solids non-fat	ND	ND	ND	11.0
- Protein	2.8	4.4	3.0	ND
- Ash	1.2	1.0	1.5	ND
- Carbohydrates*	6.2	8.3	5.5	ND

ND = not reported, * = by difference calculated from [100-water-fat-protein-ash]
Source: [a] cited in Seow and Gwee (1997)

From Table 2.4, typical fresh undiluted coconut milk comprises about 50-55% water, 32-40% fat, 3-4% protein and 11% solids non-fat by weight. The solids include proteins, carbohydrates (mainly sucrose), minerals (phosphorus, calcium and potassium) and vitamins (B and C) (Seow and Gwee, 1997).

As described above, coconut milk is a dispersion of coconut oil in water. The oil droplets in the extracted coconut milk are naturally stabilised by coconut proteins and phospholipids (Monera and del Rosario, 1982). The average droplet size of coconut oil droplets in natural coconut milk is reported to be about 10 μm (Hagenmaier et al., 1972) or 11-12 μm in volume weighted mean diameter (d_{43}) with a bimodal size distribution (Tangsuphoom and Coupland, 2008a). The surface electrical charge of coconut oil droplets at pH 6.1 to 6.3 is -16 mV, which is mainly contributed by the major acidic amino acid, glutamic acid, present in coconut milk proteins (Tangsuphoom and Coupland, 2008a).

Natural coconut milk is very unstable, readily separating by gravitational force into two distinct phases: lighter creamy and heavier water phases. However, when coconut milk is centrifuged at high speed, it separates into three phases: a light liquid phase which is mainly cream containing a high concentration of protein-stabilised fat droplets; a heavy liquid phase consisting of water and water-soluble materials; and a solid phase comprising of undissolved solids and fibre (Hagenmaier et al., 1972).

Coconut milk has pseudoplastic rheological properties (Chiewchan et al., 2006, Simuang et al., 2004, Vitali et al., 1985). Flow behaviour of coconut milk depends on the oil content and stabilisers (if added) (Chiewchan et al., 2006, Peamprasart and Chiewchan, 2006, Simuang et al., 2004, Tangsuphoom and Coupland, 2005). Homogenisation of coconut milk affects flow behaviour of coconut milk (Chiewchan et al., 2006). The homogenisation pressure ranging from 15 to 27 MPa is usually applied in the production of canned coconut milk after heat treatment at temperatures between 109.3 and 121.1°C. Heat-treated homogenised coconut milk shows pseudoplastic behaviour with its flow behaviour index (n) between 0.719 and 0.971 differing from a

Newtonian fluid that has a flow behaviour index equal to 1.0 (Daubert, 2003). After the high pressure homogenisation, the flow behaviour index becomes decreased (Chiewchan et al., 2006).

Prior to homogenisation, coconut milk is usually subjected to heat treatment. Globular proteins in coconut milk become denatured by heat treatment and hydrostatic high pressure, as a consequence, protein aggregation could take place (Dumay et al., 1994). The size of oil droplets in homogenised coconut milk varies depending on the addition and amount of small molecule surfactants. The more the surfactants are added, the smaller of the oil droplets in diameter result in. It has been shown that mean droplet diameter could range from 16.0 to 0.3 μm depending on concentration of added surfactants ranging from 0 to 1.0 wt%, respectively (Tangsuphoom and Coupland, 2009).

2.4 Coconut oil and coconut milk proteins

2.4.1 Coconut oil

Coconut oil found in coconut meat and coconut milk is often referred as lauric oil as almost half of the total fatty acids in coconut oil is lauric acid which is a twelve carbon containing medium chain fatty acid (Gunstone, 2004). The physical characteristics of coconut oil are shown in Table 2.5. Coconut oil comprises mainly of medium chain triglycerides (deMan, 1998, Siew, 2001), consisting of different saturated or unsaturated fatty acids esterified to the glycerol backbone of coconut oil triglycerides, but lauric acid being predominant, which result in hundreds of different molecular composition of triglycerides in coconut oil (Gresti et al., 1993). In addition to triglycerides, there are substantial amounts of mono- and diglycerides and small amounts of free fatty acids, phospholipids and sterols present in coconut oil (deMan, 1998, Siew, 2001).

The molecular composition of triglycerides determine the physical properties of fats and oils, such as melting, crystallisation and polymorphism behaviour, which are directly responsible for the properties of products containing oils or fats (Nor Hayati et al., 2008). The differences in triglyceride compositions between anhydrous dairy milk fat

(AMF) and coconut oil are shown in Table 2.6. The melting temperature of coconut oil is in the range between 23°C and 26°C (Gunstone, 2004). At the temperature above 26°C, coconut oil is liquid. In a study using differential scanning calorimetry (DSC) reported by Granger et al. (2005c), the refined coconut oil formulated in ice creams was shown to begin to crystallise at a temperature of about 10°C and become fully crystallised when the temperature reaches about -18°C.

Coconut oil has a similar fatty acid profile to palm kernel oil. Both have more than 50% lauric acid (C12:0) and 15% myristic acid (C14:0) (Azeez, 2007, Che Man et al., 1997, deMan, 1998, Pamela, 1999). Fatty acid compositions of coconut oil, palm kernel oil and their fractionated oils are shown in Table 2.7. Coconut oil is composed of 93.85% saturated fatty acid (SFA), 5.36% monounsaturated fatty acid (MUFA) and 0.79 polyunsaturated fatty acid (PUFA) (Marina et al., 2009). When coconut oil is fractionated with acetone (1:3 w/v) at 8^{o}C for 3 hours, it yields 2 parts: a 84% liquid fraction (coconut olein); and a 16% solid fraction (coconut stearin) (Ghosh and Bhattacharyya, 1997). Coconut oil in coconut milk tends to form orthorhombic crystalline platelets upon crystallisation, whereas palm kernel oil and milk fat form triclinic crystals. The latter form is the most desirable type of crystals which contributes to a smooth texture in products such as margarine (Young, 1985).

Lipids can be modified by blending, fractionation, hydrogenation and inter- or intra-esterification (Nor Hayati et al., 2008). The modification of lipids leads to differences in their physicochemical properties, such as slip melting point (SMP), solid fat content (SFC) and polymorphic behaviour (Nor Hayati et al., 2008, Norizzah et al., 2004). A blend of one oil with other oils could alter the polymorphic form of the resulting oil blend, for example, a blend of palm oil with low trans-fatty acids led to a transition of the polymorphic form from β′- to β-form (Konno et al., 2012). A number of studies on the physicochemical properties of blended lipids and related products have been investigated (Chu et al., 2002, Liew et al., 2001b, Nor Hayati et al., 2008, Norizzah et al., 2004, Shen et al., 2001).

The properties of ice creams, such as droplet size, melting resistance, overrun, viscosity and viscoelastic properties, are largely affected by the different types of oil blend used in ice cream formulations (Goh et al., 2006, Liew et al., 2001b, Méndez-Velasco and Goff, 2011, 2012a, b, Sung and Goff, 2010). For example, oil blends of a highly saturated fatty acid oil (palm kernel oil) mixed with a highly unsaturated oil (sunflower oil) or milk fat mixed with palm kernel oil or flaxseed oil, have been used to investigate their effects on the properties of ice cream. Liew et al. (2001b) showed that ice cream emulsions containing a mixture of anhydrous milk fat (AMF) and enzyme-transesterified palm kernel olein had greater elastic properties than those containing a mixture of AMF and normal palm kernel olein. Méndez-Velasco and Goff (2011, 2012a, b) and Sung and Goff (2010) showed that when saturated oil in fat blends was about 60 to 80%, the ice cream had bigger oil droplets in size, reflecting more pronounced partial coalescence of fat droplets and more melting resistance.

Table 2.5 Physical characteristics of coconut oil

Characteristics	Typical	Range
Specific gravity, 30°C	-	0.915- 0.92
Reflective index, 40°C	-	1.448 - 1.449
Iodine value	10	7.5 - 10.5
Kinematic viscosity at 40 ◦C ($mm^2 s^{-1}$)*	27.96	-
Saponification number	-	248 - 264
Melting point (°C) (Mettler Droping Point)	26.5	25 - 28
Solidification point (°C)	-	14 - 22
Crystal habit	β′	-
Solids fat index (%) at		
10.0 °C	54.5	-
21.1 °C	26.6	-
26.7 °C	0	-

Source: O'Brien (2004) and *Benjapornkulaphong et al. (2009)

Table 2.6 Triglyceride species of anhydrous milk fat and coconut oil (wt%)

Oil or fat	Monoglyceride	Diglyceride	Triglyceride	Complex lipids
Anhydrous milk fat[a]	ND	1.7	98.3	ND
Coconut oil[b]	3.5	6.6	85.4	4.6
Coconut oil[c]	4.0	12.0	84.0	ND

ND = not reported
Source: [a] Relkin et al. (2004), [b] Pham et al. (1998) and [c] O'Brien (2004)

2.4.2 Coconut milk proteins

The protein content of coconut milk is about 5-10% by weight on dry basis (Seow and Gwee, 1997). The main proteins found in coconut milk consist of 11S globulin (also called cocosin), 7S globulin and albumins (Balachandran and Arumughan, 1992, Rasyid et al., 1992). Globulins are soluble in diluted salt solutions (e.g. 0.5M NaCl) while albumins are water soluble proteins. Globulins and albumins constitute about 75% and 25% of the total coconut milk protein, respectively, and the two globulins, 11S globulin and 7S globulin constitute about 86% and 14%, respectively, of the total globulins (Garcia et al., 2005). The 11S and 7S coconut globulins are storage proteins which are also found in other plant seeds but have been named differently depending on their sources. For instance, 11S globulins correspond to glycinins found in soybean (Adachi et al., 2003, Riblett et al., 2001) or legumins in cupin (Mills et al., 2002). Likewise, 7S globulin is referred to correspond to soybean β-conglycinin (Adachi et al., 2003) or cupin euvicilins (Mills et al., 2002). Coconut milk proteins have minimum solubility at pH between 4 and 5 but are soluble with increasing pH with the maximum solubility at pH between 6 and 8 (Onsaard et al., 2005, Onsaard et al., 2006). At pH 4, coconut milk proteins are highly coagulated compared with coconut milk at pH 6 (Tangsuphoom and Coupland, 2008a).

Table 2.7 Fatty acid composition of coconut oil, palm kernel oil, their fractions, anhydrous milk fat and sunflower oil

Type of fats or oils	Iodine Value [d]	Saturated fatty acid (wt%)									Unsaturated fatty acid (wt%)				
		Butyric: C4:0	Caproic: C6:0	Caprylic: C8:0	Capric: C10:0	Lauric: C12: 0	Myristic: c14:0	Palmitic: C16:0	Stearic: C18:0	Total	Palmitoleic: C16:1	Oleic: C18:1	Linoleic: C18:2	Linolenic: C18:3	Total
Coconut oil [a]	8.5	n.d.	0.9	4.9	6.2	50.3	19.2	8.3	1.3	**91.1**	n.d.	6.3	2.6	n.d.	**8.9**
Coconut oil[b]	ND	n.d.	0.5	8.4	6.1	52.5	16.9	7.2	1.2	**93.8**	n.d.	5.4	0.4	n.d.	**6.2**
Coconut olein [a]	10.0	n.d.	1.0	5.4	6.8	48.2	18.1	7.8	1.4	**88.7**	n.d.	7.8	3.5	n.d.	**11.3**
Coconut stearin [a]	4.0	n.d.	0.4	2.2	3.1	57.3	23.9	10.9	0.7	**98.5**	n.d.	7.8	0.7	n.d.	**8.5**
Palm kernel oil [c]	17.5	n.d.	0.3	3.9	3.4	49.9	16.5	8.1	2.8	**84.9**	n.d.	12.9	2.1	n.d.	**15.0**
Palm kernel olein [c]	21.0	n.d.	0.1	4.0	3.6	45.6	14.4	8.2	2.3	**78.2**	n.d.	18.6	3.1	n.d.	**21.7**
Palm kernel stealin [c]	9.0	n.d.	0.1	2.0	2.8	58.1	22.1	7.4	1.6	**94.1**	n.d.	5.0	0.8	n.d.	**5.8**
Anhydrous milk fat [e]	ND	4.2	2.4	1.4	3.0	3.5	11.2	28.7	11.6	**66.0**	1.4	21.5	1.2	0.6	**24.7**
Anhydrous milk fat [e]	ND	3.9	2.2	1.2	2.6	3.1	10.5	31.9	10.0	**65.4**	2.0	21.5	1.4	0.5	**25.4**
Sunflower oil [f]	ND	-	-	-	-	-	-	6.0	4.0	**10.0**	-	14.0	76.0	-	**90.0**

n.d. = not detected; ND = not reported

Source: [a] Ghosh and Bhattacharyya (1997), [b] Marina et al. (2009), [c] Siew (2001), [d] Rossell (1985), [e] Shen et al. (2001) and [f] deMan and deMan (2002).

Major types of charged amino acids found in coconut endosperm proteins are glutamic acid, arginine, and aspartic acid (Kwon et al., 1996). Coconut globulins possess a high level of hydrophobic amino acids, such as valine, leucine and methionine, while coconut albumins contain a relatively high level of hydrophilic amino acids, such as lysine and glycine, compared to globulins (Kwon et al. 1996). The ratio of polar to nonpolar amino acids (P), the frequency of non-polar hydrophobic residues (NPN) (calculated as the fraction of the sum of hydrophobic amino acid residues over the total number of residues) and the average hydrophobicity (Hϕ) of these two major coconut milk proteins based on their amino acid compositions are 3.46, 0.18 and 765, respectively, for albumins, and 1.71, 0.29 and 965, respectively, for globulins (Kwon et al., 1996). From these values, albumins have the higher P value, indicating albumins is comprised of a higher proportion of polar amino acids which confers the high water solubility, whereas globulins is composed of a relatively higher NPS and Hϕ, thus more hydrophobic than albumins. In terms of the protein structure, the amount of proline residues adversely influences the secondary and tertiary structures of proteins (Damodaran, 1994) which hinders the formation of a well-ordered conformational structure, resulting in a random disordered protein tertiary structure. The percentage of proline was reported to be 2.7% and 3.4% of the total amino acids in globulins and albumins, respectively (Kwon et al., 1996). The low level of proline in both proteins indicates that albumins and globulins may exist as compact globular proteins with well-defined 3-dimensional structure.

2.5 Characterisation of coconut milk proteins by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method to quantify and identify the composition of proteins by separating them on the basis of molecular weights (Walker, 2009). A number of studies have been attempted to pull out the protein profile from defatted coconut meal (the coconut fresh residue after solvent extraction of coconut oil) (Carr et al., 1990, DeMason and Sekhar, 1990, Teuber and Peterson, 1999, Voigt et al., 1994) and coconut milk (Rasyid et al., 1992, Tangsuphoom and Coupland, 2009) by SDS-PAGE. Under a non-reducing condition, proteins in coconut meal revealed polypeptide bands with the

estimated molecular weights in the range from 18 kDa to greater than 100 kDa (Kwon et al., 1996, Sjögren and Spychalski, 1930). On the other hand, under a reducing condition, the bands of protein subunits of coconut meal proteins were resolved into seven prominent bands with the molecular weights spanning between 14 kDa and 52 kDa (Kwon et al., 1996). Those protein bands represent the presence of the two species of proteins, including globulins which exhibited four protein bands with the estimated molecular weights of 17, 25, 27 and 56 kDa and albumins with the dissociated protein bands at 15, 28 and 52 kDa (Kwon et al., 1996).

In general, the migration pattern of some polypeptide bands observed from coconut meal proteins quite resemble the dissociation pattern of 11S globulin proteins of soybean (Rasyid et al., 1992). The results of the polypeptide bands corresponding to globulins showing greater band ranges under the non-reducing condition than the reducing condition suggest the presence of disulfide bonds that link the polypeptide subunits of globulins together. The contrast phenomenon was however observed with albumin proteins. There was no obvious difference in the polypeptide bands for albumins under two conditions, indicating that the polypeptide subunits in albumins are not linked via the disulfide bonds (Kwon et al., 1996).

The polypeptide bands of proteins in coconut milk were variously reported in the literature that they were resolved into three major bands with the estimated molecular weights ranging from 16 kDa to 52 kDa (Rasyid et al., 1992) or seven major bands with the estimated molecular weights ranging from 18 kDa to 50 kDa (Tangsuphoom and Coupland, 2009). The estimated molecular weights of the dissociated polypeptides bands in coconut milk reported by Tangsuphoom and Coupland (2009) were quite close to the subunits of protein bands obtained from coconut meal flour which were identified as globulin and albumin proteins according to the study reported by Kwon et al. (1996). Coconut 11S globulins (cocosin) have been also varyingly reported to have a molecular weight of about 208 kDa (Osborne, 1924, Sjögren and Spychalski, 1930), 300 kDa to 360 kDa (Carr et al., 1990) and 326 kDa (Garcia et al., 2005). The 7S globulin has the molecular weight of about 156 kDa (Garcia et al., 2005) or in the range from 150 kDa to

180 kDa (Reeve and Sherman, 1988). Water soluble albumins have been estimated to have a molecular weight of about 100 kDa (Garcia et al., 2005, Kwon et al., 1996).

It has been reported that the content as well as the quality of natural proteins in coconut milk are not enough to make the oil droplets in coconut oil-in-water emulsions stable, causing a phase separation into two portions (Monera and del Rosario, 1982). In several studies, the ability of coconut proteins to form and stabilise oil-in-water emulsions have been shown (Onsaard et al., 2005, Onsaard et al., 2006, Tangsuphoom and Coupland, 2009). Proteins isolated from coconut skim milk could produce emulsions containing corn oil with a monomodal distribution with high stability (Onsaard et al., 2005). Similarly, proteins that were fractionated from the cream phase of coconut milk could also form small droplets in corn oil-in-water emulsions (Onsaard et al., 2006). In another study reported by Tangsuphoom and Coupland (2009), the only protein species responsible for stabilising emulsions in coconut milk were shown to be cocosin, the 11S globulins, as globulins were only found at the interfacial layer of droplets when coconut milk was homogenised. This infers that albumins were left behind in the serum phase of the homogenised milk without being adsorbed to the interface, probably due to less affinity to bind to the oil-water interface than globulins. About one fourth of the total coconut globulin proteins was reported to absorb at the oil-water interfaces after homogenisation with the surface protein load of 7 mg/m^2 at the interface (Tangsuphoom and Coupland, 2009).

2.6 Oil-in-water emulsion and its stability

An oil-in-water (O/W) emulsion is defined as a colloidal dispersion in which small droplets of oil are dispersed in another immiscible aqueous phase (Wilbey, 2003). In general, a high mechanical shear force using a high pressure homogeniser is employed to produce small oil droplets which are dispersed in the aqueous continuous phase containing emulsifiers which are surface active substances, including amphiphilic biopolymers (i.e., some proteins and polysaccharides) and small molecule surfactants (McClements, 2004a, Schultz et al., 2004).

During homogenisation, the surface active molecules present rapidly adsorb at the oil-water interface of small oil droplets formed from bulk oil or newly created from existing big oil droplets, resulting in a substantial reduction in the interfacial tension at the oil-water interface (McClements, 2004a). In addition, those emulsifiers would form a protective membrane around droplet surface to protect those droplets from aggregation (i.e., flocculation and coalescence) (McClements, 2004a). In general, the chemical structure of emulsifiers consists of a hydrophilic head and a hydrophobic (lipophilic) tail. Upon an adsorption at the interface, the emulsifier molecule orientates the hydrophobic part to be located in an aqueous phase and the lipophilic part to be in the lipid phase, thus minimising the contact area between the aqueous and lipid phases and reducing the interfacial tension between the two phases (Dickinson, 1992b, McClements, 2004a).

Several factors determine the surface load and composition of the interfacial membrane of the dispersed droplets in emulsions such as type and concentration of emulsifiers, temperature, pH, ionic strength and homogenisation conditions (McClements, 2004a). Emulsifiers, for example, globular proteins, that are present at a high concentration lead to an increase in the surface protein load, which can be due to their interaction at the interface leading to a formation of more dense or multiple layers rather than a single layer at the droplet surface (Dalgleish, 1996, McClements, 2004a, Norde, 2003). The surface protein load of some protein-stabilised droplets was reported to increase with increasing temperature presumably due to protein unfolding and with increasing salt concentration possibly due to the screening effect of the electrostatic repulsion between adsorbed and unadsorbed proteins (Dalgleish, 1996, McClements, 2004a).

The use of multiple emulsifiers is common in preparing food emulsions. The composition of the interfacial membrane will be more complex with a mixture of added emulsifiers. In a case that there are two or more than two different types of emulsifiers present in the system, the competitive adsorption between those surface active materials at the interface usually occurs (Dickinson and Tanai, 1992). During homogenisation under turbulent conditions, emulsifiers with a high diffusion rate will tend to be initially

adsorbed and form a protective membrane at the droplet surface, however, during storage, emulsifiers that have greater affinity for the interface may displace the initially adsorbed emulsifiers, resulting in desorption of some emulsifiers and a change in the interfacial membrane composition.

The stability and rheological properties of the emulsions formed can be quite different, depending on the composition of the droplet surface membrane, in terms of type, concentration and structure (Dickinson et al., 1993, Dickinson and Tanai, 1992). Emulsions are not thermodynamically stable and undergo destabilisation through various phenomena, including droplet flocculation and coalescence (Aronson, 1989, Dickinson, 1992c). Flocculation is a phenomenon where oil droplets dispersed in emulsions become clustered without losing their original shape but with maintaining their individual identity, thus can be reversed into their original droplets (Aronson, 1989). Coalescence is a phenomenon that two or more oil droplets merge together through the rupture of interfacial layers between approaching droplets, thus forming a single larger droplet (Damodaran, 2005, Goff, 2000). The flocculated or coalesced droplets generally lead to creaming (Aronson, 1989, Dickinson, 1992c). Creaming is a phenomenon that oil droplets move upwards to the top surface of emulsion (Damodaran, 2005). Creaming occurs faster with increasing particle size due to an increase in the density difference between the dispersed phase and the continuous phase (Damodaran, 2005).

2.7 Ice cream

Ice cream is a multiphase frozen product made up of several main ingredients including fats, proteins, small molecule surfactants, stabilisers, sugars and water (Clarke, 2004, Goff, 2006, Marshall et al., 2003, Walstra and Jonkman, 1998). Ice cream comprises of three states of substances (Figure 2.2), including solid fat and water (ice crystals), liquid concentrated sugar solution and air cells (Clarke, 2004). By volume, ice cream consists of about 50% air, 30% ice crystal, 5% milk fat and 15% matrix (Clarke, 2004, Crilly et al., 2008).

Ice cream matrix is a continuous phase of concentrated viscous liquid containing sugars, proteins, polysaccharides, salts, stabilisers and water (Goff, 1997b, Goff, 2002), in which tiny particles of fat, ice crystals, air bubbles and other insoluble substances are dispersed. The unfrozen liquid viscous syrup constitutes about 30% of the total mass of ice cream (Marshall, 2002). Approximate sizes of the dispersed elements are: fat droplets, diameter 0.5-2.0 μm; lactose crystals, length 20 μm; ice crystals, average diameter 35-100 μm; air cells, diameter between 60-160 μm (Marshall, 2002) and 20-50 μm (Goff, 2002).

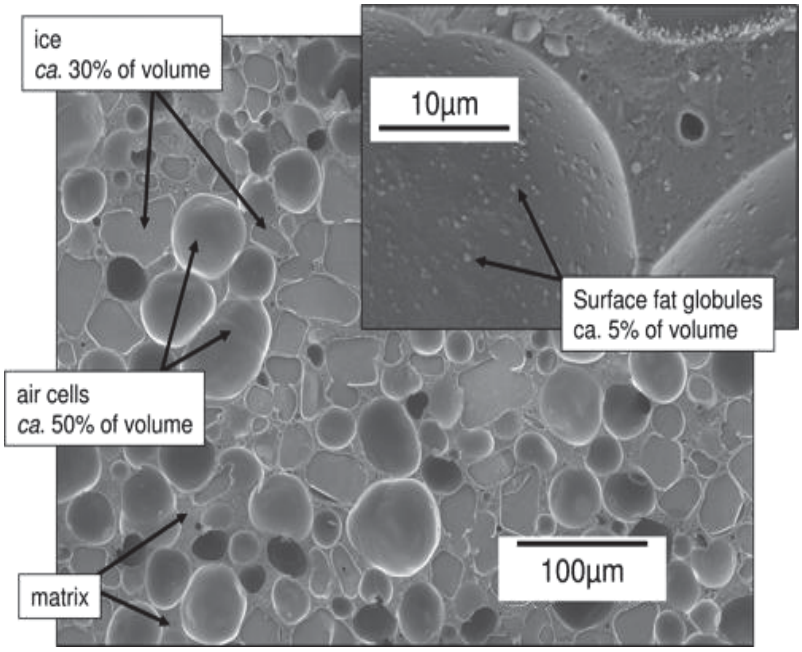

Figure 2.2 Microstructure of ice cream by scanning electron microscopy (SEM). Source: Crilly et al. (2008).

2.8 Manufacturing process of ice cream

An ice cream production involves multi-stage operations starting from the formulation and preparation of an ice cream mix which is an oil-in-water emulsion. Several processing unit operations involved include blending of ingredients, pasteurisation (for example at 85°C for 30 seconds), homogenisation, cooling, ageing and freezing and churning processes. The ice cream mix is a cold aged homogenised mixture of pasteurised ingredients, in which protein-stabilised oil-in-water emulsions are present. Ageing involves storing the homogenised ice cream mix at about 4°C for a few hours or

overnight prior to the subsequent freezing which also involves the whipping and churning of the aged ice cream mix (Clarke, 2004, Davies et al., 2000, Marshall et al., 2003). During freezing of the aged ice cream mix in an ice cream freezer with temperatures at approximately -26°C to -28°C (Hartel, 1996), air is usually introduced into the ice cream mix to create air cells or overrun (Clarke, 2004). The frozen aerated ice cream is then drawn from the ice cream freezer at a temperature about -5°C to -6°C for packing and then stored at -30°C for hardening (Hartel, 1996). Figure 2.3 shows the schematic diagram of ice cream manufacturing process.

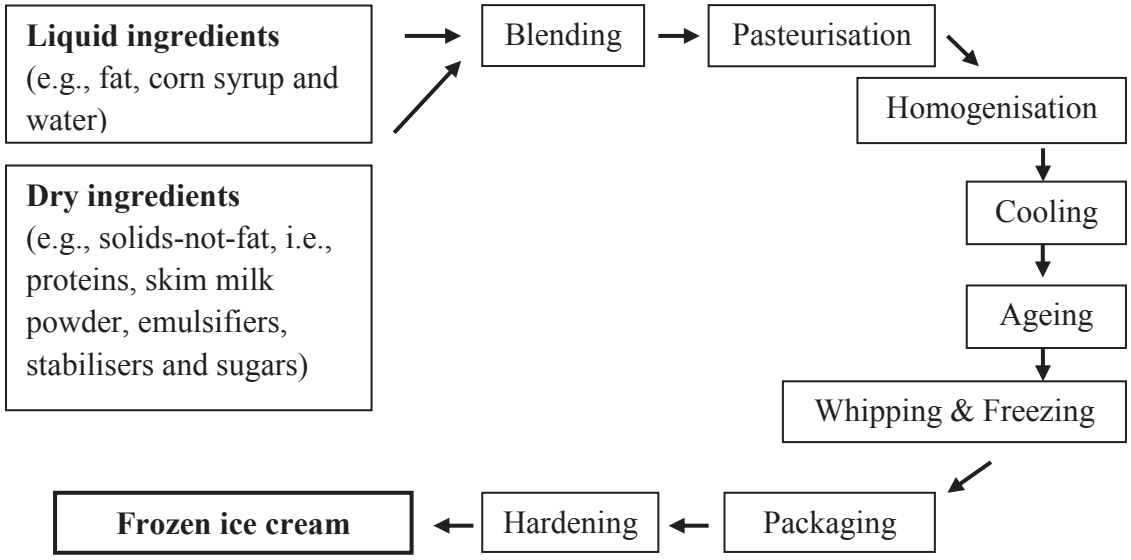

Figure 2.3 Schematic process flow diagram of manufacturing ice cream. Adapted from Goff (2002, 2007).

2.9 Major unit operations in ice cream manufacturing processes

In ice cream or similar frozen dessert production, homogenisation, ageing and freezing are the main processing steps that determine the quality and properties of finish frozen products.

2.9.1 Homogenisation

In ice cream making process, homogenisation is a process by which an ice cream premix is forced by pressures through a very small orifice in the homogeniser to breakdown bulk oil in small droplets and/or reduce the size of existing fat globules from milk or cream formulated in the ice cream mix (Marshall et al., 2003, Wilbey, 2003). The size of oil droplets is normally reduced to smaller than 2 μm in diameter, which makes an ice cream mix emulsion uniform and stable without phase separation (Marshall et al., 2003). As the fat droplets reduce in their size, the surface active materials available in the ice cream mix such as proteins and small molecule surfactants tend to competitively adsorb at the newly formed droplet surface to reduce the interfacial tension at the oil-water interface (Dickinson and Tanai, 1992, Marshall et al., 2003).

Homogenisation pressure has a significant influence on the number, size and particle size distribution of fat globules formed in oil-in-water emulsions. In ice cream making, it is ideal to have the large number of small fat droplets be formed by homogenisation in order to enable its contribution to the body and structure of ice cream through their partial coalescence (Walstra and Jonkman, 1998). As a result of homogenisation, the homogenised small fat droplets increase their ability to cover air bubbles and crystals from 6 m^2 to 150 m^2 per litre at a later stage of the ice cream making process (Walstra and Jonkman, 1998). According to the Kolmogorov's theory of isotropic turbulence, during homogenisation, large molecules, such as proteins, are more preferably adsorbed to the newly created fat droplets than small molecule surfactants added in the ice cream formulations (Gelin et al., 1994).

Currently, the homogenisation pressure up to 350 MPa (3,500 bar) can be applied using a high-pressure homogeniser in order to prepare emulsions (Floury et al., 2000). In general, an increase in homogenisation pressure decreases the size of fat globules being formed in the homogeniser (Floury et al., 2000). The homogenisation pressures normally used for the ice cream mix containing 10-14 wt% fat are 130-180 bar and 34 bar for the 1st and 2nd stage of homogenisation, respectively (Marshall et al., 2003).

Koxholt et al. (2001) showed the homogenising pressures at 100/20 bar ($1^{st}/2^{nd}$ stage) were high enough to prepare a stable ice cream containing 10 wt% fat with acceptable meltdown properties. Innocente et al. (2009) also showed the effect of different homogenisation pressures ($1^{st}/2^{nd}$ stage) at 970/30 bar and 150/30 bar on the properties of ice creams containing 5-8 wt% fat. The higher pressure resulted in a change in the particle size distribution of oil droplets in the ice cream mix from bimodal to monomodal with smaller droplet diameter, which in turn led to an increase in viscosity and more pronounced solid-like ice cream.

2.9.2 Ageing

Ageing is one of the crucial steps in an ice cream production. It is a process by which a homogenised ice cream mix is kept undisturbed at about 4°C for at least 4 hours or a longer time, normally 24 hours (Marshall et al., 2003). During the ageing step, proteins and other hydrophilic colloidal materials become fully hydrated fat becomes crystallised and the rearrangement of oil droplet membranes occurs (Marshall et al., 2003). The fully hydrated proteins and stabilisers lead to a marked increase in the viscosity of the aged mix after at least 4 hour ageing (Marshall et al., 2003, Minhas et al., 2000a). The minimum ageing time for at least 4 hours is required to ensure that the fat droplets in ice cream mix containing 10 wt% fat almost become crystallised because the homogenised fat droplets undergo crystallisation at slower rates than the droplets in the state before homogenisation (Marshall et al., 2003).

Another important phenomenon occurring in the ice cream mix during the ageing is that in this quiescent step, small molecule surfactants become more active than proteins and migrate from the serum phase to the oil-water interface, displacing a substantial amount of adsorbed proteins at the fat droplet surface (Bolliger et al., 2000a, Davies et al., 2000, Gelin et al., 1994). This phenomenon makes the resulting membrane thinner and more fragile which in turn facilitates the formation of partially coalesced fat droplets during the subsequent treatment of the aged ice cream mix with whipping and freezing. It was reported that ageing of ice cream mix did not change the droplet size distribution of particles in aged ice cream mix (measured in terms of volume surface mean diameter)

(Gelin et al., 1994), indicating the droplet aggregation and coalescence do not take place during ageing. The properly aged mix provides the resulting ice cream with an optimal extent of fat destabilisation (partial coalescence) which is important for the proper melting rate and shape retention of ice creams (Marshall et al., 2003).

2.9.3 Whipping and freezing

At a temperature above the melting point of fat, fat droplets are in a liquid form. When an oil-in-water emulsion in the aged ice cream mix is subjected to shear forces introduced by agitation during churning (whipping) and freezing stage, the fat globule membrane of two colliding droplets is disrupted by the penetration of fat crystals from one partly crystalline fat droplet into approaching another fat droplet (Fredrick et al., 2010, Vanapalli and Coupland, 2001). This leads to the joining of two droplets through the protruding fat crystals piecing into neighbouring droplet interface which are partly enclosed by some liquid fat flowing to the fat crystals from the droplets, resulting in the partially coalesced droplets (Fredrick et al., 2010, Vanapalli and Coupland, 2001). In an ice cream making freezer, the concurrent churning and freezing are introduced to ice cream mixes. During freezing, temperature of the system employed is lower than the melting temperature of fat used, in this case, fat droplets that start crystallisation from the ageing step become more crystallised in the freezer (Eisner et al., 2005).

During the freezing and whipping of ice cream mix at this low temperature range about -28°C, full coalescence (true coalescence) of fat droplets by their collision is obstructed by the presence of a network of fat crystals inside both of the colliding droplets (Davies et al., 2000). As a result, the collision of crystallised droplets instead leads to the formation of irregularly aggregated droplets or partial coalescence of fat droplets throughout the whole ice cream structure (Goff, 1997b). It is worthy to note that for partially coalesced droplets, the identity of individual droplets is still retained in the aggregates (Davies et al., 2000, Goff, 1997b). These aggregated droplets are reportedly important to stabilise air cells generated via whipping in ice creams by adsorbing and building up a network structure (indicated by an arrow) at the surface of air cells as

illustrated in Figure 2.4 (Eisner et al., 2005). In the droplets, the size of fat crystals is governed by the crystal form of fat, e.g., β form leads to granular crystals and β′ form leads to smaller crystals when fat becomes crystallised (Harada and Yokomizo, 2000, Kawamura, 1979, 1980).

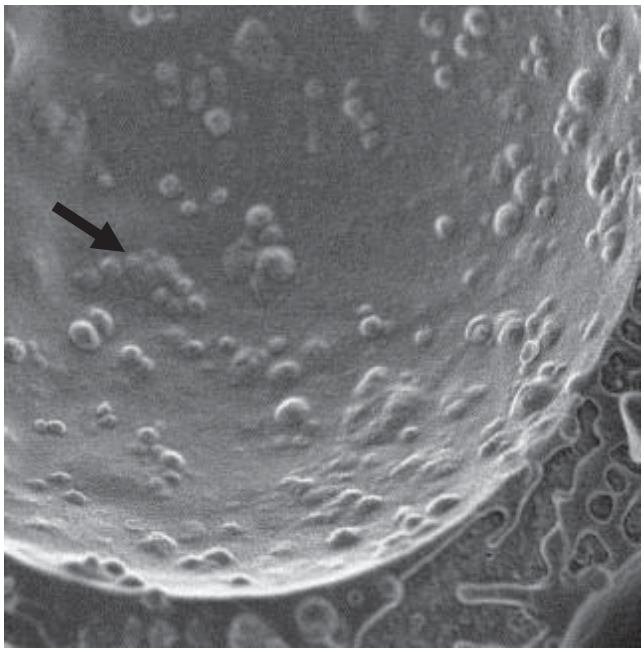

Figure 2.4 Micrograph obtained from the low-temperature scanning electron microscopy (LT-SEM) of an air cell in ice cream with fat droplets adhered at the surface. Source: Eisner et al. (2005).

During the freezing stage, air is incorporated into the ice cream structure by direct entrainment driven by scraper blades of the manual ice cream freezer or direct injection through orifices into ice cream in the continuous ice cream freezer (Chang and Hartel, 2002, Hartel, 1996). In normal standard ice creams, air cells account for 50% of the whole volume of ice cream (Thomas, 1981, Walstra and Jonkman, 1998). Chang and Hartel (2002) demonstrated in their study that the size of air cells decreased as the churning time of ice cream mix in the ice cream freezer increased but the size remained stable without changing after a certain time. From their study, the size of air cells progressively decreased from the first 1 minute to about 10 minutes before ice cream was drawn from the freezer and remained constant until 22 minutes of drawing time. They also reported no dependency of air cell size on the fat level and small molecule

surfactant content but instead the size of air cells depended largely on the content of stabiliser which was attributed to its effect for an increase in the apparent viscosity of the mix as it was frozen. Eisner et al. (2005) underlined the importance of ice cream mix viscosity for its influence on air cell size by demonstrating a substantial reduction in the size of air cells in the ice cream with a high viscous matrix.

2.10 Roles of ingredients in ice cream

2.10.1 Fats and fat blends

Fats are the main constituent element of partial coalescence and contribute to the formation of ice cream structure via the clusters of crystalline fat droplets (Walstra and Jonkman, 1998). They become partially coalesced with an aid of two other elements that usually contribute to the oil-water interface of emulsions, including amphiphilic macromolecules which are predominantly proteins and low molecular weight surfactants such as lecithin and mono- and diglycerides (Burgaud et al., 1990, Floury et al., 2000, Walstra, 1983). Although fat droplets are one of the most essential elements for partial coalescence, however, not all fat droplets are partially coalesced in ice cream (Walstra and Jonkman, 1998).

Small fat droplets are obtained from a homogenisation process of crude fats. Although dairy fats are normally used in ice cream making, vegetable fats, such as fractionated vegetable fat of lauric oil (e.g. coconut oil and palm kernel oil) and non-lauric oil origins, can be utilised as ingredients for creating partial coalescence (Benjamins et al., 2009). Fats from plant origins may be used as frozen dessert ingredients, such as coconut oil, flaxseed oil, palm and palm kernel oil (Goh et al., 2006, Granger et al., 2005b, Granger et al., 2005c, Liew et al., 2001b, Soler, 2005, Thaiudom, 2005).

Fats and oils have different melting properties with a broad range of melting temperatures according to their solid fat contents at the particular temperatures because of their composition of a number of different triglyceride molecules. For example, fractionated vegetable fat from lauric acid oil origins, for example, coconut oil, has the

solid fat content in the range between 64% and 76% at 20^{o}C, whereas refined winterised sunflower oil contains 0% solid fat content at the temperature range between 20^{o}C and 35^{o}C (Benjamins et al., 2009). Fats become crystallised as temperature decreases below their melting points. The crystallisation onset temperature of fats decreases when fats are emulsified or mixed with other emulsions (Vanapalli and Coupland, 2001).

A difference in the fatty acid composition and arrangement in lipids also results in assorted fat droplet structures, sensory properties and flavour perception of colloidal food emulsion systems (Nazaruddin et al., 2008, Relkin et al., 2004). Such a difference is also responsible for the difference in the stability and destabilisation of fat droplets which consequently affects the melting behaviour of fats (Nazaruddin et al., 2008, Relkin et al., 2004). In addition, the degree of saturated or unsaturated of fatty acids and their relative composition in fats and oils contribute significantly to the properties of the microscopic structures of frozen products containing them (Sung and Goff, 2010).

The amount of fat droplets in emulsions also determines the magnitude of partial coalescence. Partial coalescence rises as the volume fraction of fat droplets in the dispersed phase increases (Vanapalli and Coupland, 2001). Fat droplets undergo partial coalescence when they are in a semi-solid droplet state. In ice cream making, fat droplets are partially crystallised at low temperature about 4-5°C while ageing and the partially crystallised fat droplets become partially coalesced by shear forces and air incorporation concurrently applied during freezing (Vanapalli and Coupland, 2001). Afterwards, partially coalesced fats are completely crystallised during hardening in a cold room (Dalgleish, 2006). Therefore, fat droplets should not be fully crystalline before partial coalescence takes place (Dalgleish, 2006). In addition, emulsified fat droplets could undergo partial coalescence when emulsions are subjected to alternatively cool and reheat treatments (Vanapalli and Coupland, 2001).

The proportion of solid fat in fat droplets also influences the shear stability of emulsions (Walstra, 1987). If the percentage of solid fat in fat droplets is high, the partial coalescence of droplets is not affected by the applied shear forces. Likewise, a low proportion of solid fat in fat droplets also leads to the inhibition of partial coalescence. A high rate of partially coalesced fat droplets usually occurs when the solid fat content (SFC) in oil droplets is between 10 and 50% (Davies et al., 2000, Marshall et al., 2003).

It is thus important to consider the content of solid fats in a system in order to obtain the desired structure and properties of foods through the establishment and optimisation of the appropriate level of SFC for the particular application (Vereecken et al., 2009). The SFC becomes lower when fats contain a lesser amount of saturated fatty acids. The physiochemical characteristics of an oil can be altered by hydrogenation or refining process and also by blending oil with other oils (Idris and Dian, 2005, Noor Lida et al., 2002, Young, 1983). The SFC of hydrogenated and refined coconut oil is 91.6% and 51.3% at 5^{o}C and 86.4% and 36.3% at 20^{o}C, respectively (Granger et al., 2005a). On the other hand, the SFC of dairy cream at 5°C, 20°C and 35°C is 47%, 13% and 0%, respectively (Dufour and Riaublanc, 1997).

Blending the different proportions of triglyceride oils can change the SFC of oil blends (Vereecken et al., 2009). The more oil with unsaturated fatty acids is mixed with oil with saturated fatty acids, the more is the reduction in the solid fat content of the blend (Mat Dian et al., 2006, Noor Lida et al., 2002). The change in the composition of fat corresponds to the change in the properties of products containing new fat blends. For example, the waxy mouth feel effect of monostearin that has a melting point at 81.5 °C could be reduced by blending with vegetable oil or other low melting oils (Vereecken et al., 2009). Therefore, blending of fats with liquid oils could make the correct solid fat content for making a right level of partial coalescence. Blending a highly saturated fatty acid oil like palm oil with a highly unsaturated fatty oil like sunflower oil was shown to contribute to the spreadability of a reduced fat spread at low temperature (Lida and Ali, 1998).

The fat blend added into ice cream formulations has been shown to affect the properties of ice creams (Goh et al., 2006, Liew et al., 2001b, Méndez-Velasco and Goff, 2011, 2012a, b). For instance, Liew et al. (2001b) showed the effects of fat blend between anhydrous milk fat and palm kernel oil on the difference in the viscoelasticity of aged ice cream mixes and ice cream emulsions. In a recent study reported by Méndez-Velasco and Goff (2012b), the properties of ice cream made from fat blend of palm kernel oil and sunflower oil had the highly aggregated structure and melting resistance when the ice cream was produced with intermediate ratios of blends.

2.10.2 Proteins

Some proteins such as milk proteins are amphiphilic macromolecules (Burgaud et al., 1990, Floury et al., 2000, Walstra, 1983). They are added in ice creams for aiding emulsifying activity, increasing emulsion viscosity and raising emulsion stability (Goff, 1997b). When proteins are present in a preparation of emulsions, they form thick absorption layers around fat droplets during homogenisation, which help stabilising emulsions by lowering the interfacial tension of the oil and water interface and forming a film around fat droplets, hence providing the droplets with stability and preventing them from coalescence (van Aken, 2003, Walstra, 1983).

The stability of emulsions increases when the mean droplet size becomes smaller and the amount of protein adsorption at the interface increases (Goff, 1997b, Williams and Dickinson, 1995). Therefore, a sufficient amount of proteins is essential for creating stable oil-in-water emulsions before partial coalescence occurs (Benjamins et al., 2009, van Aken, 2004, van Aken and Zoet, 2000) even though adsorbed proteins are later displaced by small molecule surfactants added in ice cream mix and released into the serum phase. The displacement of adsorbed proteins by small molecule surfactant promotes partial coalescence of fat droplets in ice cream (Goff, 1997b, Goff and Jordan, 1989).

The protein content at approximately 4% is usually present in ice cream mix emulsions (Vega and Goff, 2005). Marshall (2002) stated that in a normal ice cream containing 10% milk fat and 10% milk solids non-fat (SNF), the SNF of about 3% is derived from proteins in the mixture of ice cream formulation. As described earlier, the displacement of proteins at droplet surfaces by added small molecule surfactants occurs in an ice cream mix during the ageing process. Even after its displacement, a small amount of proteins still remain at the fat droplet surface. In the unfrozen serum phase of aged ice cream mix, the discharged proteins play their new role as a part of solids-not-fat components which contributes to the increased viscosity of ice cream mix (Segall and Goff, 2002).

Although proteins used to formulate ice creams are derived from dairy sources (Vega and Goff, 2005), vegetable proteins such as wheat protein isolate can be used to produce an acceptable ice cream (Ahmedna et al., 1999). Vegetable proteins are produced from a variety of plants, including leguminous seeds such as lupine, pea, soybean, wheat and coconut. These proteins and their isolates could be used to form a gel network and stabilise fat droplets in several oil-in-water emulsion products (Franco et al., 2000, Nunes et al., 2003, Onsaard et al., 2005, Onsaard et al., 2006, Puppo et al., 2003, Puppo et al., 2008).

2.10.3 Small molecule surfactants

Small molecule surfactants are the substances used to lower the interfacial tension and stabilise fat droplets (Dickinson and Tanai, 1992, Hasenhuettl, 2008a). Some small molecule surfactants are added into ice cream formulations mainly to partly destabilise (demulsify) protein-stabilised fat droplets and promote air cell entrainment at a later point when the ice cream mix emulsion is processed into ice creams (Davies et al., 2001, Marshall et al., 2003, Segall and Goff, 2002). The amount and nature of small molecule surfactants determine the magnitude of demulsification (partial destabilisation) of fat droplets (Granger et al., 2004a). As the amount of surfactants increases, the more increase in protein desorption which in turn favours an increase in fat droplet destabilisation for partial coalescence in ice cream emulsions (Granger et al., 2004a).

Two main types of small molecule surfactants are usually used in ice cream making, including water-soluble surfactants such as sorbitan esters (Tween or polysorbates) and oil-soluble surfactants such as mono- and diglycerides (Dickinson and Tanai, 1992, Marshall et al., 2003). Mono- and diglycerides (MDG) are obtained from the partial hydrolysis of fats derived from animals or vegetables (Marshall et al., 2003). MDG usually has the hydrophilic and lipophilic balance (HLB) values less than 5, which makes MDG well solubilise in the oil phase (Stauffer, 1999) or be better used to stabilise water-in-oil emulsions (Dickinson et al., 1993). The structure of MDG is composed of a glycerol molecule which is esterified with a fatty acid molecule. The two free hydroxyl groups in monoglycerides make them more surface active than diglycerides (Hasenhuettl, 2008b). Several types of mono- and diglycerides used in ice cream formulations such as glycerol monostearate (GMS) and glycerol monooleate (GMO), each of which has a HLB value at 3.8 and 2.8, respectively (Goff and Jordan, 1989). The most common use of water soluble surfactants, such as polyoxyethylene sorbitan esters, in ice cream is polyoxyethylene (20) sorbitan monooletae (also known as Tween 80 or polysorbate 80) (Goff and Jordan, 1989, Marshall et al., 2003). This surfactant has the HLB value of 15 which makes it a good oil-in-water surfactant (Goff and Jordan, 1989, Stauffer, 1999). The structure of sorbitan esters is composed of a fatty acid molecule, such as stearate and oleate, esterified to a sorbitol molecule in which polyoxyethylene groups are attached to it. For Tween 80, oleic acid is attached to the sorbitol molecule (Hasenhuettl, 2008b).

These two different species of water and lipid soluble surfactants work differently in ice cream, the water-soluble surfactants are involved at the oil-water interface, leading to the displacement of substantial amount of proteins which promotes the formation of partially coalesced fat network (Dickinson and Tanai, 1992). The lipid-soluble surfactants adsorb at the oil phase as well as the air-water interface which helps promoting the adsorption of fat to air cells and air incorporation of ice cream (Marshall et al., 2003, Zhang and Goff, 2005). The suggested amount of both surfactants in ice cream formulation is at 0.02-0.04 wt% and 0.1-0.2 wt% of water-soluble and liquid-soluble surfactants, respectively (Marshall et al., 2003).

In ice cream mix, small molecule surfactants do not play a role as a dominant emulsifying agent in the formation of an ice cream mix emulsion (Walstra and Jonkman, 1998). This means that before ageing, the small molecule surfactants added are not directly providing stability to droplets in emulsions. During the ageing step at 4^{o}C, those surfactants however move from a liquid phase to the oil-water interface and cause the reduction in the surface proteins of protein-stabilised fat droplets, resulting in a weak thinner membrane formed on the droplets which is easier to become partially coalesced (Segall and Goff, 2002). The reason for the water-soluble surfactants to better adsorb to and displace proteins at the interfacial layer of oil droplets than the oil-soluble surfactants (Euston et al., 1995, Pelan et al., 1997b) can be due to the difference in their HLB values as described above (Hait and Moulik, 2001, Krog, 1977). Tween 80 adsorbs at the interface in the form of expanded monolayers around the droplet interface whereas MDG and PUMDG tend to form loose monolayers at the oil phase of the interface (Krog, 1977).

It should be mentioned that the presence of lipid-soluble small molecule surfactants such as mono- and diglycerides is also required to destabilise ice cream emulsions during ageing and freezing steps, leading to a greater partial coalescence of fat droplets (Davies et al., 2000, Goff and Jordan, 1989). In other words, lipid-soluble surfactants increases the crystallisation rate of homogenised fat droplets (Marshall et al., 2003). Therefore, lipid-soluble surfactants aid partial coalescence of fat droplets and at the same time induce displacement of some proteins from the fat droplet surface and air interface (Goff, 2008) although its ability to displace the adsorbed proteins from the interface is lower than that of water-soluble surfactant (e.g. Tween 80) as described above. The synergistic effect has been shown in ice cream mixes when both species of surfactants presented (Goff, 1997a). It was shown that the substantial amount of proteins was displaced from the droplet surface as the oil-soluble species was used together with water-soluble surfactants in 20 wt% soya oil-in-water emulsions (Dickinson and Tanai, 1992).

The degree of saturation of surfactants also affects partial coalescence and protein displacement as well as fat crystal behaviour within the fat droplets (Davies et al., 2000, 2001). Granger et al. (2003) demonstrated that the nature of fat and surfactants caused the different properties of oil-in-water emulsions in the ice cream mixes and the resulting frozen ice creams. Their further study pointed out that the presence of unsaturated fatty acids in the partially unsaturated mono- and diglyceride mixtures led to greater fat agglomeration than the saturated mono- and diglyceride mixtures, resulting in a greater extent of an increase in the particle size diameter greater than 2 μm in ice creams containing coconut oil (Granger et al., 2004a, Granger et al., 2005c).

2.11 Role of other ingredients on the properties of ice cream

Another important part in the structure of ice creams is the unfrozen aqueous phase or serum phase in which sugars, proteins, stabilisers and polysaccharides are solubilised, dispersed and concentrated (Dalgleish, 2006, Goff, 2002). These components also affect the stability and partial coalescence of ice cream emulsions (Dalgleish, 2006).

2.11.1 Stabilisers

Stabilisers which are a group of water-soluble polysaccharides or hydrocolloids are normally used in ice cream formulation, including sodium alginate, carrageenans (e.g. κ-carrageenan), locust bean gum (LBG), guar gum, xanthan gum, pectin, carboxymethylcellulose (CMC) and gelatine (Marshall et al., 2003). The proper use of stabilisers in ice cream making primarily is to provide an ice cream mix with increased viscosity and enhance the properties of frozen ice creams with smoothness, small ice crystals, product uniformity and melting resistance (Goff, 1997a). In addition, stabilisers can mask the perception of large ice crystals and help lubricating the ice crystals (Marshall et al., 2003). They can also prevent shrinkage and retard the moisture migration in ice cream (Clarke, 2004). Stabilisers should be used with a proper amount to avoid too excessive viscosity of the matrix and the heavy and soggy texture (Marshall et al., 2003).

A small amount of stabilisers about 2% by weight can elevate the matrix viscosity, reduce the melting down rate which is the rate at which frozen ice creams and desserts lose their mass as they melt, and decrease the number of ice crystals in finished frozen products (Buyong and Fennema, 1988, Clarke, 2004). It was shown that no serum separation of whipped dairy cream emulsions occurred as the mixtures of locust bean gum and λ-carrageenan were employed (Camacho et al., 1999). Minhas et al. (2002) showed that some plant origin stabilisers, such as sodium alginate, karaya, guar gum, acacia gum and ghatti gum, affected the viscosity, flow behaviour index (n) and consistency coefficient (m) of the ice cream mixes made from buffalo milk. All mixes with those exhibited a pseudoplastic fluid with specific m values in the range between 1.17 and 0.36 and showed n values less than 1.

LBG, also known as carob bean gum, is a galactomannan polysaccharide comprising of a mannose backbone and galactose side chains (Marshall et al., 2003, Sworn, 2004). It was shown to be able to form a gel-like network around ice crystals after freezing a model solution made from sucrose with or without skim milk powder (Goff et al., 1999a). The similar gel network could be formed by carrageenan and gelatine in the presence of proteins, but such a gel network like network formed by LBG was not observed from guar gum, another type of a galactomannan stabiliser (Goff et al., 1999a). Besides, carrageenan added to sucrose solutions could decrease the extent of protein phase separation, but increase the recrystallisation rate which was not induced when LBG was used (Goff et al., 1999a, Regand and Goff, 2003). The ability of delaying recrystallisation in frozen desserts depends on molecular interactions between polysaccharide stabilisers and proteins (Regand and Goff, 2003). The texture of ice cream added with LBG is smooth with a slow meltdown rate. LBG can also prevent accretion of ice crystals, that is the physical contact of two ice crystals in an unfrozen phase, because of its gel-like properties (Donhowe and Hartel, 1996).

Guar gum as a polysaccharide thickener is obtained from the endosperm of the legume *Cyamopsis tetragonolobus* L. (Sworn, 2004). It is a cheap polysaccharide that is usually used in a combination with LBG in ice creams. Guar gum forms a

pseudoplastic gel with shear thinning behaviour in cold water which can provide body to ice cream (Sharma and Hissaria, 2009). Guar gum is more soluble at cold temperatures than LBG which is fully hydrated at the temperature about 80°C (Sharma and Hissaria, 2009).

2.11.2 Sweeteners

A number of sweeteners are present and used in ice cream formulations such as lactose, dextrose, fructose, corn syrup and sucrose (Bhandari, 2001). Sucrose is one of the most commonly used sweeteners in frozen dessert formulations (Fernández et al., 2007). Sugars are added in the range between 13 and 16% on the sucrose basis in frozen desserts for the enhancement of their sweetness, palatability, smooth body and texture and they are dissolved in the serum phase of frozen desserts (Bhandari, 2001). Sugars have a direct effect on the freezing point depression of the ice cream mix (Bhandari, 2001). They are also main sources of solids in frozen desserts and are responsible for crystallisation in the frozen products.

When dairy products are used, excessive lactose content leads to lactose crystallisation and sandiness in most frozen desserts. As sucrose is used, sucrose crystallisation can also occur. Sucrose crystallisation results in a defect known as white spot which is an incidence when a high sucrose content is used in frozen products like water ices (Clarke, 2004). These effects can be reduced by using other sugars such as corn syrup instead of lactose and sucrose (Clarke, 2004).

2.12 Coconut milk ice cream

In general, cows' milk and its products, i.e., dairy cream, are used as the main source of fats and proteins and ingredients for making ice creams, but fats and proteins from different sources, such as plants, can also be used. Fats and oils from plants can be obtained from plant seeds, germs and husk or directly extracted from a solid endosperm of particular plant fruits, including coconut and palm tree. In recent years, various vegetable fats and oils are gaining greater interest to substitute all or some part of

bovine milk fats. These include rice bran oil, flaxseed oil, palm oil, palm kernel oil and coconut oil (Goh et al., 2006, Granger et al., 2005c, Hyvönen et al., 2003, Kailasapathy and Sellepan, 1998, Liew et al., 2001b, Nazaruddin et al., 2008, Soler, 2005). However, most studies have still been using bovine milk or related milk products, such as skim milk powder, whey proteins and sodium caseinates, as raw materials to make and enhance some properties of ice creams.

By definition based on the standard identity of ice cream, coconut milk ice cream is referred to as a frozen dessert formulated with coconut milk as it is not based on dairy milk (Food and Drug Administration Thailand, 2001). Coconut milk ice cream is one of the favourite frozen desserts in Thailand and some other tropical Asian countries (Surapat and Rugthavon, 2003). This frozen dessert is made predominantly from fresh coconut milk and/or coconut milk products, sweeteners, small molecule surfactants and stabilisers. Its original formula contains vegetable oil but has no milk fat, milk proteins or lactose. However, several recipes available currently are adapted by adding some dairy milk products for the enhancement of texture and flavour qualities. Although there are several formulations for making coconut milk ice creams, the processing methods are the same as the conventional ice cream manufacturing process used in dairy ice cream (Surapat and Rugthavon, 2003).

Soler (2005) developed a formula of a non-dairy frozen dessert comprising of coconut milk and soy protein isolate and investigated the sensory characteristics on the consumer acceptance and purchase intention. Thaiudom (2005) has compared the rheological properties between frozen dessert mixes containing coconut milk and anhydrous milk fat. The mix containing coconut milk showed less increase in the apparent viscosity (Pa.s), resulting from the low rate of partial coalescence, than the mix composed of anhydrous milk fat. However, both mixes exhibited the non-Newtonian flow behaviour with time dependent shear thinning (Thaiudom, 2005). In addition, several imperfections associated with coconut milk ice creams have been reported including coarse texture with large ice crystals and a little coconut flavour in the finished product (Thaiudom, 2005). Those defects may be caused by several factors

such as an improper mix viscosity, inappropriate formation and growth of ice crystal nuclei during freezing, the failure of the aeration process and recrystallisation during storage or transportation (Thaiudom, 2005).

Although some research on the coconut milk emulsions and ice creams has been shown in the literature (Soler, 2005, Thaiudom, 2005), there is a lack of knowledge on the emulsion and ice cream systems made from coconut milk. The research on the utilisation of coconut milk and its fractions as a raw material in making emulsions and ice creams has not been well studied. The scientific knowledge on the stability and properties of coconut milk emulsions which is fundamental to the understanding of the formation and properties of coconut milk-based ice cream is scarce.

2.13 Partial coalescence of emulsion fat droplets in ice cream

The development of structure in ice cream can be divided into two stages, in the mix production step and during the concurrent steps of freezing and aeration (whipping) (Goff, 1997b, Goff, 2002). During the mix preparation step, bulk fat or large fat globules of milk or cream added as ingredients become a small size of fat droplets by homogenisation which are then stabilised by proteins in the ice cream mix by homogenisation of the heated mix. Ageing allows time for small fat droplets to undergo crystallisation as well as the formation of a new interfacial membrane by displacement of proteins from the interface by small molecule surfactants. During the freezing and aeration, the semi-liquid crystalline fat droplets are partially coalesced into a continuous network and spread throughout the whole product (Segall and Goff, 2002).

It is generally referred that a process called "partial coalescence" is responsible for the formation of a three-dimensional network in ice cream and other related products such as whipped cream (Dalgleish, 2006, Davies et al., 2000, 2001, Goff, 1997b, Méndez-Velasco and Goff, 2012a, b). Partial coalescence plays an important role in the structure formation in many frozen products. The clusters of partially coalesced fat droplets are usually introduced into several aerated frozen products, such as ice cream

and whipping cream, for the enhancement of textural and sensorial properties such as stiffness, dryness, melt resistance, smoother texture, fattiness and creaminess (Goff, 2000, Granger et al., 2004a). The fat clusters are also crucial for the stability of air cells in ice cream for the smooth texture of frozen products (Benjamins et al., 2009, Bolliger et al., 2000a, Goff, 1997b, Goff, 2002).

Fat droplets in ice cream exist in two different forms, such as discrete fat droplets and partially coalesced crystalline fat clusters (Clarke, 2004). Partial coalescence is a process occurring in oil-in-water emulsions comprising of tiny fat droplets in which a network of crystalline fats is present (Goff, 1997b, Walstra and Jonkman, 1998). This process can be divided into three stages, including crystallisation of droplets, contact between droplets and mixing of oil around the contact point through the protruding fat crystals (Benjamins et al., 2009, Goff, 1997b, Vanapalli and Coupland, 2001). Partial coalescence begins with the small homogenised fat droplets stabilised by proteins in the mix that are partly crystallised by low temperature during the ageing step. Simultaneously, small molecule surfactants added in the mix displace some of proteins from the fat droplet interface (Dickinson and Tanai, 1992, Zhang and Goff, 2005). This makes the newly formed fat droplet membrane (surfactant membrane) prone to destabilisation (de-emulsification) which is needed for a partial coalescence process (Goff, 1997b).

Partial coalescence is facilitated by a shear force employed during the freezing process to induce the contact between semi-liquid crystalline fat droplets to share some parts together. The shear forces generated in an ice cream machine by agitation during freezing and churning process leads to the direct collision between a semi-solid droplet to another semi-solid droplet (Boode and Walstra, 1993a, Vanapalli and Coupland, 2001). During shearing while freezing, some fat crystals in the semi-solid fat droplets protrude out of the fat droplet surface and pierce the thin film of closely approaching droplets, thereby leading to the connection between fat droplets by the protruding crystals (Walstra and Jonkman, 1998). However, due to the presence of a network of fat crystals in the collided droplets, this limits the mobility of the droplets and obstructs the

complete coalescence of those droplets into a single big droplet (Dalgleish, 2006). As a result, irregular fat clusters are created and later form the three-dimensional continuous structure in ice cream (Figure 2.5) (Goff, 1997b, Walstra and Jonkman, 1998).

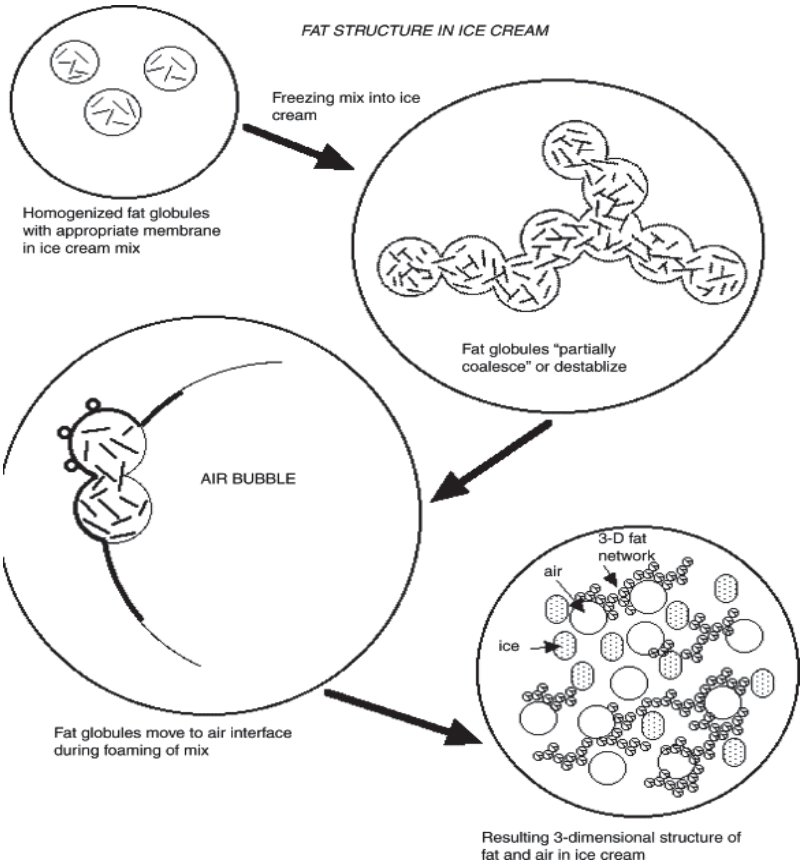

Figure 2.5 Schematic representation of the development of partially coalesced fat droplets in ice cream. Source: Eisner et al. (2005).

When fat crystals in a semi-crystalline fat droplet penetrate into another semi-crystalline fat droplet, the liquid oil in the droplets will flow and wet the fat crystals in the collided droplets aiding the linkage between the two droplets, resulting in the partially coalesced fat droplets (Benjamins et al., 2009, Clarke, 2004, Vanapalli and Coupland, 2001, Walstra and Jonkman, 1998). When the ice cream mix with partially coalesced fat clumps is continuously frozen and aerated in an ice cream machine freezer, the partially coalesced fats become a continuous fat crystal network in the aqueous phase of a frozen ice cream throughout the whole product (Benjamins et al., 2009, Goff, 1997b, Walstra and Jonkman, 1998). The aggregated crystalline fat globules could be determined by

the presence of particles with diameter larger than the initial size of droplets (Granger et al., 2005c).

In addition, air incorporation or whipping during freezing enhances the formation of fat clumps (Goff et al., 1995) which means that the high rate of partial coalescence occurs when air is incorporated during the freezing and whipping process of ice cream mix (Goff, 1997b). Although the mixing of air, while shear force is applied during freezing, into the ice cream mix emulsions increases the rate of partial coalescence, however, the partial coalescence may still occur without air via a calcium bridging mechanism (Agboola and Dalgleish, 1995) if a certain amount of calcium is present and the adsorbed proteins at the interface are sensitive to interact with calcium ions. Two mechanisms, i.e., perikinetic (Brownian motion) or orthokinetic (shear-induced) mechanism, have been reported to be an effective means for the partial coalescence in emulsions (Goff, 1997b). Partial coalescence of fat clumps can retain their structure unless the crystals are melted (Dalgleish, 2006).

The degree of agglomeration or partial coalescence of fat droplets is significantly influenced by many other factors including ingredients (Benjamins et al., 2009, Bolliger et al., 2000a, Goff, 1997b, van Aken et al., 2003, Vanapalli and Coupland, 2001) such as nature, type and concentration of fat, and emulsifiers and surfactants (proteins and low molecular weight emulsifiers) (Benjamins et al., 2009, Boode et al., 1993, Granger et al., 2003, Granger et al., 2005c, Herrera, 2002, Palanuwech and Coupland, 2003, Segall and Goff, 1999, van Aken et al., 2003, Walstra and Jonkman, 1998); amount, size and shape of crystallised fat (Davies et al., 2001); the orientation of crystals at the interface (Granger et al., 2005c); and manufacturing parameters, such as preheating temperature prior to homogenisation and/or pasteurisation, homogenisation pressure, velocity gradients, ageing time, shear rate during agitating and freezing, air incorporation and freezing conditions, such as time, temperature and method (Benjamins et al., 2009, Bolliger et al., 2000b, Goff, 1997b, Koxholt et al., 2001, van Boekel and Walstra, 1981a, c, Walstra and Jonkman, 1998). In addition, the type and concentration of stabilisers contribution to the viscosity and properties of ice cream mix

also affect the partial coalescence of fat droplets (Granger et al., 2005c, Minhas et al., 2000b).

Due to the fact that the partial coalescence involves the protruding of fat crystals into another droplets, besides the effects of the number, dimension and arrangement of crystals at the interface as mentioned above, the ratio of solid to liquid fat content in fat globules is another very important factor that responds directly to the degree of fat crystal piercing and partial coalescence (Benjamins et al., 2009, Boode et al., 1993, Herrera, 2002, Palanuwech and Coupland, 2003, van Aken et al., 2003). The more fat crystals being wetted by the liquid fat or other aqueous phase do not impair the stability of emulsions but promote the partial coalescence (Benjamins et al., 2009, Boode and Walstra, 1993b).

2.14 Characterisation of emulsions and ice creams

The rheological and textural characteristics of emulsions or ice creams containing partially coalesced fats vary depending on some factors, such as ingredients including fats, proteins, emulsifiers, stabilisers and sweetener and various process parameters including ageing time, freezing temperature and dasher speed (El-Nagar et al., 2002, Minhas et al., 2000b, Minhas et al., 2002). Emulsions containing partially coalesced fats differ from those with native or stabilised fat droplets. Fat clumps in emulsions generally exhibit a broader droplet size distribution than stabilised fat droplets containing individual discrete droplets, hence increasing the viscosity of emulsions (Benjamins et al., 2009, Goff, 1997b). Emulsions containing fat clusters may show a yielding behaviour or the increase in the volume fraction occupied by the clusters of fat droplets (Benjamins et al., 2009). The viscosity of emulsions rises when there is an increase in the content of crystalline fats.

Partial coalescence is a process contributing to the formation of a three-dimensional fat network in aerated frozen products which is important for stabilising air cells in ice cream (Segall and Goff, 1999, Zhang and Goff, 2005). In ice cream, this process leads

to substantial changes in ice cream characteristics such as an increase in viscosity and overrun of ice cream (Benjamins et al., 2009, Goff, 1997b). Several measurements were carried out to characterise the existence of partial coalescence in ice cream emulsions including particle size diameter and particle size distribution (Gelin et al., 1994, Méndez-Velasco and Goff, 2012a, b), turbidity (Goff and Jordan, 1989), solvent extractable of fat (Pelan et al., 1997a, Pelan et al., 1997b), low-temperature scanning electron microscopy (LT-SEM) (Goff, 1997a, Goff et al., 1999b), meltdown test (Innocente et al., 2009, Koxholt et al., 2001), and overrun (Méndez-Velasco and Goff, 2012a, b).

2.14.1 Particle size analysis

Size of particles as well as the particle size distribution can be measured by a laser diffraction instrument using a Mastersizer instrument (Benjamins et al., 2009), however, a laser diffraction machine is not suitable for analysing droplet sizes bigger than 100 μm (Benjamins et al., 2009). Generally a particle size analyser is usually used for measuring the size of fat droplets in emulsions and partial coalescence could be assigned as a percentage of particles greater than 2 μm (Granger et al., 2005c) or particles greater than d_v (0,9) (Méndez-Velasco and Goff, 2012b). This may not be applied for the emulsions prepared form coconut milk as it is generally known that coconut milk is unstable with a very large mean particle diameter. The distribution pattern of particles obtained from the measurement of particle size analysis could also be used to predict the presence of partial coalescence in emulsions. It is generally measured in ice cream mix after homogenisation and ageing. The droplets in ice cream mix emulsion usually exhibit a monomodal particle size distribution (Gelin et al., 1994, Innocente et al., 2009, Segall and Goff, 2002). The presence of the new population of greater droplet size in the distribution curve or the change in the distribution from monomodal to bimodal or multimodal observed from the frozen ice cream after the mix has undergone whipping/aeration may correlate to the existence of larger droplets due possibly to partial coalescence (Bolliger et al., 2000a, Davies et al., 2001).

2.14.2 Rheological properties

The analysis of viscosity and viscoelastic properties such as storage modulus (G′), loss modulus (G″) and loss tangent (Tan δ) can provide useful information to identify the properties and structure of a fluid system such as emulsions and ice creams. The response of materials to the applied mechanical force can be classified into two opposing forms including the elastic properties of a solid and the viscous flow properties of a liquid (Rao, 1992b). As emulsions are usually fluid composed of both liquids and suspended or dissolved solid compounds, they usually exhibit a non-Newtonian behaviour. These non-Newtonian fluids normally show both elastic and viscous properties known as viscoelastic behaviour (Rao, 1992a).

A number of published data measured from samples such as ice creams and related products have shown the analysis of rheological properties by an oscillatory test with small deformation (Granger et al., 2004a, Granger et al., 2004b, Liew et al., 2001b, Méndez-Velasco and Goff, 2011, 2012b). Oscillatory test is a test aimed to investigate the linear and nonlinear properties of materials (Fang and Choi, 2012). The frequency dependence test, e.g. a frequency sweep test, is a test aimed to measure the relative motion of molecules in the bulk of samples (Rohn, 1995). The data obtained can inform about the relaxation times, entanglements and cross-linking of molecules. The temperature dependence test, e.g. a temperature sweep test, gives data about the thermal activities or temperature dependency of materials (Rohn, 1995).

For fluid material, the stress varies depending on the rate of change of strain or shear rate with time (Ross-Murphy, 1994). Generally, in order to conduct an oscillatory test, viscoelastic samples are to be measured in the linear viscoelastic range (LVR) which is defined as a region where the material response, e.g. strain, is directly proportional to the value of force, e.g. stress, being applied to materials (Shoemaker, 1992) or being as a region where storage and/or loss moduli are independent of the applied force. Hence, measuring samples in the LVR region ensures no significant change in a material's structure (Kim et al., 2001). The measured viscoelastic parameters, e.g. G′ and G″, are intrinsic properties of material structure (Vlachopoulos and Polychronopoulos, 2012)

which later can be used to correlate to molecular structure and morphology of the material (Rohn, 1995). A set of viscoelastic parameters can be obtained from a non-oscillatory test such as viscosity (η) and from an oscillatory test including storage or elastic modulus (G′), loss or viscous modulus (G″), and loss tangent or tan δ which is a measure of the ratio of the viscous modulus (G″) to elastic modulus (G′) at frequency ω (Ross-Murphy, 1994). Loss tangent is a dimensionless parameter but is very useful to inform about stage and structure of materials. As this values are logarithmically plotted against frequency, if at low frequencies, large tan δ means uncross-linked structures (Ferry, 1980). Materials which are classified to have the elastic properties have tan δ < 1 (Ross-Murphy, 1994).

The measurement of G′ is documented to be the most reliable mean to determine the elasticity of material. The higher G′ at low frequencies means that material composes of longer high molecular weight tail, thus having higher elasticity (Vlachopoulos and Polychronopoulos, 2012). From the oscillatory measurement, material is classified as liquid or showing the liquid-like behaviour if G″ is much greater than G′ with low-frequency dependent for both moduli, but if material is gel or showing the solid-like behaviour, G′ is much greater than G″ and both values are slightly frequency dependent (Ross-Murphy, 1994).

In case of a sweep test, an increase in G′ and G″ moduli shows the formation of chain-like structure in materials, whereas the decline shows the breakdown of chain structures (Fang and Choi, 2012). If at the low frequency range, G″ is greater than G′, then G′ α ω^2 behaviour dominates as frequency increases, the crossover in materials' structure occurs (Ross-Murphy, 1994). If at high frequency range, G′ and G″ become much less-frequency dependent, resulting in a formation of a plateau region or the presence of entanglement networks (Ross-Murphy, 1994).

2.14.3 Overrun in ice cream

As it was mentioned earlier that partially coalesced fat droplets play an important role in stabilising air cells in ice cream during the whipping/freezing process (Zhang and Goff, 2005), therefore, the measurement of overrun can provide indirect information on the partially coalesced fat droplets formed during the freezing and whipping process. Overrun is the measurement of amount of air which is incorporated into ice cream structure after ice cream mix undergoes freezing and aeration. Air cells are entrained into ice cream mixes by the scraper blades which is rotating in the batch ice cream freezer, whereas air is injected through orifices into ice cream mix in a continuous ice cream freezer (Chang and Hartel, 2002). Ice cream with 100% overrun means, at the same weight, the resulting ice cream has two times the volume greater than the volume of the original ice cream mix (Marshall et al., 2003). The overrun percentage is calculated by a comparison of weight of an ice cream compared to the weight of the corresponding ice cream mix at the same volume using the following equation.

$$\text{Overrun} = \frac{\text{weight of mix(g) - weight of ice cream(g)}}{\text{weight of ice cream (g)}} \text{ x } 100$$

2.14.4 Meltdown rate of ice cream

The extent of partially coalesced fat network ice cream determines shape retention and the melting rate of ice cream (Marshall et al., 2003). Partially coalesced fat droplets play an important role in stabilising air cells (Clarke, 2004, Zhang and Goff, 2005), therefore ice cream with high melt resistance may determine the high extent of partial coalescence. The measurement of the rate that ice cream melts is a good indicative of the extent of partially coalesced fat clump in ice cream (Marshall et al., 2003). The meltdown rate of ice cream is done by putting ice cream on a mesh screen and leaving it to melt in a controlled-temperature cabinet (usually at 20°C) for a given period of time (Marshall et al., 2003). The meltdown curves plotted as a function of the weight or percentage of dripping portion against time are created.

2.14.5 Fat destabilisation index of ice cream

Fat destabilisation index is a calculation based on the turbidity measurement. The principle of this method is based on the fact that the size of partially coalesced fat droplets is bigger than individual droplets that are not partial coalesced, which in turn make the coalesced droplets have less total surface area to scatter light, hence less absorbance value reading (Marshall et al., 2003). Generally, ice cream emulsions of the aged mix and ice cream are measured for the absorbance values at 540 nm in the diluted state. Before the measurement, emulsions of aged ice cream mix and ice cream are diluted with water at a concentration of 1:500 (Goff and Jordan, 1989, Marshall et al., 2003).

2.15 Literature review conclusions

Natural coconut milk is very unstable and separates into two fractions, cream and serum phases. Three main species of proteins are known to be present in coconut milk, including 11S globulins (cocosin), 7S globulins and albumins. Among these proteins, 11S globulins are reported to be predominantly present at the interface of coconut oil droplets. In some studies concerning the emulsifying capacity of fractionated proteins from coconut milk including cream and coconut skim milk, small droplets of emulsions were shown to be formed but the stability of coconut emulsions stabilised by different proteins of coconut milk has not been well studied. In order to use coconut milk as a major ingredient for making oil-in-water emulsions and other frozen products, the properties and stability of coconut milk emulsions, in terms of their ability to produce a small size of coconut oil droplets with a high stability against phase separation, need to be investigated.

Normally, dairy milk is widely used as an ingredient in the production of various food products. For example, frozen desserts and ice cream are normally produced from dairy milk, cream and dairy-based dry powders as a main ingredient base. There is a growing interest in the development of ice creams made from vegetable oils as well as plant proteins without using dairy ingredients. Some research studies have shown the

potential of using a single or blends of vegetable oils on the properties of emulsions and ice creams. However, studies concerning the use of coconut oil or coconut milk in making emulsions and/or ice cream have not been carried out. A study on the properties of coconut milk emulsions as well as ice cream based on coconut milk needs to be conducted. From many literatures, fats, proteins and small molecule surfactants are primary ingredients that play vital roles in the formation of the three-dimensional network in ice creams. The process called "partial coalescence" is mainly mentioned as the main de-stabilisation mechanism involving in the formation of ice cream structures. Ice cream with good fat structures usually has high overrun, melting resistance and shape retention. Several parameters such as type and quantity of surface active materials and properties of fat, such as composition and solid fat content, are well defined to have significant impacts on partial coalescence. A number of studies have been conducted and well explained about the functionalities of those ingredients in ice cream based on dairy milk but very few researchers have focused on the properties of ice cream based on coconut milk. The current research was therefore aimed to gain the understanding of characteristics and stability of oil-in-water emulsions based on coconut milk and properties of ice creams made based on coconut oil-in-water emulsions.

Chapter 3

Characterisation of oil-in-water emulsions prepared with coconut milk and coconut skim milk at different ratios of coconut oil to protein

3.1 Abstract

The two liquid bases, coconut milk (CM) and coconut skim milk (CSM), were used to prepare coconut oil-in-water emulsion with varying coconut oil to protein ratios (O/P) (5:1, 11:1 and 16:1) by using homogenisation pressures at 220/20 bar and 550/50 bar ($1^{st}/2^{nd}$ stage). The results showed that two different homogenisation pressures had no significant effect on the size of emulsion droplets formed in both emulsion systems made from CM and CSM. One of the main differences between the two emulsion systems was their particle size difference observed from emulsions prepared from two different emulsion based on CM and CSM. The volume weighted mean diameter (d_{43}) of particles was significantly larger in the emulsions prepared from CM than from CSM. The mean particle diameter for the former was in a range of about 75-160 μm, depending on the O/P ratio, whereas it was in a range of 8-17 μm for the latter. The particle size was significantly reduced at the ratios of O/P decreased in the emulsions made from CM but its effect was less consistent for the CSM-based emulsions. The electrical charges of droplets in both emulsions were around between -22 mV and -38 mV depending on O/P ratios and types of emulsion bases. All emulsions prepared revealed phase separation with a cream layer separated from the serum phase, indicating emulsion instability due to their initial large particle size and/or droplet aggregation (flocculation and/or coalescence). The microscopic observations show that the droplets of emulsions made from CM were highly flocculated. Overall the results provide an insight into some differences in the properties and stability of coconut oil-in-water emulsions made from CM and CSM, both of which were not very stable to phase separation. This could be resulting from the nature of surface-active coconut milk proteins that do not possess the high emulsifying and stabilising properties.

3.2 Introduction

Coconut milk is a natural oil-in-water emulsion which is extracted from the endosperm (flesh) of mature coconut nut (*Cocos nucifera* L.) normally by physical forces (e.g. squeezing and pressing) with or without an addition of water (Seow and Gwee, 1997, Tangsuphoom and Coupland, 2005). The natural coconut milk is unstable, readily separating into two layers, an opaque cream phase and an aqueous transparent serum phase (Monera and del Rosario, 1982). The instability can be due to the large size of oil droplets and the properties and composition of interfacial layer of natural oil droplets that do not sufficiently confer the stabilisation against droplet aggregation and coalescence (Monera and del Rosario, 1982).

Several different protein species are present in coconut endosperm including 11S globulins (cocosin), 7S globulins, prolamines, glutelin-1, glutelin-2 and albumins (Kwon et al., 1996). The properties of 11S globulins and albumins are different, based on their solubility and chemical properties (Garcia et al., 2005, Kwon et al., 1996). 11S globulins are salt-soluble proteins and more hydrophobic than albumins that are soluble in water (Tangsuphoom and Coupland, 2008b, 2009). Among those proteins, two proteins reported as main constituent proteins in coconut milk are 11S globulins (cocosin) and albumins (Tangsuphoom and Coupland, 2009). It was reported that after homogenisation of coconut milk, the proteins found on the surface of the homogenised oil droplets in coconut milk were only 11S globulins, based on the analysis of interfacial proteins of washed creams from the homogenised milk separated by SDS-PAGE (Tangsuphoom and Coupland, 2009).

The functionalities of coconut milk proteins to emulsify and stabilise oil have been shown by several research groups in recent years. In some studies by Onsaard et al. (2005) and Onsaard et al. (2006), the emulsifying capacity of two fractionated proteins from different fractions of coconut milk such as cream and coconut skim milk was compared by using them in the preparation of in 10% corn oil containing emulsion (Onsaard et al., 2005, Onsaard et al., 2006). The fractionated proteins from the latter fraction showed better effects by producing the smaller droplets (d_{32}) in the range

between 0.5 and 2 μm which were smaller than the size of droplets stabilised by proteins in the former fraction which ranged between 2 and 8 μm (Onsaard et al., 2005, Onsaard et al., 2006). This infers that the proteins from two different fractions of coconut milk had different emulsifying properties. Fat droplets in emulsions become smaller after the disruption of big droplets within a homogeniser (Freudig et al., 2003, Wilbey, 2003). Since the size of droplets (d_{32}) stabilised by coconut proteins was reported to be smaller than 8 μm which is not considered to be small enough to make an emulsion stable against phase separation (Dickinson, 1992b, McClements, 2004a). The size of the newly created droplets depends on homogenisation pressure and the droplets becomes smaller with increasing homogenisation pressure (Innocente et al., 2009, Koxholt et al., 2001).

In the studies by Onsaard et al. (2005) and Onsaard et al. (2006) described above, the physicochemical properties of protein samples used in the preparation of emulsions might have been changed from their native state because of the chemicals and methods used in the fractionation and extraction of proteins. The type of oil used was corn oil which is liquid. The properties of coconut milk proteins to emulsify and stabilise coconut oil which is a semi-solid at low temperature (e.g. 4^{o}C) can be different from their behaviour to form and stabilise the liquid oil like corn oil. The emulsion stability after the emulsion preparation over time during storage at different temperatures can also be different when the oil droplets contain different types of oils with different melting properties and solid fat content. The main objectives of this study were to investigate the properties of coconut oil-in-water emulsions prepared from coconut milk or coconut skim milk at different ratios of oil to protein by using two different homogenisation pressures.

3.3 Materials and Methods

3.3.1 Materials

Commercial frozen coconut milk (Sagana delights, The Philippines) packed in a PE plastic bag was bought from a local distributor and used to prepared two different continuous phases for the preparation of oil-in-water emulsion samples. In selected emulsions, virgin coconut oil (Zanian organic, Thailand) which was bought from a local store was used as a dispersed phase in those emulsions.

3.3.2 Preparation of coconut milk and coconut skim milk

Frozen coconut milk was thawed at 4°C overnight and then heated to 35°C in a water bath (55°C) to ensure that all fat in the frozen milk was melted. The thawed coconut milk was divided into two portions. One portion was homogenised using a high-shear mixer (Silverson L4RT, Silverson Machine ltd, Waterside, England) for 2 minutes. The other portion was treated with a cream separator (Model LWA 205, Westfalia separator AG, Germany) operating at 12,000 rpm to prepare coconut skim milk by separating coconut oil from the coconut milk. The coconut milk (CM) and coconut skim milk (CSM) prepared were then added with sodium azide (0.02%, w/v) as an antimicrobial agent and kept at 4°C for further experiments.

3.3.3 Preparation of emulsions

Six different base mixtures for the preparation of emulsions were prepared based on CM and CSM to contain three different ratios of oil to protein (O/P) by mixing different proportions of CM, CSM and virgin coconut oil (CO) (Table 3.1). Distilled water was used to make a final balance of composition in some mixtures. The contents of fat, protein and water and the ratios of O/P in emulsions are shown in Table 3.2. The amounts of these components including ash and carbohydrate in the emulsion formulations derived from three base ingredients (CM, CSM and CO) including water are shown in Table 3.3. The concentrations of both protein and oil in the emulsions prepared from CM were about two times higher than the emulsions based on CSM but

the O/P ratios (about 16:1, 11:1 and 5:1) in the emulsions between two groups of samples were fixed constant.

Table 3.1 Emulsion formulations prepared with CM or CSM at three different ratios of oil to protein

Ingredient (%wt)	Emulsion					
	C16	**C11**	**C5**	**S16**	**S11**	**S5**
CM	50.6	50.6	31.2			
CSM			68.5	92.2	92.8	92.8
CO	7.0	2.0		7.8	5.2	2.6
Water	42.4	47.4	0.4		2.0	4.7
Total	100	100	100	100	100	100

Letters C and S in the names of emulsions mean the emulsions based on coconut milk (CM) and coconut skim milk (CSM), respectively. The numbers 16, 11 and 5 represent the ratios of coconut oil (CO) to protein in emulsions at 16:1, 11:1 and 5:1, respectively.

Table 3.2 Contents of fat, protein and water in emulsions and the ratios of oil to protein

Emulsion	Fat (%)	Protein (%)	Water (%)	O/P
C16	15.0	0.9	82.4	16
C11	10.0	0.9	87.4	11
C5	5.0	0.9	91.4	5
S16	7.9	0.5	89.4	16
S11	5.3	0.5	92.0	11
S5	2.6	0.5	94.7	5

Table 3.3 Amounts (wt%) of fat, protein, ash, carbohydrate and water in emulsions derived from CM, CSM, CO and water.

Emulsion	Fat			Protein			Ash			CHO			Water
	CM	CSM	CO	CM	CSM	CO	CM	CSM	CO	CM	CSM	CO	
C16	8.0		7.0	0.9			0.3			1.4			42.4
C11	8.0		2.0	0.9			0.3			1.4			47.4
C5	4.9	0.1		0.9	0.4		0.2	0.3		0.9	1.9		0.4
S16		0.1	7.8		0.5			0.5			2.6		
S11		0.1	5.2		0.5			0.5			2.6		2.0
S5		0.1	2.6		0.5			0.5			2.6		4.7

Letters C and S in the names of emulsions mean the emulsions based on coconut milk (CM) and coconut skim milk (CSM), respectively.

The mixtures were heated to 35°C in a water bath to ensure that all oil was liquid. The heated mixtures were then blended with a high shear mixer (Silverson L4RT, Silverson Machine ltd, Waterside, England) at 6,000 rpm for 2 min to prepare a coarse emulsion. The coarse emulsions were then homogenised by a two-stage high pressure homogeniser (APV 2000, APV, Rannie/Gaulin, Albertslund, Denmark) with four passing times at two different pressures at 220/20 and 550/50 bar ($1^{st}/2^{nd}$ stage). This resulted in a total of 12 samples of oil-in-water (O/W) emulsions.

3.3.4 Proximate analysis of CM and CSM

3.3.4.1 Moisture content

The content of moisture (or total solids) was analysed by air oven method using an AOAC Official Method 990.20. Samples (about 2 g) were weighed in a pre-dried aluminium moisture dish fitted with a lid. The dishes were placed in an air oven (Contherm, model 250M, New Zealand) at 105°C overnight. The dishes covered with lids were transferred to a desiccator to cool down to room temperature for 15 min and then weighed. The moisture content was expressed as the amount of sample's weight loss as a percentage.

3.3.4.2 Ash content

The content of ash in samples was analysed by using an AOAC Official Method 940.26. About 5 g of sample was placed into a pre-weighed aluminium crucible (Volcan A 550, CA, USA) and then charred over a Bunsen burner. The samples in crucibles were placed in a muffle furnace at 550°C for 4 hr until the samples were completely combusted. Then, the crucibles were cooled down in a desiccator before weighing. The amount of ash was calculated as a percentage of ash in samples.

3.3.4.3 Protein content by Kjeldahl method

The analysis of protein content was conducted by determining the total nitrogen content using Kjeldahl method following an AOAC Official Method 991.20. This method consisted of three main steps, including digestion, distillation and titration steps. Approximately 2 g of samples were placed into a Kjeldahl digestion flask. For digestion, two Kjeltabs tablets of catalyst (3.5 g K_2SO_4/3.5 mg Se) and 25 ml H_2SO_4 were added into each flask. The flasks were digested on a digestion block (Tecator digestion block DS20, Tecator, Sweden) at 400°C for 4 hrs. After cooling, 75 ml of distilled water were added into each flask. The flasks were then put in a distillation unit (Kjeldahl system 1026, Tecator, Sweden) and added with 75 ml of 50% (w/w) NaOH without agitation. Steam was then introduced into the digestion flask for the distillation of ammonia (NH_3) into a receiving flask containing 50 ml of 4% boric acid (H_3BO_3) added with a few drops of phenolphthalein. After distillation, the boric acid solution was titrated with 0.1 M HCl to a first trace of pink as indicative of the end point. The volume of HCl (ml) used for titration was recorded. Recovery of nitrogen was tested with L-tryptophan (0.16 g) and sucrose (0.67 g) using the same reagents under the same conditions which was higher than 98% in all cases. The percentages of nitrogen and protein in samples were calculated using the following equations.

$$\text{Nitrogen (\%)} = \frac{1.4007 \text{ x } \left(\text{ml of HCl for test portion-blank portion}\right) \text{ x Molarity of HCl solution}}{\text{weight (g) of sample}}$$

Protein (%) = % Nitrogen x 6.25

3.3.4.4 Fat content by Mojonnier method

The content of fat in samples was determined by the Mojonnier fat extraction following an AOAC Official Method 989.05. About 10 g of samples warmed at 38°C were weighed into a Mojonnier flask. To each flask, 1.5 ml ammonium hydroxide (NH_4OH) solution was added and mixed thoroughly. After the addition of three drops of phenolphthalein indicator, 10 ml of 95% ethanol was added to the flask and mixed thoroughly. For the extraction of fat from the samples, 25 ml of diethyl ether were added followed by the addition of 25 ml petroleum ether. After the addition of each of two ether solvents, the flask was shaken vigorously for 1 min. The flask was then centrifuged using a centrifuge (Model Super Vario-N, Funke Gerber, Labortechnik, Germany) at 600 rpm for 30 sec to obtain the separation of aqueous phase from non-polar solvent phase. The solvent ether phase containing extracted fat was decanted into a pre-weighed aluminium dish. The solvent decanted into the aluminium dish was evaporated on a hot plate in a fume hood. The fat extraction was done two more times by repeating the procedures described above. The second extraction was done with 5 ml of 95% ethanol, 15 ml diethyl ether and 15 ml petroleum ether whereas 95% ethanol was not used in the third extraction. After the evaporation of solvent on the hot plate in a fume hood, the dishes containing fat were placed in an air oven at 100° ± 1°C for 15 min to remove the solvent completely. The dishes were then transferred into a desiccator to cool down to room temperature. The amount of extracted fat in the aluminium dish was weighed and expressed as a percentage of fat.

3.3.5 Particle size of emulsions

The mean particle diameter and the size distribution of particles in emulsion samples were measured by a static light scattering technique using a particle size analyser (Mastersizer 2000MU (A), Malvern Instruments Ltd, Malvern, Worcestershire, UK). The refractive indices of 1.449 and 1.33 for coconut oil and water phases, respectively, and the absorbance value of 0.001 for the oil droplets were used to determine the diameters of particles in emulsions. The average particle size of emulsion droplets measured were reported as d_{43} (volume weighted mean diameter) defined as $\sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles with diameter d_i. The mean diameter

as d_{43} was selected to express all the mean of particle diameter measured in this study as the d_{43} is more sensitive to the presence of large particles or flocculated droplets than d_{32} (surface weighted mean diameter) which is defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of particles with diameter d_i (McClements, 2004a). The particle size analysis was carried out at a room temperature in triplicate with three times reading per loading. The diameter of particles in emulsions was measured by adding samples directly into the instrument sample chamber, in which the loaded samples were diluted to approximately 1:1000 in water. With the size measurement, the data of specific surface area (m^2) were also obtained.

3.3.6 Zeta potential of emulsion droplets

The electrical charge or zeta potential (ζ-potential) of the oil droplets in emulsion samples was measured using a multi-angle light scattering instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Prior to the measurement, all emulsion samples were diluted with reverse osmosis (RO) water to prevent the multiple light scattering effect. The samples were measured at 25^{o}C in triplicate on the basis of running at least 12 times per each replication.

3.3.7 Microscopic examination of emulsions

Emulsion samples were visually observed for their stability after storage overnight at 4°C and 35°C. Prior to the analysis, the emulsions stored at 4°C and 35^{o}C were warmed to ambient temperature. Each sample was then gently mixed by inverting the tubes containing samples. A small drop of samples was placed on a slide and covered with a cover slip. The droplets of emulsions were visually observed using an optical light microscope (Carl Zeiss, Model Axiostar plus, Goettingen Germany). Digital images were taken with a digital camera (Carl Zeiss, Model AxioCam MRC, Goettingen Germany) attached to the microscope.

3.3.8 Emulsion stability against phase separation

The stability of emulsions to phase separation (creaming) was visually determined by monitoring phase separation of emulsions occurring during storage. Each emulsion sample (10 ml) was placed in a 15 ml glass test tube and sealed tightly with a cap. In this study, the test tubes were then left to stand for one day at 4°C and 35°C. The total height of emulsions (HE) and the height of droplet depleted layer (HD) (i.e. serum layer) from each tube were measured. The stability of emulsions was calculated in a term of percentage of phase separation using a following equation.

$$\textbf{Phase separation (\%)} = \frac{\text{Height of droplet depleted layer (HD)}}{\text{Height of emulsion (HE)}} \times 100$$

3.3.9 Analysis of emulsion turbidity

The stability of emulsions against droplet aggregation and flocculation was determined by a turbidity measurement using spectrophotometer (Reddy and Fogler, 1981). The principle is that the absorbance of emulsions changes inversely with the particle size of suspended particles in emulsions (Reddy and Fogler, 1981). Prior to the measurement, emulsions were diluted with distilled water to oil concentrations in the suitable ranges that gave the absorbance values not higher than 2.0. The absorbance of diluted emulsions was measured at 600 nm by a UV-visible spectrophotometer (Shimadzu, Shimadzu Corporation, Japan). The absorbance values were plotted as a function of oil concentrations.

3.3.10 Statistical analysis

All experiments and measurements were carried out at least in duplicate. The results obtained from the measurements were reported as the mean and standard deviation. The data were statistically analysed using a SAS (version 9.3 for window) (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range tests were used to determine the significance of means at the minimum of 95% confidence level ($P < 0.05$).

3.4 Results and Discussion

The chemical composition of CM and CSM determined by the proximate analysis is shown in Table 3.4. As a result of removal of some coconut fat globules, the CSM which was fractionated from CM had a lower protein and fat content than the CM.

Table 3.4 Composition of CM and CSM analysed by the proximate analysis

Milk	Composition[a], wt%				
	Moisture	Protein	Fat	Ash	CHO[b]
Coconut milk (CM)	79.1 ± 0.1	1.8 ± 0.1	15.9 ± 0.1	0.5 ± 0.1	2.8 ± 0.1
Coconut skim milk (CSM)	96.8 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.5 ± 0.1	2.1 ± 0.1

[a]Values are means of three replicate measurements from duplicate samples (n = 6).
[b]Carbohydrate (CHO) was calculated from (100 - moisture - fat - protein - ash).

A set of three emulsion samples differing in their O/P ratios (5:1, 11:1 and 16:1) were prepared from either CM or CSM by using two different homogenisation pressures, 200/20 bar or 550/50 bar ($1^{st}/2^{nd}$ stage). This resulted in a total of 12 different emulsion samples which were analysed and compared for their particle size and zeta potential and the stability of emulsions against phase separation and droplet aggregation.

3.4.1 Particle size and particle size distribution of emulsions

The particle size distributions (PSDs) of emulsion droplets show either bimodal or multimodal distribution (Figure 3.1). Two different homogenisation pressures used led to a few minor but noticeable differences in the PSD of emulsions. The high pressure of 550/50 bar used in the preparation of emulsions tended to result in a more pronounced single defined population with a narrower size distribution. The PSD curves shown in Figure 3.1 also illustrate that the major percentage of volume frequency of droplet population had the particle size of approximately 5 μm for the emulsions prepared from CSM while it was much larger at around 100 μm for the emulsions based on CM.

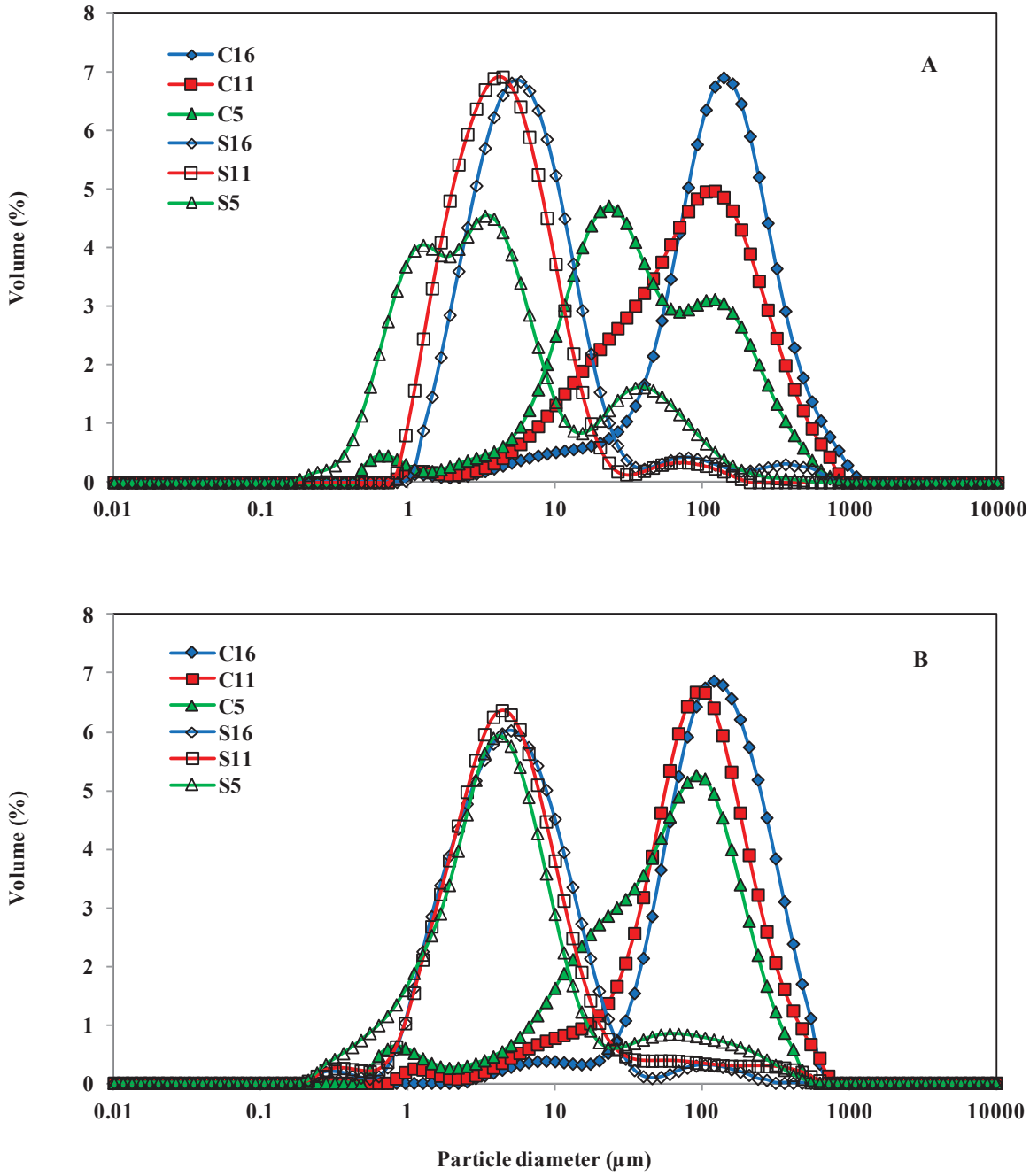

Figure 3.1 Particle size distributions of emulsions prepared by two different homogenisation pressures ($1^{st}/2^{nd}$ stage): (A) 220/20 bar and (B) 550/50 bar. Abbreviations: C and S mean the emulsions prepared from CM and CSM, respectively; 16, 11 and 5 represent the ratio of O/P at 16:1, 11:1 and 5:1, respectively.

The mean particle diameters (d_{43}) of droplets in emulsion samples are shown in Figure 3.2. The results show that type of liquid bases (CM and CSM) used in the preparation of emulsions had a significant influence on the difference in the mean particle diameters between two groups of emulsions made with CM and CSM ($P < 0.05$). In all cases, the average mean particle diameter was much larger in emulsions prepared with CM than with CSM, regardless of the homogenisation pressures and the ratios of O/P, indicating that proteins in CSM had a better ability to form and stabilise coconut oil droplets with smaller size than proteins in CM. This finding was in agreement with some studies that reported that in a range of protein concentrations between 0.1% and 1%, the size of corn oil droplets stabilised by the proteins derived from the serum (skim milk) phase of coconut milk was smaller than the size of droplets stabilised by the proteins fractionated from the cream phase of coconut milk (Onsaard et al., 2005, Onsaard et al., 2006).

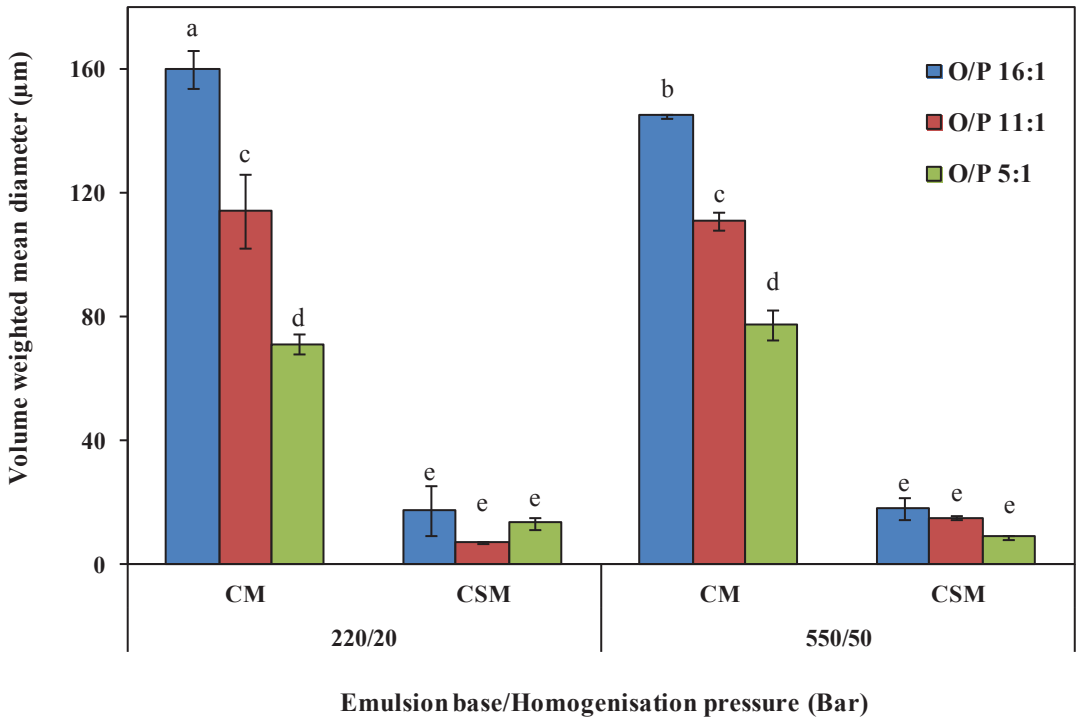

Figure 3.2 Effects of oil to protein (O/P) ratios on the mean particle diameter (d_{43}, µm) (n =6) of the emulsions prepared based on CM and CSM by homogenisation at 220/20 bar ($1^{st}/2^{nd}$ stage) or 550/50 bar ($1^{st}/2^{nd}$ stage).

It can be considered that the surface active materials present in the CM-based emulsions are the constituent proteins (e.g. globulins) and phospholipids naturally present at the surface of coconut oil droplets in coconut milk (Monera and del Rosario, 1982) and some water-soluble proteins from the serum phase of coconut milk, whereas in the CSM-based emulsions, the available surface active compounds are predominantly the water-soluble serum proteins of coconut milk. Coconut milk has been known to contain two major groups of proteins: a) coconut globulins (also called cocosin) which are salt-soluble hexameric proteins consisting of six pairs of acidic and basic polypeptides linked by disulfide bonds (Balasundaresan et al., 2002) and predominantly found at the interface of coconut oil droplets and b) albumins which are water soluble and consist of three polypeptide subunits (Garcia et al., 2005, Kwon et al., 1996, Tangsuphoom and Coupland, 2009). According to Tangsuphoom and Coupland (2009), after the homogenisation of coconut milk, cocosin was the only type of proteins that adsorbed at the oil droplet surface. This suggests that cocosin may have a better ability to be adsorbed at the interface of oil droplets formed during homogenisation than albumins but their ability to stabilise the oil droplets against droplet aggregation and coalescence may be lower than albumins, resulting in the formation of large oil droplets. This may be due to their chemical nature as proteins being more hydrophobic and some other physical chemical properties different from albumins. One example, the CSM serum proteins could lower the interfacial tension of oil droplets better than the proteins (mainly cocosin) present in CM, hence providing better stability to the oil droplets (more details in Section 3.4.4).

Two different homogenisation pressures, 220 bar (22 MPa) and 550 bar (55 MPa), used in this study did not show a noticeable difference in the size of particles between emulsions. In general, the size of particles produced is smaller when the pressure applied is higher (Floury et al., 2000, Floury et al., 2004, Hogan et al., 2001, Lethuaut et al., 2002). The reasons for the observed no particle size difference may be due to the extent of pressure difference not high enough to show a significant difference and/or the concentration of proteins that may be insufficient to stabilise all droplets formed during homogenisation. In a study reported by Hogan et al. (2001), no reduction in the particle

diameters of whey protein stabilised soy bean oil emulsions was shown when the homogenisation pressures increased from 30 MPa to 50 MPa.

Regarding the different ratios of O/P in emulsions, its effect was only observed to be significant for the emulsions based on CM as shown in Figure 3.2. The decreasing ratio of O/P in the CM emulsions led to a smaller mean droplet diameter at the homogenisation pressures of both 220/20 bar and 550/50 bar. On the other hand, a pattern of changing particle size attributable to the O/P ratio could not be seen among the emulsions based on CSM because there was no consistent trend in changing the size of particles prepared under two different pressures. A similar trend was reported in the literature that the mean particle diameter (d_{32}) was decreased from 8 μm to 2 μm in 10% corn oil emulsions stabilised by proteins fractionated from coconut cream as the ratio of O/P was decreased from 50 to 10 (Onsaard et al., 2006) but the size of particles stabilised by proteins derived from coconut skim milk was smaller than 2 μm, regardless of a ratio of O/P (Onsaard et al., 2005). It was also reported in the literature that the size of particles stabilised by dairy milk proteins decreases with decreasing mass ratio of O/P but remains the same without further reduction at above a certain level of protein concentration at a given amount of oil (Hogan et al., 2001, Tomas et al., 1994b).

It should be mentioned that the mean particle sizes (d_{43}) of emulsions based on CM in this study was much larger in a range of 75-160 μm than the size range of 2-8 μm (d_{32}) shown in the study reported by Onsaard et al. (2006) although the type of emulsified oil and the emulsion ingredient formulation and preparation conditions are not identical. It should be noted that this large difference and also a relatively big difference between the two emulsion systems prepared from CM and CSM may be resulted from a difference in the physical nature of coconut oil present between two systems before they were homogenised, which may affect the efficiency of homogenisation process, in terms of breakdown of coconut oil into smaller droplets. In other words, the emulsions based on CM contained a significant portion of coconut oil coming from CM which had already existed naturally in an emulsified state before homogenisation applied unlike

the CSM emulsion was prepared from coconut bulk oil (Table 3.3). The natural coconut oil-in-water droplets present in CM may not readily be broken down into smaller droplets or even they are broken down into the smaller droplets, the newly created droplets stabilised by proteins in CM (believed to be cocosin) may not provide the stability against droplet coalescence. It has been reported in the literature that the surface of natural coconut oil droplets in homogenised CM is predominantly surrounded by salt soluble globulins (cocosin) (Tangsuphoom and Coupland, 2009).

3.4.2 Zeta potential (ζ-potential) of emulsion droplets

The electrical charge **(**zeta potential) of emulsion oil droplets was analysed (Figure 3.3). The oil droplets in all emulsions were negatively charged between -22 mV and -38 mV. The values of ζ-potentials of droplets in the CM emulsions produced by two different homogenisation pressures ranged from -24 mV to -26 mV and from -27 mV to -38 mV when the emulsions were prepared at the pressures of 220/20 bar and 550/50 bar, respectively. A similar level of ζ-potential of oil droplets was observed in the CSM emulsions which was in the range of between -22 mV and -37 mV at 220/20 bar and between -24 mV and -36 mV at 500/50 bar. For the electrical charge of oil droplets between the two emulsion systems prepared from CM and CSM, a significant difference that could be attributed to the effect of ingredient bases (CM and CSM), which contained the same O/P ratio, could not be clearly established. This is because there was little difference in electrical charge between emulsion droplets prepared at the high homogenisation pressure of 550/50 bar and there was no consistent trend between the samples prepared at the low pressure of 220/20 bar. However, among the samples prepared at the high homogenisation pressure (550/50 bar), a trend of more negatively charged droplets with increasing the ratio of O/P was shown. In theory, the zeta potential of emulsion droplets should be the same if the chemical compositions of emulsions are the same, regardless of a difference in their particle size. However, the zeta potential is measured differently to a certain extent by a method based on the dynamic light scattering technique when the emulsion particle size is different. From some other experiments (data not shown), the zeta potential was observed to increase with increasing particle size. Another possible reason for the observed zeta potential

decrease with increasing ratio of proteins relative to coconut oil from the emulsions prepared with both CM and CSM could be due to differences in the ionic strength between samples resulting from the different ingredient formulations.

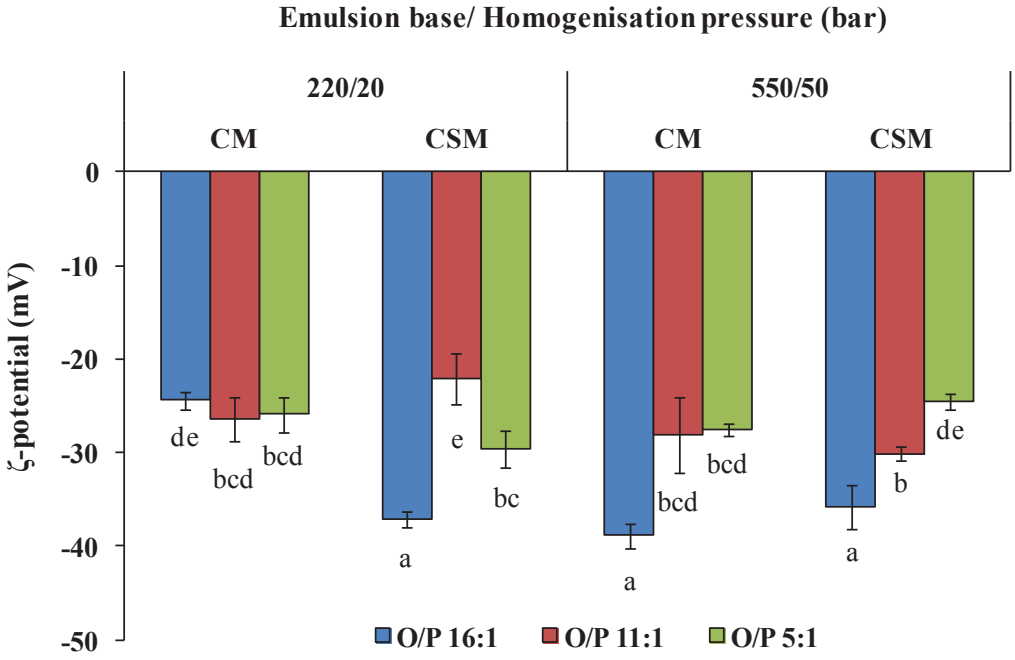

Figure 3.3 Droplet surface charges of emulsions containing different ratios of O/P prepared by two different pressures ($1^{st}/2^{nd}$ stage). Abbreviations: CM and CSM mean emulsions based on CM and CSM, respectively. Average values (n=6) with the same letter are not significantly different (P > 0.05).

The zeta potential values observed in this study were similar to the previously reported values of the oil droplets in 10% corn oil emulsions stabilised by the proteins derived from cream or coconut skim milk of coconut milk which were in the range between -25 mV and -38 mV (Onsaard et al., 2005, Onsaard et al., 2006). In a recent study by Tangsuphoom and Coupland (2008a), it was reported that the ζ- potential of droplets in coconut milk was -16 mV which is not very high. In general, the zeta potential value higher than -30 mV or -60 mV is required to provide good or excellent, respectively, stability of emulsions against droplet flocculation and coalescence (Riddick, 1968). The zeta potentials of emulsion droplets stabilised by the proteins in CM and CSM could

thus be not very high enough to provide the emulsion stability via the electrostatic repulsive force.

3.4.3 Microscopic images of particles in emulsions

The emulsion droplets were observed using an optical microscope at room temperature after storage at 4^{o}C and 35^{o}C overnight. This was to examine the physical state and appearance of oil droplets, in terms of droplet flocculation, coalescence and clustering. At both storage temperatures, the emulsions based on CM showed some flocculated big oil droplets compared to the emulsions based on CSM (Figure 3.4).

From the microscopic images, it can be seen that as the ratio of O/P in the CM emulsions decreased, their droplets became smaller. The extent of droplet flocculation in these emulsions also seemed to be more pronounced in droplet flocculation when the samples were prepared at the low pressure of 200/20 bar. The particle size of the CM-based emulsion with an O/P ratio of 5:1 that was stored at 4^{o}C was shown to be very big but it was not observed from the same sample stored at 35^{o}C. This might have resulted from the nature of samples collected that was not homogeneous due to creaming as shown in Figure 3.5.

For the CSM-based emulsions, their emulsion droplet size was relatively much smaller with less flocculation unlike the droplets in CM-based emulsions which were bigger in their size. However, no significant differences were observed between the samples with different O/P ratios, regardless of storage temperatures and homogenisation pressures. This suggests that the predominant proteins in CSM may have a better ability to stabilise the droplets against droplet flocculation and aggregation than ones in CM.

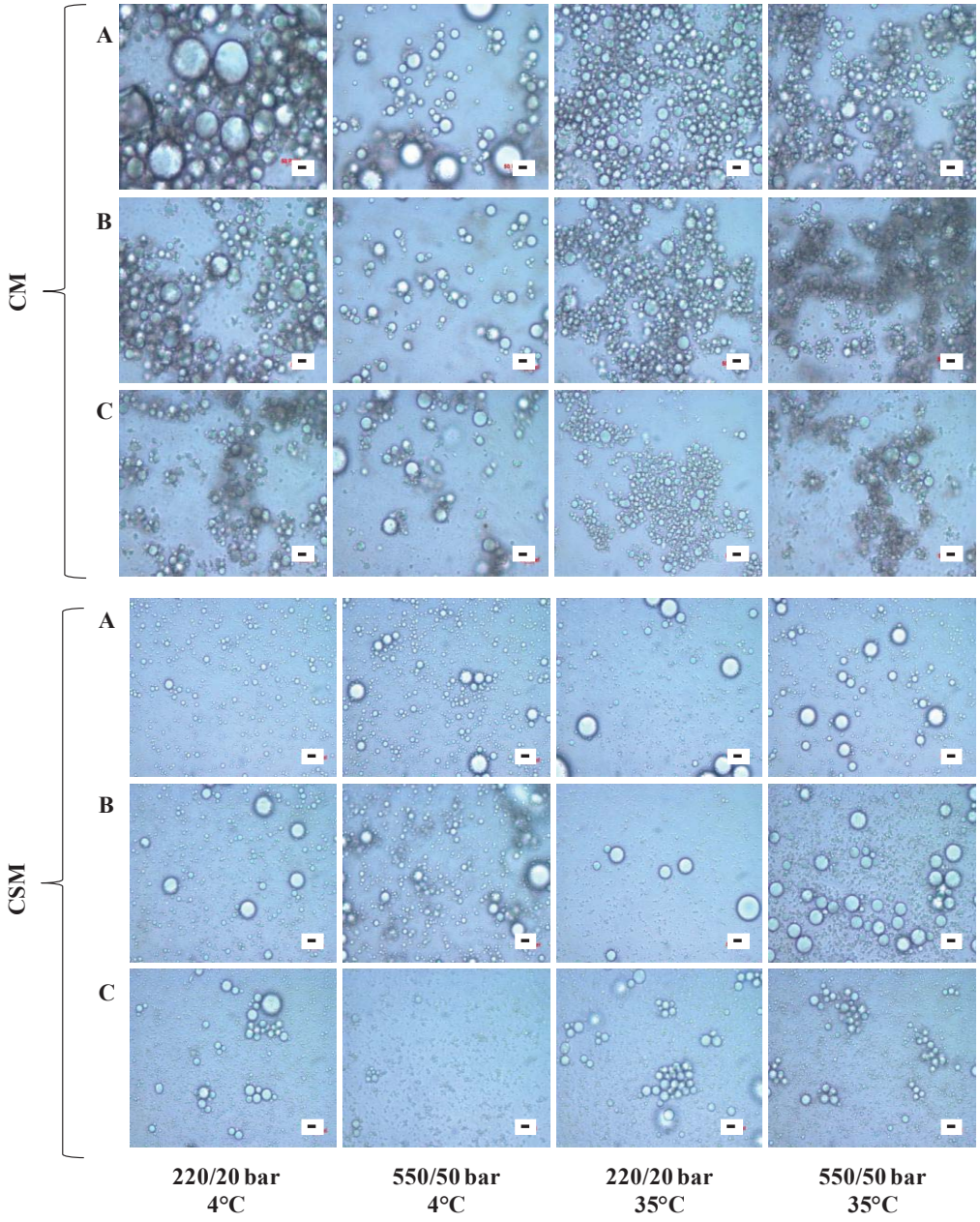

Figure 3.4 Optical micrographs (x400 magnification) of coconut oil-in-water emulsions prepared from CM and CSM with O/P ratios at 16:1 (A), 11:1 (B) and 5:1 (C). Images were taken after one day storage at 4°C and 35°C. The scale bar represents 15 µm.

3.4.4 Emulsion stability against phase separation

The emulsions stored at 4°C and 35°C for one day after the preparation were compared for their stability against phase separation and creaming that could occur resulting from droplet aggregation and/or coalescence. All emulsions exhibited relatively quick gravitational separation on the same day after the preparation (pictures not shown). After one day at 4°C and 35°C, emulsions exhibited phase separation to some different extents as shown in Figure 3.5.

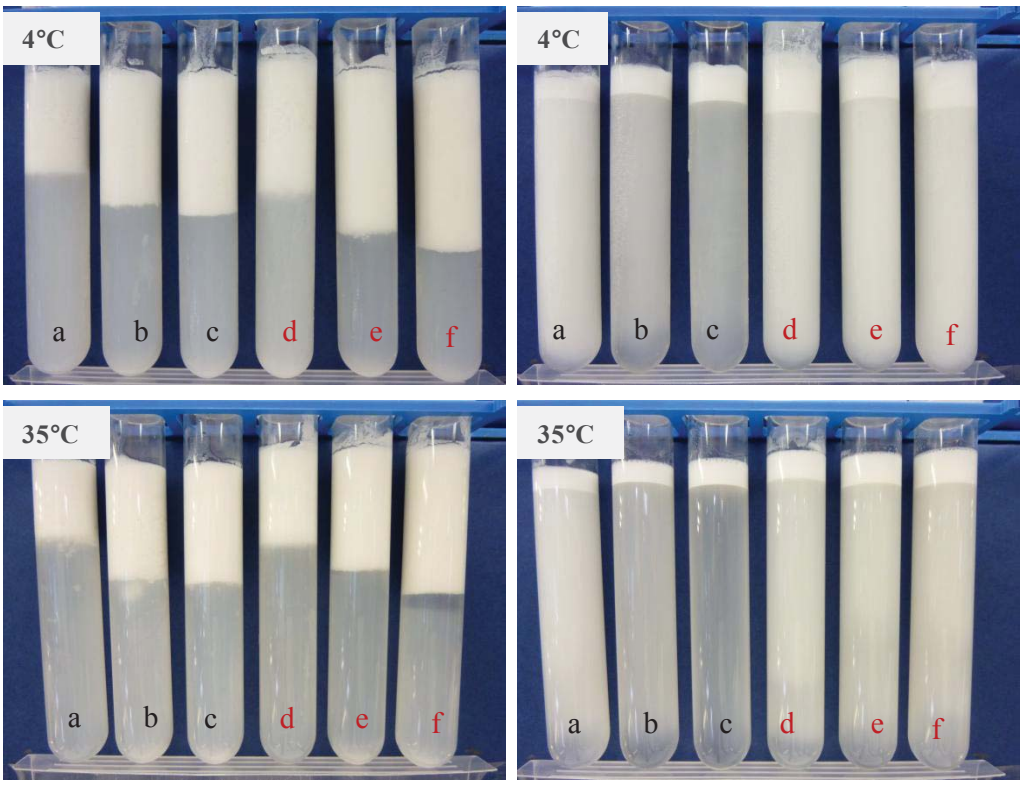

Figure 3.5 Pictures of emulsions after 1 day storage at 4°C and 35°C that compare the stability of emulsions against phase separation. A ratio of O/P in emulsions is 5:1 (a, d), 11:1 (b, e) and 16:1 (c, f).

All emulsions based on CM separated into two distinct phases, a cream layer at the top and a clear serum layer at the bottom, regardless of the homogenisation pressures used. The CSM-based emulsions also revealed phase separation but to a less extent. In other words, CSM emulsions had relatively a small amount of cream layer at the top and some droplets suspended in the serum phase, thus making turbid underneath the cream phase. The relatively small amount of creaming was due partly to the less amounts of coconut oil formulated into the CSM emulsions than in the CM emulsions (Table 3.2). Among the CSM-based emulsions with different ratios of O/P, a noticeable visual observation was that the emulsions prepared at a high pressure of 550/50 bar were more stable against phase separation (creaming) than the same emulsions prepared at the low pressure of 220/20 bar. Another pronounced observation was that among the emulsions prepared at 220/20 bar, the serum phase was more turbid (less clear) at both storage temperatures as the ratio of coconut oil decreased. This indicates that the emulsion stability was higher with decreasing the ratio of O/P which should be due to the presence of smaller particles but this conclusion could not be clearly drawn from the results of the particle size analysis.

In summary, the difference in the extent of creaming observed between the two groups of emulsion systems made from CM and CSM was apparently due to the difference in their particle size which was much smaller in the CSM-based emulsions than in the CM-based emulsions. Nevertheless, the particle sizes of CSM-based emulsions can be considered to be still big enough to undergo a gravitational separation, thus leading to the separation into the creaming and serum phases.

3.4.5 Emulsion stability to droplet flocculation

The absorbance of diluted emulsion samples was measured at 600 nm, in term of turbidity. The turbidity of emulsions has been shown to be inversely related to the oil droplet size, i.e., the lower the turbidity, the bigger the mean particle diameter or flocculation (Aoki et al., 2005, Reddy and Fogler, 1981). The plots of absorbance against oil concentration are presented in Figure 3.6. The results show that as the coconut oil concentration in the diluted solutions increased, the absorbance of the

emulsions also increased linearly. This increase was quite constant throughout the whole dilution range of oil concentrations between 0.185% and 0.3%. As described above, the turbidity usually decreases with increasing mean particle diameter which may be attributed to the aggregation or creaming of those droplets if this occurs during storage (Aoki et al., 2005, Ogawa et al., 2004).

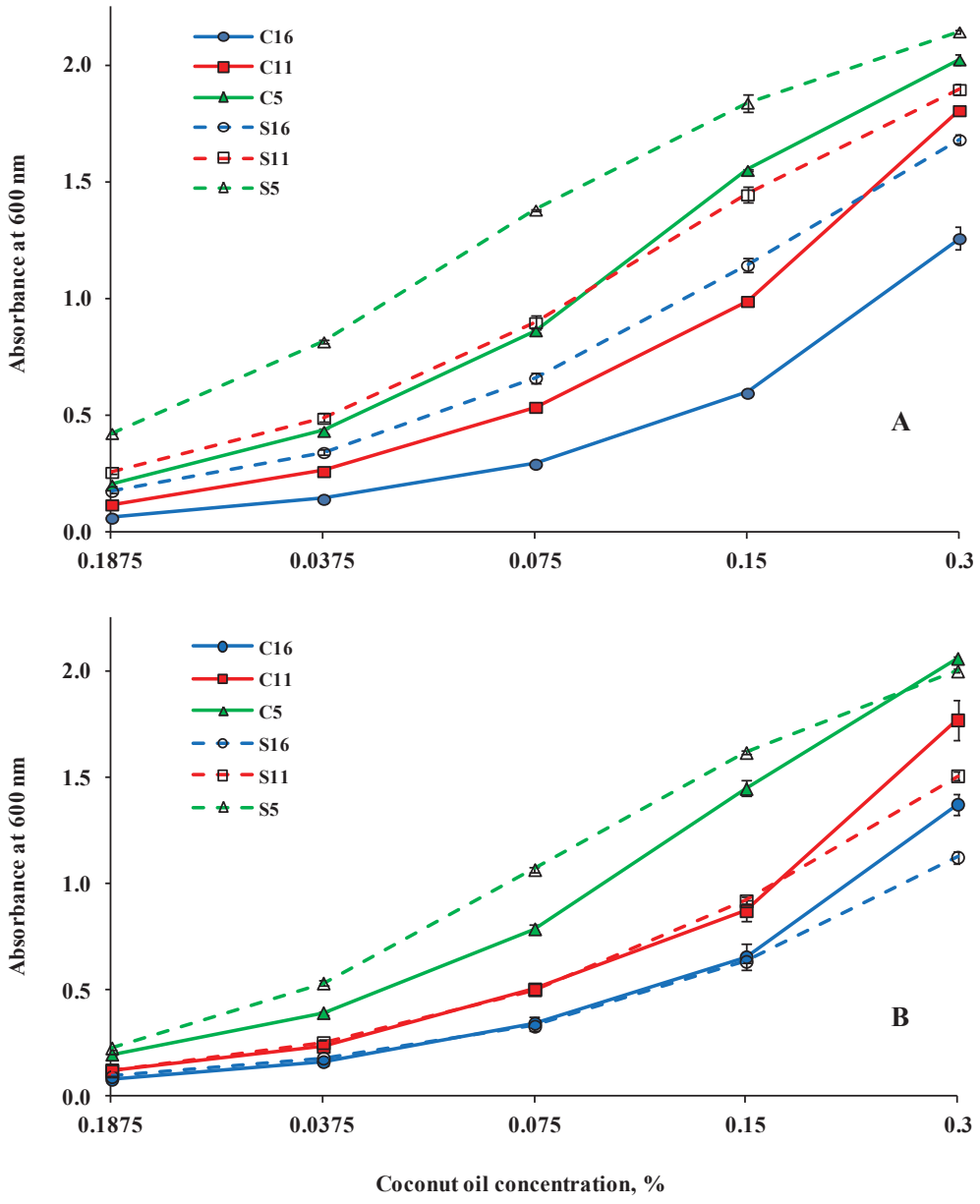

Figure 3.6 Turbidity of emulsions prepared by homogenization at 220/20 bar ($1^{st}/2^{nd}$ stage) after one day storage at 4°C (A) and 35°C (B). Abbreviations: C and S mean the emulsions prepared from CM and CSM, respectively; 16, 11 and 5 represent the ratio of O/P at 16:1, 11:1 and 5:1, respectively.

At the storage temperature at 4ºC, the turbidity measured from the emulsion based on CSM with the O/P ratio of 5:1 was higher than the other emulsions with higher O/P ratios of 11:1 and 16:1, reflecting the presence of the smaller droplets in this emulsion. For the emulsions based on CM, the turbidity decline was also observed as the O/P ratios increased being the lowest turbidity from the CM emulsion with the O/P ratio of 16:1 which was due to the presence of big droplets. It should be mentioned that the turbidity measurement conducted in this study however does not actually provide information about whether the droplet aggregation and coalescence occurred or not during storage as the differences in the measured turbidity between samples could be due to differences in their original initial particle size.

Figure 3.7 shows the comparison on the turbidity between samples made at the same oil concentration of 0.15%. Overall, the emulsion turbidity tended to be higher at 4^{o}C than 35^{o}C, suggesting the presence of smaller droplets at 4^{o}C. The tendency of lower turbidity at 35^{o}C may infer that droplets were more flocculated or coalesced at 35^{o}C. The ratios of O/P showed the clear indication of its contribution to the turbidity which could be related to the average size of particles in emulsions. The average size of droplets was quite proportional to the ratio of O/P in emulsions among the emulsions based on CM. After the storage at both temperatures, the turbidity of emulsions with the low O/P ratio, regardless of the emulsion bases, was relatively high, reflecting the presence of smaller droplets. Based on the results of the turbidity analysis, it can be indirectly concluded that some variability with an inconsistent pattern for the mean particle sizes of the CSM-based emulsions shown in Figure 3.2 may be due to some difficulties in the measurements of the samples.

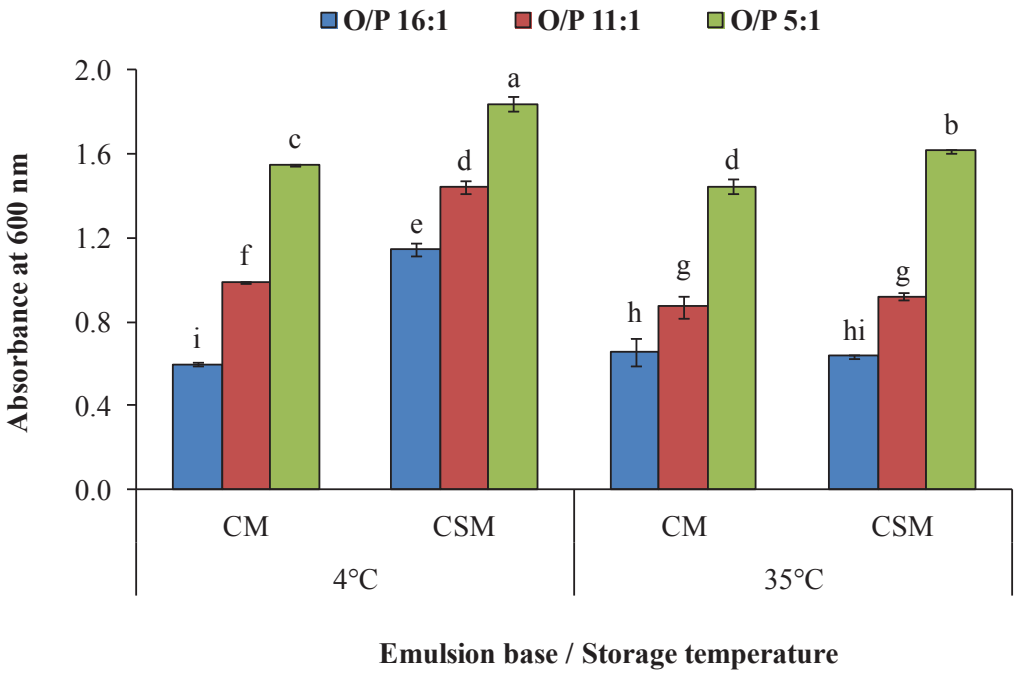

Figure 3.7 Comparison of the turbidity of emulsions at the oil concentration of 0.15%. The data were derived from Figure 3.6. The emulsion samples were prepared by homogenization at 220/20 bar ($1^{st}/2^{nd}$ stage) and the turbidity was measured after one day storage at 4°C and 35°C. Abbreviations: CM and CSM mean the emulsions prepared from CM and CSM, respectively; 16, 11 and 5 represent the ratio of O/P at 16:1, 11:1 and 5:1, respectively. Absorbance values with the same letter are not significantly different (P > 0.05).

3.5 Conclusions

Emulsions containing different coconut oil concentrations were prepared from two different fractions of coconut milk (i.e. coconut milk and coconut skim milk) at three different ratios of O/P using two different homogenisation pressures. The size of emulsion droplets formed was much smaller when the emulsions were prepared using CSM than CM. It seems that the large particle size of emulsions made based on CM was related to the oil droplets naturally present as an emulsion droplets in CM that might not have been able to be broken down readily into smaller droplets and/or that the droplets were able to be broken down but might not have been well stabilised by proteins in CM (e.g. coconut cocosin), thereby providing insufficient ability to make stable emulsions. The latter suggests that the difference in the size of emulsion droplets and emulsion stability between the two emulsion systems could be due to the different

types of proteins adsorbed to the interface of oil droplets when the emulsions were prepared from CSM and CM although the CM also contained the proteins present in CSM. The ratios of O/P had a significant effect on the size of emulsion droplets in the emulsions based on CM with decreasing particle diameter as the ratio of O/P decreased. However, no appreciable difference in droplet size attributable to the ratio of O/P was observed in emulsions based on CSM.

Regardless of the coconut milk fraction and ratios of O/P, both emulsion systems revealed high instability to phase separation (creaming) at both 4^{o}C and 35^{o}C during storage for one day although the CSM emulsions with the lowest O/P ratio of 5:1 had some better stability against creaming at both temperatures. The results of zeta potential of emulsion droplets with the range between -22 mV and -38 mV, depending on the ratio of O/P and homogenisation pressures applied, showed no strong trend of the effects of the O/P ratios on the electrical charge of droplets between the emulsions made from CSM and CM. This level of electrical charge may not be high enough to enable the droplets to repel each other and prevent them from droplet flocculation and/or coalescence. Overall the results suggest that the emulsions made from CM and CSM were very unstable and the coconut milk proteins were not highly efficient in stabilising the coconut oil droplets.

Chapter 4

Comparison of the stability and properties between coconut oil-in-water emulsions prepared from reduced fat coconut milk and coconut skim milk by particle size analysis and SDS-PAGE

4.1 Abstract

Reduced fat coconut milk (RFM) and coconut skim milk (CSM) were separated from coconut milk (CM) by using a centrifugal cream separator. Six different emulsions containing 5% or 10% coconut oil (CO) were then prepared by homogenising those three different milks and bulk oil (if required) at different ratios. Properties of all emulsions were determined by particle size and particle size distribution, droplet charges, droplet micrographs, emulsion stability (phase separation and flocculation) and interfacial protein composition by SDS-PAGE. The results show that proteins in CSM had better ability to form smaller droplets from the added bulk oil in CSM-based emulsions which was significantly smaller than those found in RFM-based emulsions ($P < 0.05$). The surface net charges of all emulsions were lower than - 20 mV which was not very high enough to provide stability to all emulsions against phase separation which was observed to occur after one day storage. The size of oil droplets in the CSM-based emulsions was not affected by the oil concentrations between 5% and 10%, whereas it was observed to increase significantly in the RFM-based emulsions. Droplets in all emulsions revealed a noticeable change in their particle size during storage at 4°C as determined through a turbidity measurement. All emulsions exhibited phase separation, due to droplet flocculation resulting possibly from a combined effect of hydrophobic interaction and low electrostatic repulsion. From the results of SDS-PAGE, the main proteins adsorbed at the emulsion interfaces in the RFM and CSM-based emulsions were identified as coconut 11S globulins with relatively small proportions of coconut 7S globulins, whereas the main proteins in CSM were coconut albumins.

4.2 Introduction

Proteins found in coconut milk were reported to consist mainly of 11S globulins (cocosin), 7S globulins and albumins while the type of adsorbed proteins at the interface of the droplets in homogenised coconut milk was only the 11S globulins (Tangsuphoom and Coupland, 2009). The electrophoretic patterns of these proteins under a reducing condition showed that coconut globulins were resolved into seven major bands of polypeptide subunits with the estimated molecular weights ranging from 18 kDa to 50 kDa (Tangsuphoom and Coupland, 2009). The globulins were characterised to exist in a polymerised state, rather than a single protein molecule, consisting of the polypeptide subunits with the molecular weights at 24, 35 and 55 kDa for 11S globulins and at 16, 22 and 24 kDa for 7S globulins (Garcia et al., 2005). Albumins were reported to consist of the polypeptide subunits at the molecular weights of 18, 26 and 55 kDa. The 11S and 7S globulins are known to be salt-soluble proteins, while albumins are soluble in water (Kwon et al., 1996). Therefore, properties of proteins between albumins and globulins to form and stabilise emulsions with oil droplets can be understood to differ from each other. Due to the similarities in molecular weights between these coconut proteins (11S and 7S globulins and albumins), the difference in the binding affinity and the presence of these proteins at the interface of oil droplets in fresh coconut milk, homogenised coconut milk or emulsions made from fractionated coconut milk seem to be rather complicated.

Some research studies investigated the effects of proteins fractionated from coconut milk, such as coconut cream and coconut skim milk, on properties and stability of emulsions containing 10% corn oil (Onsaard et al., 2005, Onsaard et al., 2006). These studies showed that small droplets (d_{32}) in the range of 2-8 μm and 0.5-2 μm could be formed when the fractionated proteins from cream and coconut skim milk by chemical and physical methods were used, respectively, and found that no emulsions exhibited stability against phase separation (Onsaard et al., 2005, Onsaard et al., 2006). From the previous studies shown in Chapter 3, the mean droplet diameter in emulsions containing 10% coconut oil made from coconut skim milk was substantially smaller than the emulsion droplets in coconut milk emulsions. In addition, two different homogenisation

pressures used between 220/20 bar and 550/50 bar ($1^{st}/2^{nd}$ stage) were also shown to have no significant effect on the size of emulsion droplets.

The main objective of the study in Chapter 3 was to understand some differences in the nature of two different emulsion systems made from CM and CSM. One of the two main variables studied was the effect of different ratios of coconut oil to protein. In order to be able to control the same ratio of oil to protein between two groups of emulsions made from CM and CSM, the content of oil had to be varied in the range of 5-15% for the emulsions based on CM and 3-8% for the emulsions based on CSM, but this resulted in some differences in the total solid contents between the two emulsion systems. This might have caused some differences in the results obtained between two different emulsion systems. Therefore, the objectives of this study were to further investigate the emulsion systems, based on CSM and CM, and also compare the properties of these emulsions with an un-homogenised coconut milk emulsion. In this study, a reduced fat coconut milk (RFM) from coconut milk was prepared and used to design the emulsion formulations.

4.3 Materials and Methods

4.3.1 Preparation of coconut milk fractions

Reduced fat coconut milk (RFM) and coconut skim milk (CSM) were prepared from frozen fresh coconut milk (CM) (Sagana delights, The Philippines). Frozen CM was thawed at 4°C overnight and warmed to 35°C in a water bath at 55°C. CM was then separated by a cream separator (Model LWA 205, Westfalia separator AG, Germany) into RFM and CSM. To produce RFM containing about 10% coconut oil (CO), CM was poured rapidly into a cream separator running at 12,000 rpm rather than feeding it slowly in a small quantity at a time to avoid the removal of too much CO from CM. On the other hand, CSM was prepared by slowly feeding through a cream separator to maximise the removal of CO from CM. During the separation of CSM, the separating disks of the cream separator were periodically disassembled and cleaned to remove the fat deposit accumulated in a clump to minimise the presence of CO in the CSM. Both

fractions (RFM and CSM) were then added with 0.05% (w/v) sodium benzoate as an antimicrobial agent and kept at 4°C until use.

4.3.2 Emulsion formulations and preparation

Six different emulsions were formulated from three liquid bases (CM, RFM and CSM) and virgin coconut oil (CO) (Zanian organic, Thailand) by mixing them in different combinations and proportions without adding any water as shown in Table 4.1. After the emulsion base materials were weighed and added together, the mixture was heated to 35°C in a water bath to liquefy all fat and then blended with a high shear mixer (Silverson L4RT, Silverson Machine Ltd., Waterside, England) at 6,000 rpm for two minutes to make a coarse emulsion. The coarse emulsion was subsequently homogenised using a two-stage high pressure homogeniser (APV 2000, Rannie/Gaulin, Albertslund, Denmark) at 220/20 bar ($1^{st}/2^{nd}$ stage) with four passing times.

Table 4.1 Formulations of six different emulsions containing 5% and 10% CO.

Emulsion	CM	RFM	CSM	CO	Total
	wt%				
S/5			95.1	4.9	100
S/10			90.1	9.9	100
RF/S/5		53.8	46.2		100
RF/S/10		95	3.8	1.2	100
C/RF/H	11.7	88.3			100
C/RF/UH	11.7	88.3			100

Abbreviation: S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM and RFM without and with homogenisation, respectively.

Different formulations were regulated to give the desired amount of fat levels (5 and 10%) and raise the protein concentration as high as possible. The selection of 10% fat content in formulations was to make the same level of fat content in ice creams with an intention of making ice creams containing 10% coconut oil at a later point to characterise the properties of ice creams made from coconut milk or fractionated portions of coconut milk. The selection of 5% fat was to compare the ability of proteins to emulsify and stabilise different concentrations of coconut oil. As a result, the fat level in some emulsions was diluted to have lower fat at 5% using CSM. Virgin coconut oil (CO) was used to increase the fat content of some emulsion samples to 10%. In selected emulsions consisting of CM and RFM without added CSM and bulk CO, the emulsion samples were prepared with and without homogenisation after mixing two portions of CM and RFM, which are denoted as C/RF/H and C/RF/UH, respectively. Table 4.2 shows the concentrations of fat and protein and the ratio of coconut oil to protein (O/P) used in the emulsion formulations. Table 4.3 illustrates the amounts (g) of fat and protein contained in each of the emulsion formulations (100 g basis) that were derived from CM, RFM, CSM and bulk CO.

Table 4.2 Concentrations (wt%) of protein and fat and the ratios of coconut oil to protein (O/P) in emulsions.

Emulsion	Protein	Fat	O/P
S/5	0.5	5	10
S/10	0.5	10	20
RF/S/5	0.9	5	6
RF/S/10	1.2	10	8
C/RF/H	1.3	10	8
C/RF/UH	1.3	10	8

Abbreviation: S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM and RFM without and with homogenisation, respectively.

Table 4.3 Amounts (g) of fat and protein in the emulsion formulations (100 g basis) that were derived from CM, RFM, CSM and bulk CO.

Emulsion	Fat (g)				Protein (g)		
	CM	RFM	CSM	CO	CM	RFM	CSM
S/5			0.1	4.9			0.5
S/10			0.1	9.9			0.5
RF/S/5		5				0.7	0.2
RF/S/10		8.8		1.3		1.2	
C/RF/H	2.1	8			0.2	1.1	
C/RF/UH	2.1	8			0.2	1.1	

Abbreviation: S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM and RFM without and with homogenisation, respectively.

4.3.3 Sample analyses

4.3.3.1 Proximate analysis

CM, RFM and CSM were determined for their compositions by the proximate analysis, including moisture, ash, crude protein and fat following the methods as described in Section 3.3.4.

4.3.3.2 Particle size diameter and size distribution

The average diameters (d_{43}) of particles and the particle size distribution of emulsion droplets were measured by a static light scattering technique using a particle size analyser (Mastersizer 2000MU (A), Malvern Instruments Ltd., Malvern, Worcester-shire, UK) as described in Section 3.3.5.

4.3.3.3 Zeta potential of emulsion droplets

The electrical charge (ζ-potential) of oil droplets in emulsions was measured as described in Section 3.3.6.

4.3.3.4 Microscopic examination of emulsions

The emulsion samples were analysed with the light microscope after preparation and also one day after storage at 4°C and 35°C as described in Section 3.3.7 with some modifications. In this chapter, the examination of emulsion droplets was carried out before and after dilution 5 times with 1.25% (w/v) SDS solution.

4.3.3.5 Emulsion stability against phase separation

The stability of emulsions to phase separation (creaming) was visually analysed by monitoring phase separation that occurred over time during storage for four days at 4°C and 35°C and the percentage of phase separation was also determined as described in Section 3.3.8.

4.3.3.6 Analysis of emulsion turbidity

The turbidity of emulsions were analysed by using the method described in Section 3.3.9. In this chapter, emulsions were diluted to coconut oil concentrations in a range of 0.0125-0.2 wt% with distilled water and the turbidity was measured after the emulsion preparation and also one day after the storage at 4°C and 35°C. Also, emulsion samples stored at 4°C and 35°C were analysed for their turbidity after dilution with 1.25% (w/v) SDS solution.

4.3.3.7 Analysis of protein composition by SDS-PAGE

The composition of proteins in samples, e.g. CM, CSM and RFM and also the proteins present in the serum phase and at interfacial layer of emulsions were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a method described by Laemmli (1970) with some modifications. All chemical reagents and solutions prepared for SDS-PAGE are shown in the Appendix A.5.

4.3.3.7.1 Sample preparation for SDS-PAGE

Some emulsions prepared in this study were analysed for the characterisation of proteins adsorbed at the surface of emulsion oil droplets. Briefly, emulsions were separated into cream and serum phases by centrifugation (Multifuge 1S-R, Heraeus, Osterode, Germany) at 4,500 x g at 4°C for 45 min. The serum phase was withdrawn and frozen at -20°C until analysis. The cream was collected and washed by re-dispersing in 20 mM phosphate buffer (pH 6.2) and centrifuged under the same condition described above. The washed cream was spread on a filter paper to drain and remove water for 10 min at room temperature and was kept frozen until use.

An aliquot of each sample (e.g. protein solutions or washed cream) was mixed with a sample buffer (62.5mM Tris-HCl at pH 6.8 containing 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) at around 1 mg proteins per 1 ml sample buffer and vortexed vigorously. The samples mixed with the sample buffer were then heated at 95°C for 10 min and centrifuged for 2 min at 10,000 rpm using a centrifuge (Labserve, model GyroSpin, Bilab Aust Pty Ltd, Australia). After cooling, the supernatant was withdrawn and analysed by SDS-PAGE. Some samples were also prepared and analysed under the same condition but in a non-reducing condition without adding 2-mercaptoethanol to the sample buffer.

4.3.3.7.2 SDS-PAGE analysis

An aliquot of each sample and molecular weight markers were loaded into SDS-PAGE gels consisting of 4% stacking gel and 12% resolving gel. The concentration of proteins from each sample loaded into gels was at approximately 10-20 μg proteins. The gel electrophoresis was conducted using a Bio-Rad Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Richmond, CA, USA) with a voltage of 110 mV for about 100 min or until the bromophenol blue bands reached down to the bottom of the gels.

After running SDS-PAGE in a chamber filled with an electrode (running) buffer (0.3% tris base, 1.4% glycine, and 0.1% SDS, pH 8.3), the gel was removed from the chamber and transferred into a Coomassie Brilliant Blue R-250 staining solution (Bio-Rad laboratories, Inc, Hercules, CA, USA). After staining for 30 min, the gel was transferred into an aqueous destaining solution (10% acetic acid and 7% methanol) and destained by gentle shaking and changing the destained solution periodically with a fresh destaining solution until the gel showed clearly separated bands with no background blue colour. The image of gels was captured using a scanner (CannoScan LiDE20, Cannon Inc.) The protein standard molecular weight markers (Bio-Rad Laboratories, Inc., Hercules, CA, USA) used for SDS-PAGE consisted of phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da) and lysozyme (14,400 Da).

4.3.3.8 Surface protein load of emulsions

The washed cream was analysed for the surface protein load of emulsion oil droplets by determining the amount of proteins adsorbed at the interface using Kjeldhal method as described in Section 3.3.4.3. The surface protein load was calculated using an equation described by Tangsuphoom and Coupland (2009) as follows.

$$\textbf{Surface protein loaded } (\Gamma) \textbf{ (mg m}^{-2}\textbf{)} = \frac{\text{Protein content of washed cream (mg ml}^{-1})}{\text{Surface area (m}^2\text{) x fat fraction by volume of emulsion}}$$

4.3.4.9 Statistical analysis

All experiments and measurements were carried out at least in duplicate and analysed as described in Section 3.3.10.

4.4 Results and Discussion

4.4.1 Proximate analysis of CM, RFM and CSM

The chemical compositions of CM and two coconut milk fractions derived from CM (RFM and CSM) were analysed by the proximate analysis as shown in Table 4.4. Based on the composition of emulsion ingredients analysed by the proximate analysis, six different emulsions containing 5% or 10% CO at protein concentrations of 0.5-1.3%, depending on emulsion liquid bases, were formulated and prepared by homogenising the mixtures of CM, RFM, CSM and CO at different proportions (Table 4.1).

Table 4.4 Composition of CM, RFM and CSM determined by proximate analysis and the theoretical compositions of RFM and CSM calculated based on an assumption of the removal of only coconut oil (not emulsified coconut oil droplets) from CM that would render 9.2% fat in RFM and 0.1% fat in CSM.

	Material	Composition[a], wt%				
		Moisture	Protein[b]	Fat	Ash	CHO[c]
Actual	CM	79 ± 0.1	1.7 ± 0.1	15.9 ± 0.1	0.5 ± 0.1	2.8 ± 0.1
	RFM	86 ± 0.2	1.3 ± 0.2	9.2 ± 0.1	0.5 ± 0.1	3.1 ± 0.1
	CSM	97 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.5 ± 0.1	2.1 ± 0.1
Theoretical	RFM	87	1.9	9.2	0.6	3.1
	CSM	94	2.0	0.1	0.6	3.3

[a] Mean values of 6 replicates ± standard deviation
[b] A nitrogen to protein conversion factor of 6.25 was used.
[c] Carbohydrate (CHO) estimated based on calculation.

The removal of coconut oil from CM to produce CSM and RFM by centrifuge using a cream separator was also found to remove a substantial amount of some proteins and other components. There was a reduction in the protein content from 1.7% to 0.5% when almost all coconut oil was removed from CM, resulting in 0.5% protein in CSM. If only coconut oil was removed from CM without a loss of any protein and other components, the protein content of CSM should be about 2.0%. This indicates that a

significant portion of proteins removed together with coconut oil was due to the presence of a large proportion of coconut milk protein at the oil-water interface. A major type of proteins removed from CM during the preparation of CSM could be thought to be water insoluble coconut globulins (cocosin) that have been reported to be predominantly found on the surface of coconut oil droplets of homogenised coconut milk as interfacial membrane proteins which are salt-soluble proteins (Tangsuphoom and Coupland, 2009). The amount of protein remained in CSM and RFM after the removal of coconut oil was roughly estimated to be about 30% and 70% of the total protein in CM, respectively. This was in agreement with a report by Hagenmaier et al. (1972) in that about 30% of the total protein in CM was water-soluble proteins solubilised in the CM serum phase, which were determined as albumins by SDS-PAGE which is discussed below.

4.4.2 SDS-PAGE of coconut milk liquid fractions

Types of proteins present in CM, RFM and CSM were analysed by SDS-PAGE under a reducing condition (Figure 4.1). Protein compositions of both CM and RFM were resolved into a similar pattern with seven major bands ranging from the molecular weights (MWs) of 18 kDa to 55 kDa and several minor bands with the estimated MWs of 14 kDa and 16 kDa. The patterns of SDS-PAGE for CM and RFM were quite similar to the studies reported in the literature that showed the protein bands in the range between 16 kDa and 55 kDa resolved from coconut endosperm (Garcia et al., 2005, Kwon et al., 1996) and coconut milk and homogenised coconut milk (Tangsuphoom and Coupland, 2009). The observed protein bands in CM and RFM corresponded to the subunits of two different types of salt soluble proteins, such as coconut 11S globulins (also called cocosin) at the MWs of around 22, 24, 32, 35 and 55 kDa and coconut 7S globulins at the MWs of 16, 22 and 24 kDa, and the subunits of water soluble proteins, such as coconut albumins at the MWs of 18, 26 and 55 kDa. The bands of the polypeptide subunits corresponding to 11S globulins in this study was similar to the coconut 11S globulins of coconut endosperm, which consisted of basic polypeptide subunits at 21 kDa and 24 kDa and acidic polypeptide subunits at 32 kDa and 35 kDa (Angelia et al., 2010). The proteins in CSM were separated into two major bands with the MWs of 18 and 26 kDa and four faint bands at the MWs of around 16, 22, 35 and 55

kDa. The two major protein bands including a minor band of 55 kDa corresponded to the polypeptide subunits of water soluble coconut albumins that were reported to exhibit several bands at the MWs of 18, 26 and 55 kDa (Garcia et al., 2005).

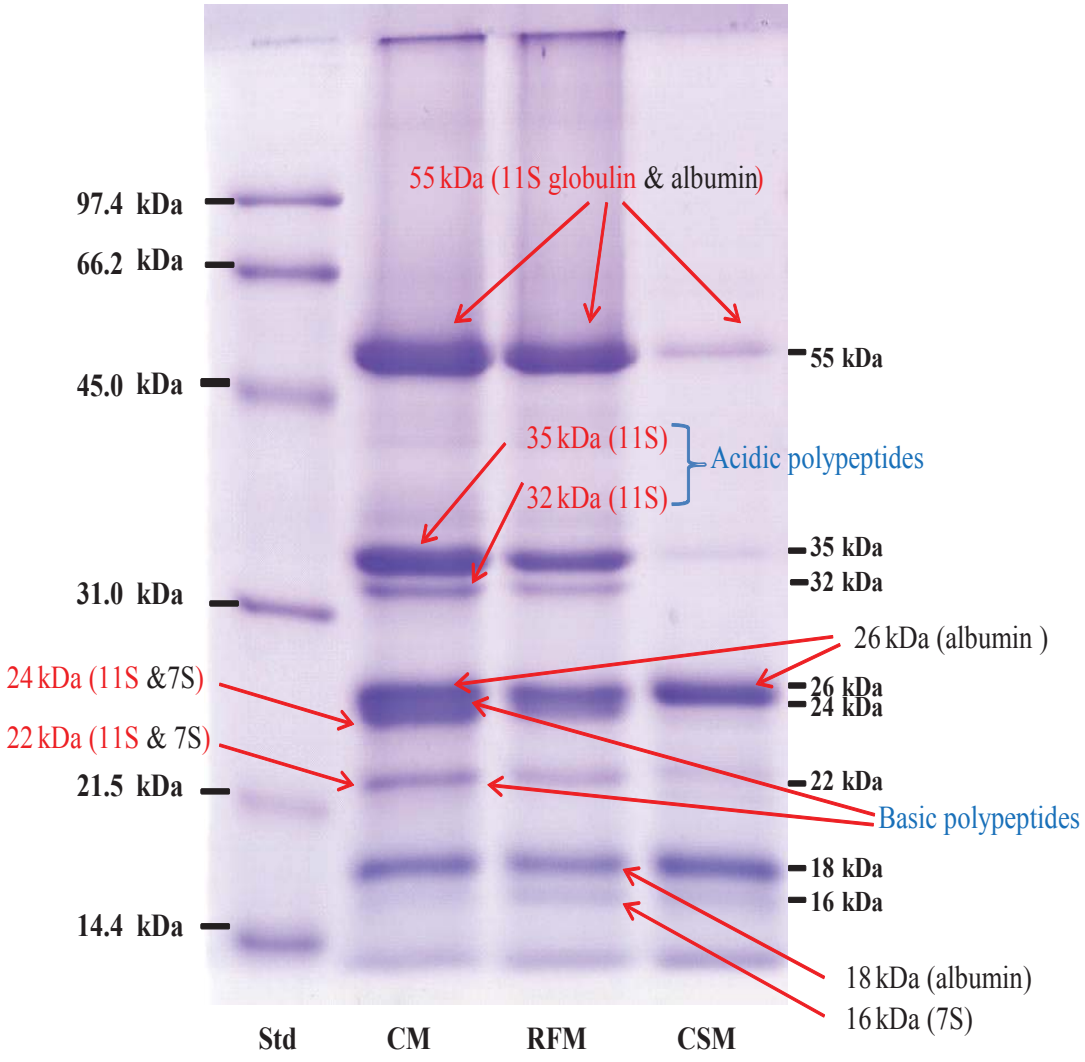

Figure 4.1 SDS-PAGE image of coconut milk (CM), reduced fat coconut milk (RFM), coconut skim milk (CSM) and standard molecular weight markers (Std).

After the separation of coconut oil (i.e. cream) from CM, the resulting CSM appeared as an opaque liquid with sedimentation when it was left undisturbed. To further analyse, the CSM was centrifuged, and the supernatant and sediment were analysed by SDS-PAGE under both reducing and non-reducing conditions (Figure 4.2).

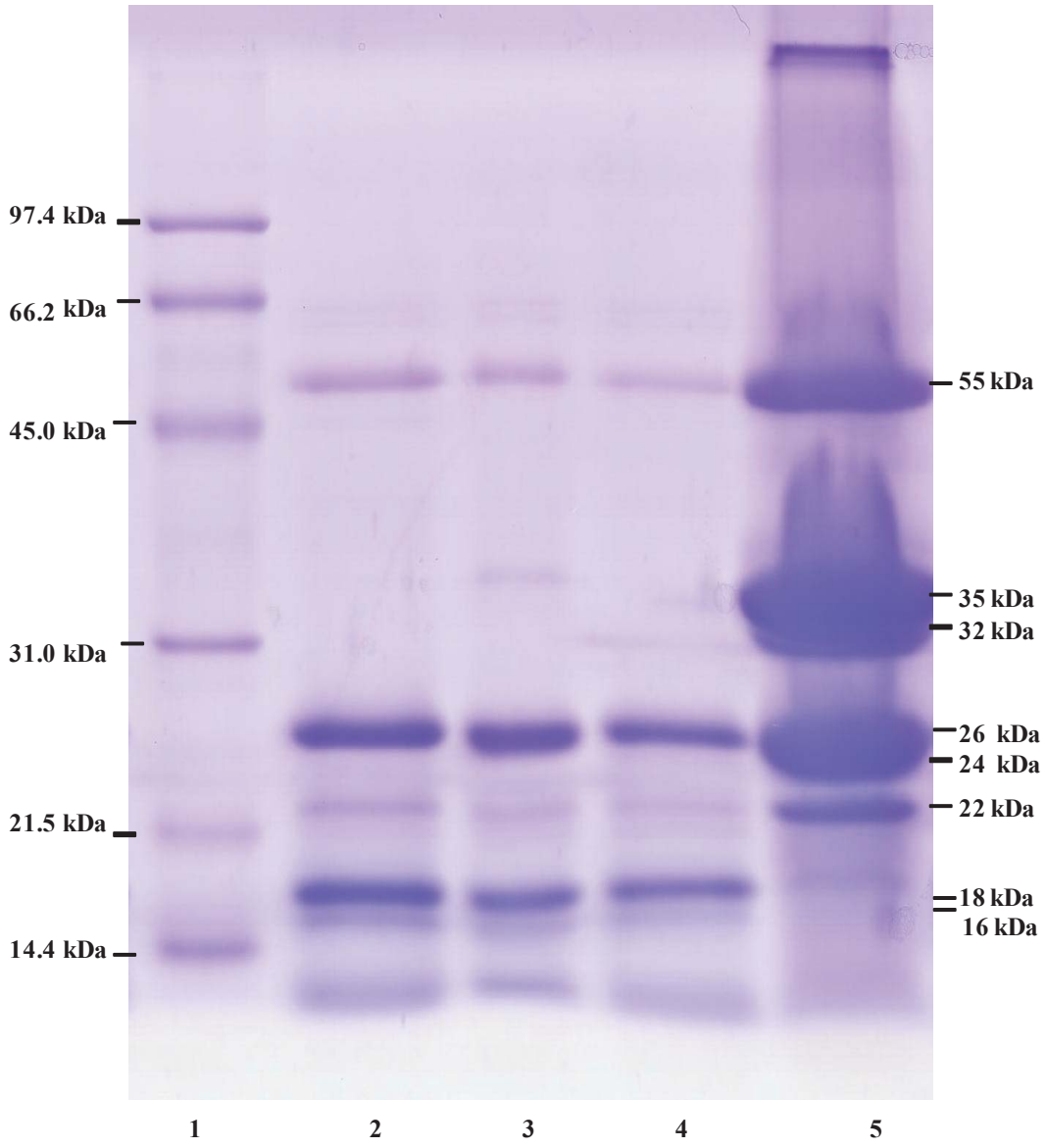

Figure 4.2 SDS-PAGE profiles of (1) standard molecular weight markers, (2) CSM with 2-mercaptoethanol (2-ME), (3) CSM without 2-ME, (4) CSM supernatant with 2-ME and (5) CSM sediment with 2-ME.

The resolved subunits of polypeptides from the original CSM without centrifugation were similar in the presence and absence of reducing agent (2-mercaptoethanol), as shown in Figure 4.2 lanes 2 and 3, indicating those polypeptide subunits (albumins) were not associated by disulphide bonds. The same electrophoretic patterns of bands were also observed from the supernatant of CSM. In the case of sediment from CSM, the different protein bands were detected which corresponded to the polypeptide subunits of water insoluble coconut 11S globulins with the MWs of 22, 24, 32, 35 and

55 kDa. This indicates a substantial amount of water insoluble proteins was also present in the serum phase of CSM in addition to their presence at the interface of coconut oil droplets and that the significant amount of the protein loss observed during the removal of CO from CM was also due to the sedimentation of these globulin proteins.

4.4.3 Particle size and size distribution of emulsions

The particle sizes and particle size distributions (PSDs) of all emulsions were analysed one day after the preparation. The PSDs of emulsions are shown in Figure 4.3.

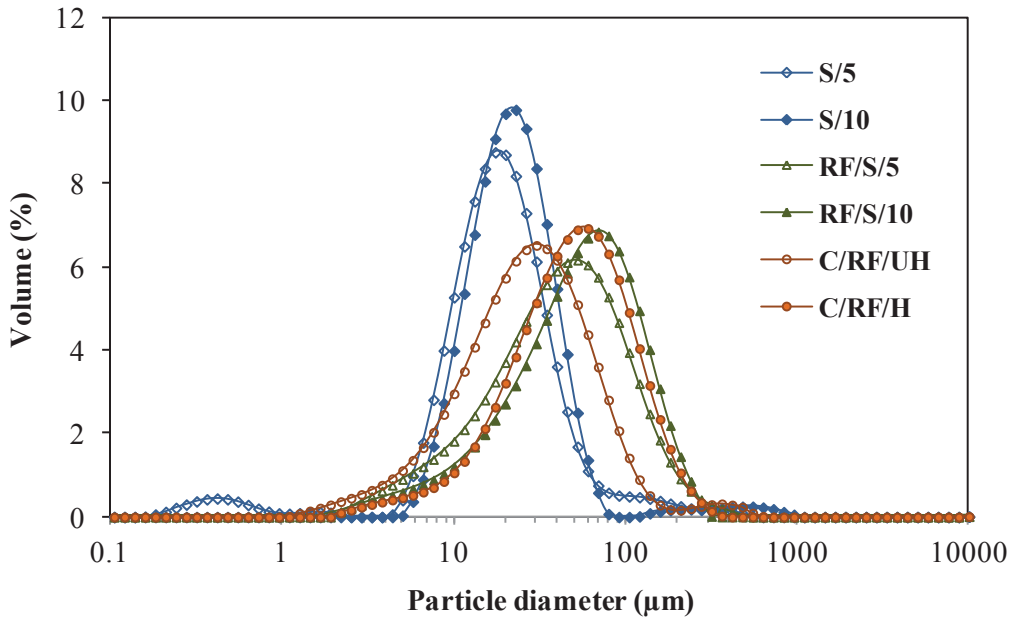

Figure 4.3 Particle size distributions of emulsions. S/5 and S/10 mean emulsions made from SM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively.

Most emulsions exhibited monomodal size distributions although one minor small peak in the small or large size regions was observed from some emulsion samples prepared with CSM at 5% or 10% CO. The major percentage of the particle volume frequency was in the range between 5 µm and 100 µm for the CSM-based emulsions. For all the other emulsions made from RFM or CM showed similar particle size distributions but

their PSDs were between 5 μm and 250 μm which was much broader than that of the CSM-based emulsions. The narrower PSD (i.e. lower span) of the CSM-based emulsions reflects that the emulsions made solely from CSM can be more stable and less sensitive to the droplet aggregation and coalescence. From the results of PSDs, it can be seen that the un-homogenised emulsions made from a mixture of CM and RF (C/RF/UH) had the PSD shift to a smaller size region compared to the corresponding homogenised emulsion (C/RF/H).

Mean particle diameters of the CSM-based emulsions containing 5 and 10% CO were around 15 μm and 20 μm, respectively (Figure 4.4). This mean particle size was much smaller than the average particle size of all other emulsions that were in the range of 40-74 μm in diameter. This confirms the results of the previous experiments shown in Chapter 3 that CSM conferred the formation of smaller oil droplets than did CM. This was in spite of the fact that the ratio of coconut oil to proteins was much higher in the CSM-based emulsions, confirming that the CSM proteins (albumins) were more effective as emulsifiers to form and stabilise smaller droplets. In addition, the observed particle size difference was related to a difference in the physical states of coconut oil initially present as bulk oil and natural emulsion droplets in the preparation of CSM-based emulsions and RFM or CM-based emulsions, respectively.

The mean particle size of natural CO emulsion droplets present in the un-homogenised sample (C/RF/UH) containing CM and RFM were around 40 μm. When this emulsion was homogenised, the particle size of the homogenised emulsion (C/RF/H) was increased to around 70 μm (Figure 4.4). This indicates that homogenisation caused the natural emulsion oil droplets present in CM to coalesce into larger droplets rather than reducing their droplet size. A similar particle size (74 μm) was also obtained from the emulsion formulation (RF/S/10) that had a similar formulation to the C/RF/H emulsion. This large mean particle size seemed to be reduced to around 51 μm when the CO concentration in the formulation (RF/S/5) was reduced to 5% with an addition of additional CSM proteins.

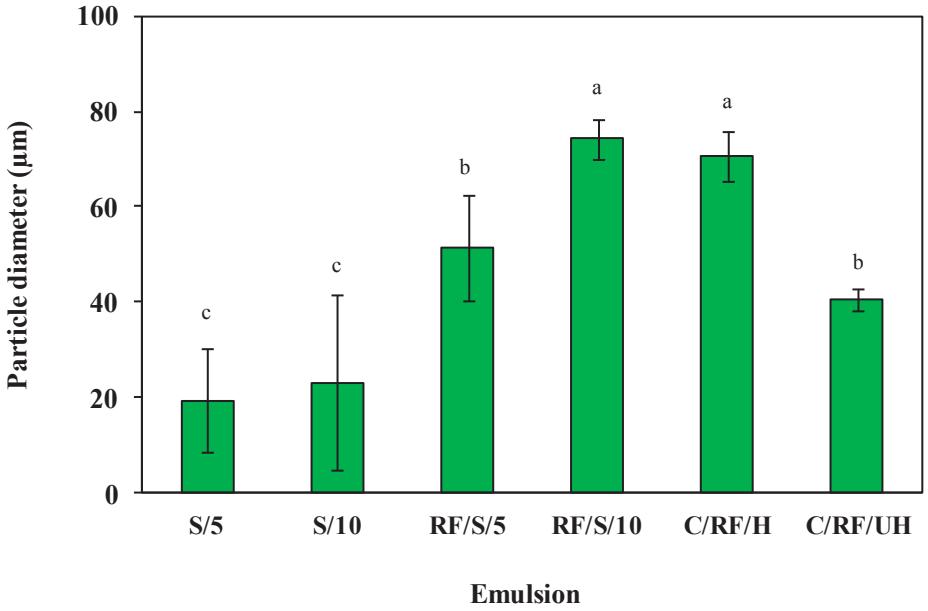

Figure 4.4 Mean particle diameter (d_{43}) of emulsions. S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

The adsorption of albumins to the interface to stabilise the broken-down oil droplets did not seem to occur in CM unlike albumins were shown to be able to stabilise the broken-down small oil droplets from bulk oil in CSM. This can be due to the low binding affinity of albumins to the interface compared to coconut globulins (cocosin). It is because cocosin is more hydrophobic than albumins (Kwon et al. 1996) and the hydrophobic sites of cocosin adsorbed to oil droplets in the homogenised RFM/CM-based emulsion become more exposure to the bulk aqueous phase which may lead to hydrophobic interactions between droplets stabilised with it, resulting in the droplet flocculation and/or coalescence.

4.4.4 SDS-PAGE of emulsions

An analysis of proteins adsorbed on the surface of oil droplets in emulsions was carried out by SDS-PAGE after separation and washing of cream from the emulsions. The

electrophoresis patterns of the interfacial proteins under non-reducing or reducing conditions are shown in Figures 4.5A and B. The serum phase of emulsions remained after the removal of cream was also analysed (Figure 4.5C). For the adsorbed interfacial proteins, the band intensity of polypeptide subunits resolved under the non-reducing condition was very weak. Another point to be made is that one protein band correcponding to a large MW of around 96 kDa was detected on gels from all samples under the non-reducing condition but this band disappered in the presence of the reducing agent, 2-mercaptoethanol, as shown in Figure 4.5B. This indicates that the polypeptide units of some proteins were linked by dissulfide bonds. Serveral studies showed that the electrophoretic patterns of proteins from coconut milk under non-reducing condtions had the bands at the MWs between 18 kDa and 98 kDa (DeMason and Sekhar, 1990, Garcia et al., 2005, Rasyid et al., 1992). According to Garcia et al. (2005), the band corresponding to the MW of 95 kDa was a result of a combination of two groups of polypeptides subunits of coconut globulins, including the basic (22 kDa) and acidic (35kDa) polypeptide subunits.

In constrast to the weakly stained or very faint bands resolved from the washed creams of emulsions under the non-reducing condition, the reduced interfacial proteins from all emulsions exhibited the polypeptide bands that were stained strongly and resolved clearly at the MWs of 22 to 55 kDa. The reduced interfacial proteins of washed creams separated from all emulsions including the emulsions made from CSM showed the same migration pattern with no distinct difference, consisting of three major bands representing protein subunits of coconut 11S globulins (cocosin) with the MWs of around 24, 35 and 55 kDa and three minor bands with the MWs of around 22, 26 and 32 kDa. Although the CSM sample exhibited the electrophoretic pattern of protein bands similar to the polypeptide bands separated from samples made from CM and/or RFM, it does not necessarily mean that the same proteins were present at the interface in both samples. Water soluble albumins consist of the polypeptide subunits with the MWs of around 18, 26 and 55 kDa as described earlier and also shown in the serum phase of emulsion samples (Figure 4.5C). Because of a similarity in their MWs of polypeptide bands between 11S globulins and albumins, these two groups of polypeptide subunits cannot be clearly distinguished on gels because of their overlapping.

However, it should also be pointed out that under the non-reducing condition, the patterns of all proteins separated between all samples were also similar, thereby it can not be ruled out that the adsorbed proteins at the interface of washed cream from the emulsions made of CSM could be mainly the salt soluble 11S globulins (cocosin) as these proteins were also found to be present in CSM from the analysis of sediment found in CSM by SDS-PAGE (Figure 4.2). As described above, there are the same or similar MWs of polypeptide subunits of different species of proteins present in CM. The band with a molecular weight of 55 kDa can represent the common subunit of either 11S or 7S or albumins (Garcia et al., 2005). The minor band with molecular weight of 22 kDa may correspond to the subunit of 7S globulin as the subunit of this globulin was shown to be present on SDS-PAGE at the MWs of 16, 22 and 24 kDa (Garcia et al., 2005).

The SDS-PAGE of proteins resolved from the serum phase after the removal of cream from emulsions appeared to be quite different from the interfacial layer proteins of washed creams (Figure 4.5C). The molecular weights of those proteins in the serum phase were all smaller than 30 kDa. Also, any noticeable difference in the patterns of protein bands between the serum phases of different emulsion samples could not be observed. The protein patterns exhibited two strong major bands with the MWs of about 18 kDa and 26 kDa which were reported to be coconut albumins (Garcia et al., 2005, Kwon et al., 1996, Tangsuphoom and Coupland, 2009).

The SDS-PAGE of washed creams was also run by loading the equal volume of samples on gels which were initially taken from the same amount of washed creams (Figure 4.6). Therefore, the difference in the band intensities stained between the samples reflected the different amounts of proteins that were present on the surface of emulsion oil droplets. It was observed that the washed cream of emulsions made from RFM and/or CM revealed more intense broad protein bands than that from CSM, indicating the surface protein load was higher in the oil droplets made from RFM and/or CM.

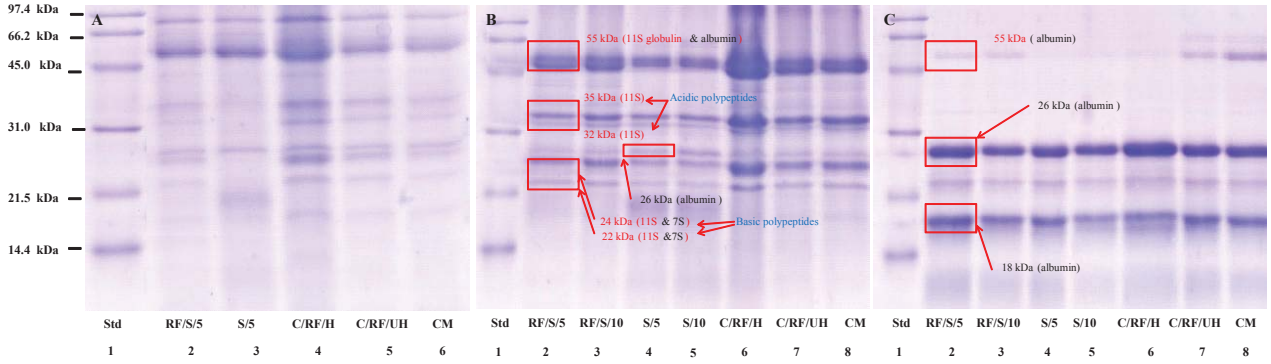

Figure 4.5 SDS-PAGE patterns for the interfacial proteins of washed creams from emulsions under non-reducing condition (A) and reducing condition (B) and the proteins in the serum phase of emulsions after the removal of cream under reducing condition (C). Std means standard molecular weight markers; S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively; and CM means original coconut milk.

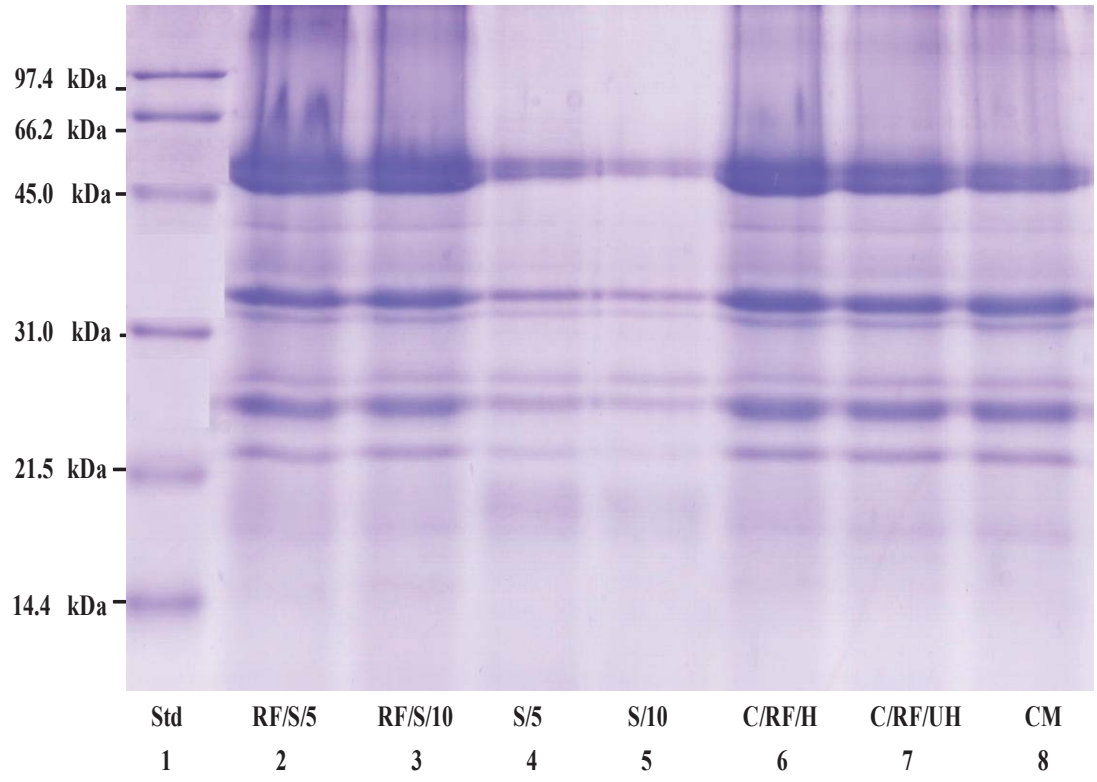

Figure 4.6 SDS-PAGE patterns of interfacial proteins separated from 1 µg washed creams of emulsions that were run under non-reducing condition. Std means standard molecular weight markers; S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively; and CM means original coconut milk.

4.4.5 Surface protein load

The amount of proteins adsorbed at the oil-water interface in emulsions was determined. The results are shown in Table 4.5. The emulsions made from a combination of RFM with CSM or CM containing 5-10% CO retained the relatively significantly larger amounts of interfacial proteins ranging from 3.1% to 4.5% than the emulsions made from CSM containing 5-10% CO that contained the amount of interfacial proteins as 0.2-04%. For the emulsions made from a mixture of CM and RFM, homogenisation led to a significant increase in the interfacial protein content from 3.1% to 4.4%.

Table 4.5 The surface protein load of oil droplets in emulsions

Emulsion	O/P	Amount of proteins in washed cream (wt%)	Surface protein load (mg m^{-2})
S/5	10	0.4 ± 0.3^{d}	3.6 ± 2.1^{d}
S/10	20	0.2 ± 0.1^{d}	1.7 ± 0.3^{d}
RF/S/5	6	4.5 ± 0.3^{a}	57.1 ± 3.3^{b}
RF/S/10	8	3.7 ± 0.4^{b}	8.3 ± 0.9^{c}
C/RF/H	8	4.4 ± 0.4^{a}	7.7 ± 0.7^{c}
C/RF/UH	8	3.1 ± 0.2^{c}	101.8 ± 5.2^{a}

Abbreviation: S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM and RFM without and with homogenisation, respectively.

Amount of adsorbed proteins at the interface of oil droplets was calculated as the surface protein load (mg m^{-2}) based on the specific surface area of oil droplets in emulsions measured by a particle size analyser. The results show a quite different pattern in the degree of the amount of adsorbed proteins between the samples when the data were compared on the basis of the percent of interfacial layer proteins per 100 g of washed cream. The surface protein load on the oil droplets of CSM-based emulsions was still observed to be low compared to the other emulsions based on RFM or CM. It was found that the emulsion made from a mixture of CM and RFM without homogenisation had the surface protein load of 101 mg m^{-2} which was significantly larger than the other samples. This may be because the measured specific surface area of droplets in the CM emulsion was rather small (data not shown), so proteins may attach to the droplet surface forming a thicker layer, rendering a high surface protein load at the droplet surface. Interestingly, homogenisation caused a significant increase in specific surface area of the homogenised droplets, thus reducing the protein surface coverage concentration of this sample from 101 mg m^{-2} to 7.7 mg m^{-2}. This was in agreement with the reported value (7 mg m^{-2}) of the protein surface load measured from homogenised coconut milk (Tangsuphoom and Coupland, 2009). From the data obtained in this study from the other samples with the surface protein loads being low or

high, a certain trend of changing the surface protein load with regard to the size of oil droplets could not be drawn.

4.4.6 Surface charge of emulsion droplets

The pH of all emulsions measured after the preparation was pH 6.5. The zeta potentials (ζ-potential) of emulsion samples were analysed (Figure 4.7). The zeta potentials obtained from some samples had a rather large variability. Overall the range of zeta potentials was between -5 mV and -20 mV and seemed to have no significant differences between the samples. A rather large variability observed could be due to various factors, including differences in droplet sizes, ionic strength and emulsion instability. The droplet charge of an unhomogenised emulsion made from a mixture of CM and RFM (C/RF/UH) was about -15 mV. This was similar to the electrical charge of oil droplets in coconut milk that was reported as -16 mV in a literature (Tangsuphoom and Coupland, 2008a). Although the emulsion made from a mix of RFM and CSM containing 5% CO had a relatively low zeta potential compared to the other samples, the possible explanation for its low zeta potential could not be drawn as no correlation could be made with the other measured parameters (e.g. particle size, surface protein load) except for its O/P ratio that was the lowest among all the samples.

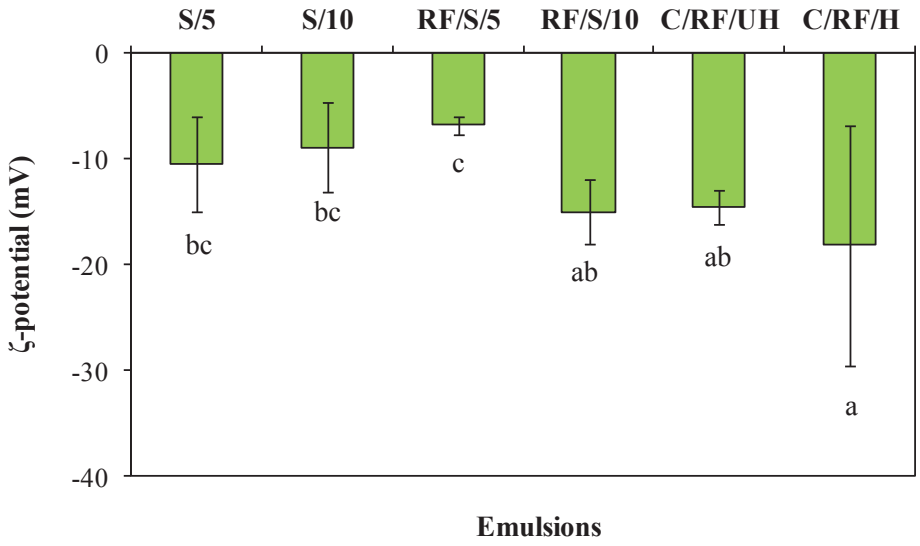

Figure 4.7 Droplet surface charges of emulsions. S/5 and S/10 mean emulsions made from SM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and SM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

4.4.7 Microscopic images of emulsions

Initially, the original samples of CM and RFM that were used to prepare the other formulated emulsions were analysed on the same day after the preparation. The micrographs of these two samples analysed are shown in Figure 4.8. The CM and RFM analysed showed that a number of natural oil droplets present in these samples were similar in appearance and present as the clusters of flocculated droplets but the droplets seemed to be discrete.

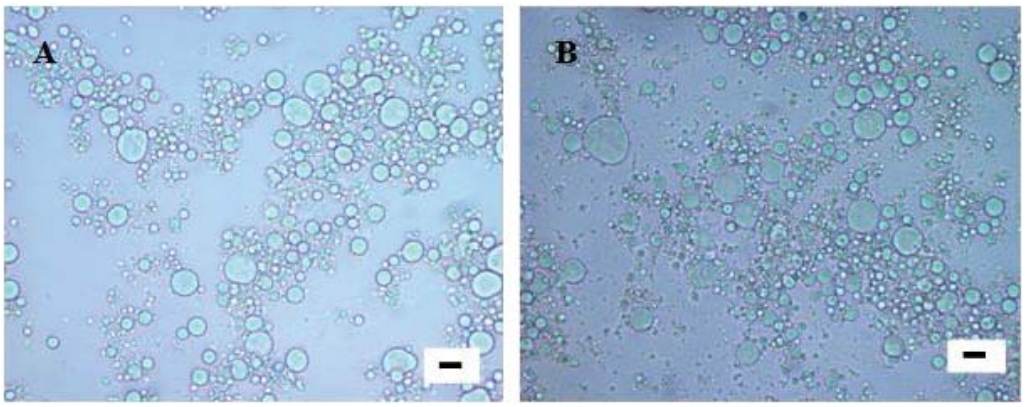

Figure 4.8 Optical images (x 400 magnification) of emulsion oil droplets in CM (A) and RFM (B). The scale bar is 15 µm.

The emulsions prepared from a mixture of CM, RFM and CSM based on six different formulations were also observed under the microscope after the preparation and also after one day storage at two different temperatures of 4^{o}C and 35^{o}C (Figure 4.9). In this case, the emulsions were analysed both in the presence and absence of SDS (dissociating agent). After the preparation, in the absence of SDS, all emulsions exhibited droplet flocculation more or less and had some big droplets mingled with small droplets. The emulsion droplets from the formulation consisting of CM and RFM without homogenisation (C/RF/UH) seemed to exist in more discrete droplets with less flocculation compared to the other emulsions. The emulsion made from CSM at 5% CO (S/5) was observed to consist of a number of small droplets with less flocculation. It should be noted that no phase separation into cream and serum phases was observed from the emulsions of S/5, S/10 and C/RF/UH on the first day of the sample preparation.

After one day storage at 4°C and 35°C, the emulsions were observed with and without dilution with 1.25% SDS solution. The microscopic images of emulsions taken are shown in Figures 4.9B-E. In the absence of SDS, the droplet flocculation could be observed in the RFM-based emulsions (RF/S/5, RF/S/10, C/RF/H and C/RF/UH) at both storage temperatures, whereas in the emulsions made from CSM (S/5 and S/10), the droplet flocculation was only observed from the sample stored at 35°C.

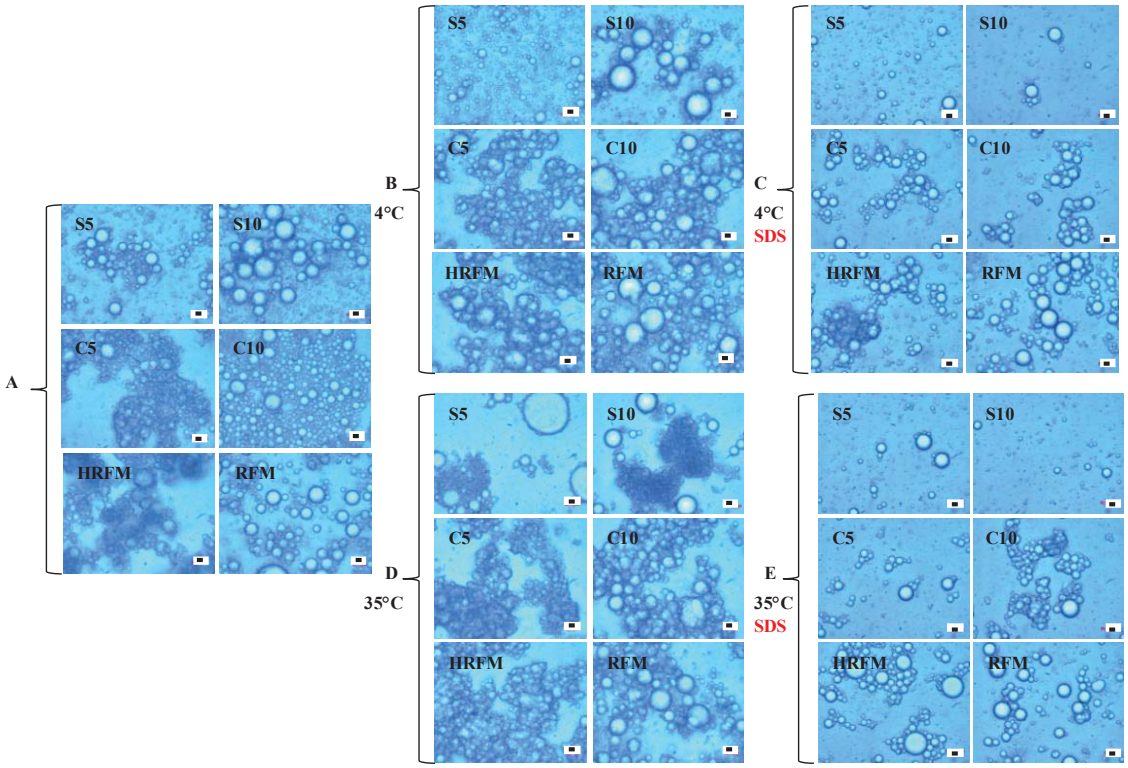

Figure 4.9 Optical images (x 1,000 magnification) of particles in emulsions that were observed after the preparation, in the absence of SDS (A) and after one day storage at 4°C, in the absence (B) and presence of SDS (C) and at 35°C in the absence (D) and presence of SDS (E). S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively. The scale bar is 5 µm.

On the other hand, in the presence of SDS, the flocculated droplets in most emulsions were dissociated, hence less clustering of oil droplets was observed. SDS is a strong anionic surfactant which can displace adsorbed proteins at the oil-water interface and break down the inter-droplet protein-protein interactions which cause droplet flocculation, agglomeration, clustering or clumps (Tangsuphoom and Coupland, 2005). Therefore, less clumping and similar oil droplet sizes were observed which infers that the destabilisation form of emulsions made from CSM, RFM and CM was due to the droplet flocculation through hydrophobic interactions. In a study by Floury et al. (2002), a high extent of aggregated droplets in 20 wt% sunflower oil-in-water emulsions stabilised by 1 and 2 wt% of 11S soy globulins was reported to be caused by

hydrophobic interaction between droplets. Under the microscope, flocs of the 11S soy proteins-stabilised droplets were observed to be dispersed into smaller individual droplets when the emulsions were diluted with SDS solution.

It was previously reported that coconut oil emulsion in coconut milk is prone to bridging flocculation of oil droplets (Tangsuphoom and Coupland, 2005). Since the protein content of the emulsion systems prepared in this study was between 0.5% and 1.3% which was not high relative to the amount of coconut oil (5 and 10%), bridging flocculation may be a prevalence in such a low protein concentration system (Dalgleish, 2004). During homogenisation, all newly formed surface areas of oil droplets may not be fully covered by the surface active proteins present in CM because the amount of available proteins is limited. As a result, a single protein molecule of coconut milk proteins may adsorb to two different fat droplets, causing them to share the interfacial layer and resulting in bridging flocculation (Dalgleish, 2004). Therefore, the droplet flocculation of coconut oil droplets can be induced by at least two different mechanisms, such as hydrophobic interaction and bridging flocculation.

4.4.8 Emulsion stability against phase separation

After the emulsion preparation, some emulsions were observed to rapidly undergo the gravitational phase separation into two distinct bulk liquid phases: cream and serum phases. Also, it was noticed that the stability of emulsions made from CM and RFM were different from the emulsions made solely from CSM. Therefore, the emulsion samples were visually monitored for their stability against phase separation. The images of samples in Figure 4.10 show that phase separation took place relatively fast in the homogenised emulsions (RF/S/5, RF/S/10 and C/RF/H) made from a mixture of RFM and CSM or RFM and CM. These samples visually exhibited a clear separation very quickly on the first day of the preparation (Figure 4.10A). On the other hand, emulsions made from CSM (S/5 and S/10) and also from a mix of CM and RFM (C/RF/UH) without homogenisation showed a high stability without creaming after their preparation, thus enabling them to remain stable without phase separation for a longer time. A relatively high emulsion stability observed from the CSM-based

emulsions containing 10% CO implies that the droplets in this emulsion were much smaller in size and could remain stable without separation at least for around half a day. In other words, the CSM-based emulsions may be feasible to use in making ice cream as it can remain stable at least for 4 hours of ageing process at 4^{o}C. This was important because one of the project objectives was to investigate the characteristics of ice cream made from coconut milk without using any dairy ingredients and to make ice cream, the ice cream mix emulsion should be reasonably stable with smaller particle size.

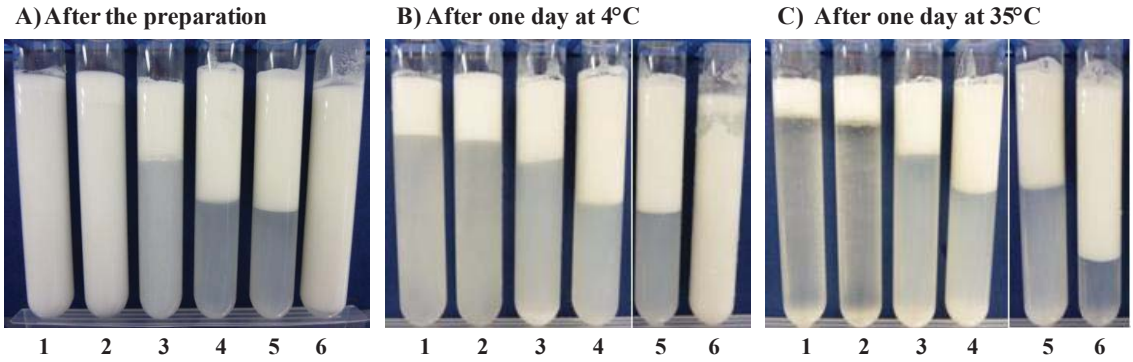

Figure 4.10 Stability of emulsions made from various fractions of coconut milk after preparation (A) and after one day storage at 4°C (B) and 35°C (C). Samples 1: S/5, 2: S/10, 3: RF/S/5, 4: RF/S/10, 5: C/RF/H and 6: C/RF/UH

After all emulsions were stored for one day at 4°C and 35°C, a clear separation was observed to occur at both storage temperatures, except for an emulsion (C/RF/UH) made from a mixture of CM and RFM without homogenisation and stored at 4°C (Figures 4.10B). This suggests that protein molecules or any other amphiphillic macromolecules (e.g. phospholipids) present in CM could not provide high stability against droplet aggregation, resulting in clear quick phase separation. However, proteins present in CSM were able to provide a short-term stability to the emulsions made from it. The emulsion instability observed in this study could be due to the properties of coconut milk proteins rendering the relatively large particle size of emulsions and their tendency to attract to each other by hydrophobic interaction leading to droplet flocculation and clustering. The visual observations of two emulsion samples (C/RF/H and C/RF/UH) made from a blend of CM and RFM with and without homogenisation revealed that as described earlier, the emulsion treated with

homogenisation became more destabilised due to their increase in the particle size. Its stability against phase separation was lower unlike the un-homogenised emulsion (C/RF/UH) remained more stable without clear phase separation after the preparation and even after storage at both temperatures compared to all other samples. At 35^{o}C, this unhomogenised emulsion containing natural coconut oil droplets exhibited a clear serum phase at the bottom but the height of serum phase was much lower than that from the respective homogenised sample (Figure 4.10C). This suggests that the surface of natural coconut oil droplets underwent some physicochemical changes via homogenisation, inducing strong attraction between droplets, thereby, the height of a cream layer formed at the top was smaller due to more packing of oil droplets in the cream phase.

4.4.9 Emulsion turbidity against droplet flocculation

The turbidity of emulsions was also measured to evaluate and compare the stability of oil droplets between samples. The turbidity of emulsions varies inversely with the oil droplet size, i.e., the lower the turbidity, the bigger the droplet size or clump (Reddy and Fogler, 1981). In this study, emulsions were diluted with water at various ratios to create a turbidity curve of absorbance against oil concentration within a linear range (Figure 4.11). The turbidity of the diluted samples was analysed on the same day after the preparation of emulsions and also after one day storage at 4°C and 35°C. On the first day, the CSM emulsion (S/5) containing 5% oil exhibited the remarkably high absorbance values, indicating this emulsion contained the smaller droplets compared to the other samples. On the other hand, the emulsion samples, such as RF/S/10 and C/RF/H emulsions, had the relatively low absorbance which indicates a number of large oil droplets retained in these emulsions. These results were in line with the results of particle size analysis shown in Figure 4.4.

As described earlier from the microscopic examination of emulsions, the emulsion (C/RF/UH) made from a blend of CM and RFM without homogenisation was shown to consist of the discrete oil droplets with less flocculation and their particle size was also relatively smaller than the other emulsions made from CM or RFM. As a result, the turbidity of this sample was also observed to be high even though the particle size did not appear very small from the microscopic examination. The turbidity of the other emulsions (RF/S/5 and RF/S/10) was measured to be low, indicating the larger particle size present in the emulsions based on the turbidity measurement, which was also in agreement with the data of the mean particle size analysed. This was in spite of the fact that the size of particles was visually observed to be smaller under the microscope. This means the particle size of clumps of small droplet agglomerates could be measured to be larger than their actual size if the agglomerates or flocs were not dissociated by gentle agitation employed during the particle size analysis. When this emulsion (C/RF/UH) was subjected to homogenisation, the turbidity of the resulting emulsion (C/RF/H) became lowered and the particle size was also measured to be larger compared to the corresponding emulsion without homogenisation. This implies that oil droplet flocs might be present but could not be dispersed and/or that oil droplets coalescence might be introduced into the emulsion.

Samples of emulsions stored at two different temperatures showed some different patterns of dispersion (Figures 4.11B and C). At 4°C, most emulsions (S/5, S/10, RF/S/5, RF/S/10, C/RF/UH and C/RF/H) showed not much change in the absorbance values relative to the initial absorbance values before storage. On the other hand, after one day at 35°C, the absorbance values indicated the presence of bigger particles in the emulsions (S/5 and S/10) made from CSM, resulting in a significant decrease in their turbidity which means these emulsions probably underwent droplet flocculation and/or coalescence. Interestingly, this phenomenon was not observed in the other emulsions as the turbidity was similar between 35°C and 4°C.

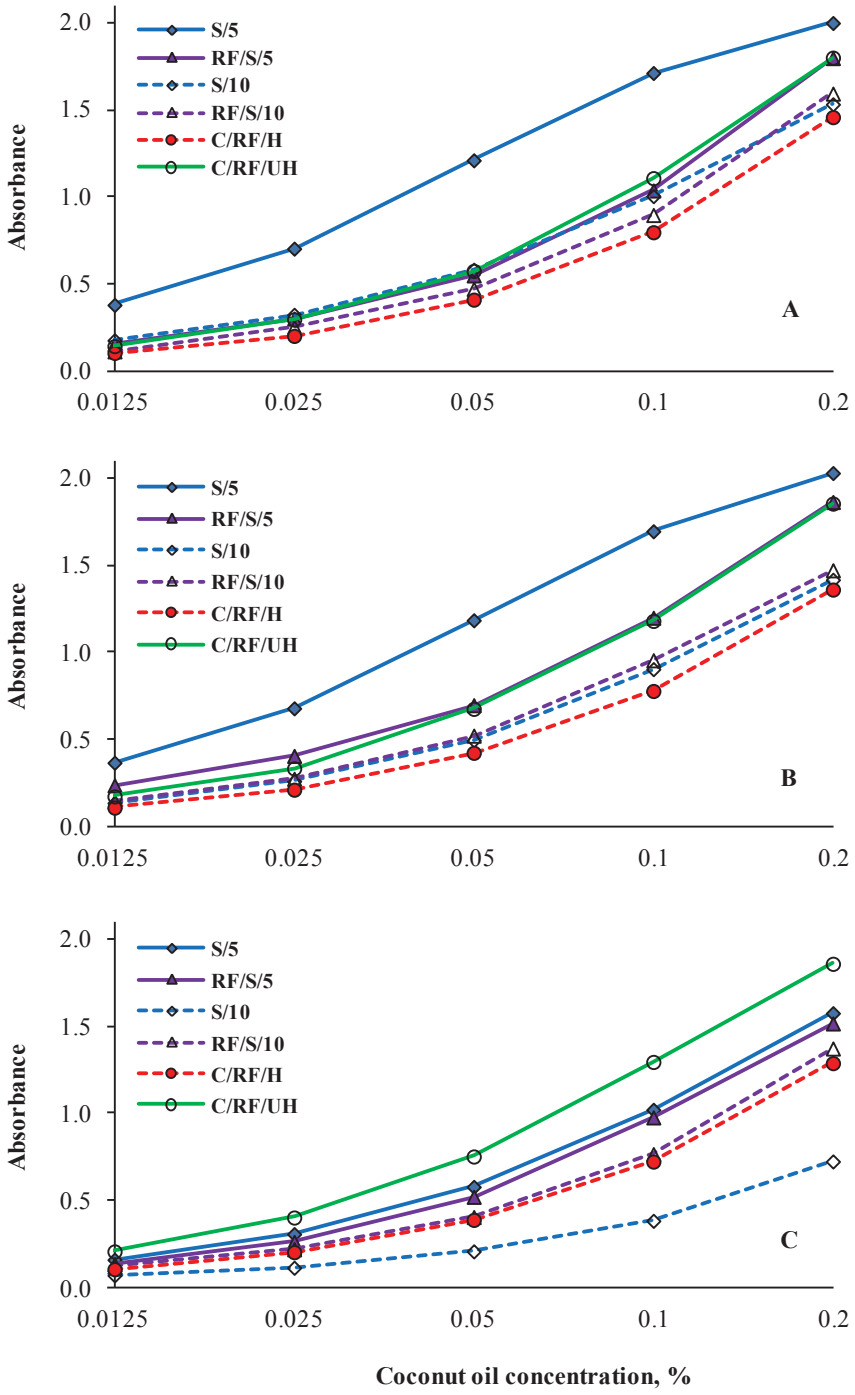

Figure 4.11 Turbidity of emulsions after dilution with water at different oil concentrations that was measured on the same day as the emulsion preparation (A) and after one day storage at 4°C (B) and 35°C (C). S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively. Data points are mean values from 6 measurements.

It is thought that the destabilisation observed at 35°C in emulsions made from CSM could be due to the composition and structural integrity of their interfacial layer differing from the other emulsions which might not be rigid and thick and be less compact and dense. As a consequence, the interface could probably be more readily disrupted and broken, thus leading to the droplet destabilisation such as coalescence, particular at a higher temperature. The other emulsions also showed lower turbidity after they were kept at 35°C but were not very different as compared to those at 4°C as already described.

In order to investigate whether the droplet flocculation or coalescence formed in the emulsions after storage at both temperatures, the samples of all emulsions were dispersed and diluted at the same oil concentration of 0.1% in water or 1.25% (w/v) SDS solution at room temperature (20°C). From the absorbance measurement shown in Figure 4.12, it appears that the storage at 4^{o}C did not cause any significant change in the turbidity of most emulsion samples when it was measured in the absence of SDS as their absorbance values were similar before and after storage at 4°C. A similar pattern was also observed at 35^{o}C in the absence of SDS, except for the two emulsions (S/5 and S/10) made from CSM. At 35°C, the turbidity of the latter two emulsions was much lower in the absence of SDS, indicating that there was a significant increase in the oil droplet size. The increase in the droplet size was due to the droplet coalescence rather than the droplet flocculation since the turbidity of these emulsions in the presence of SDS only rose slightly. This indicates that the droplet coalescence took place in these samples during storage at 35°C rather than the flocculation of droplets.

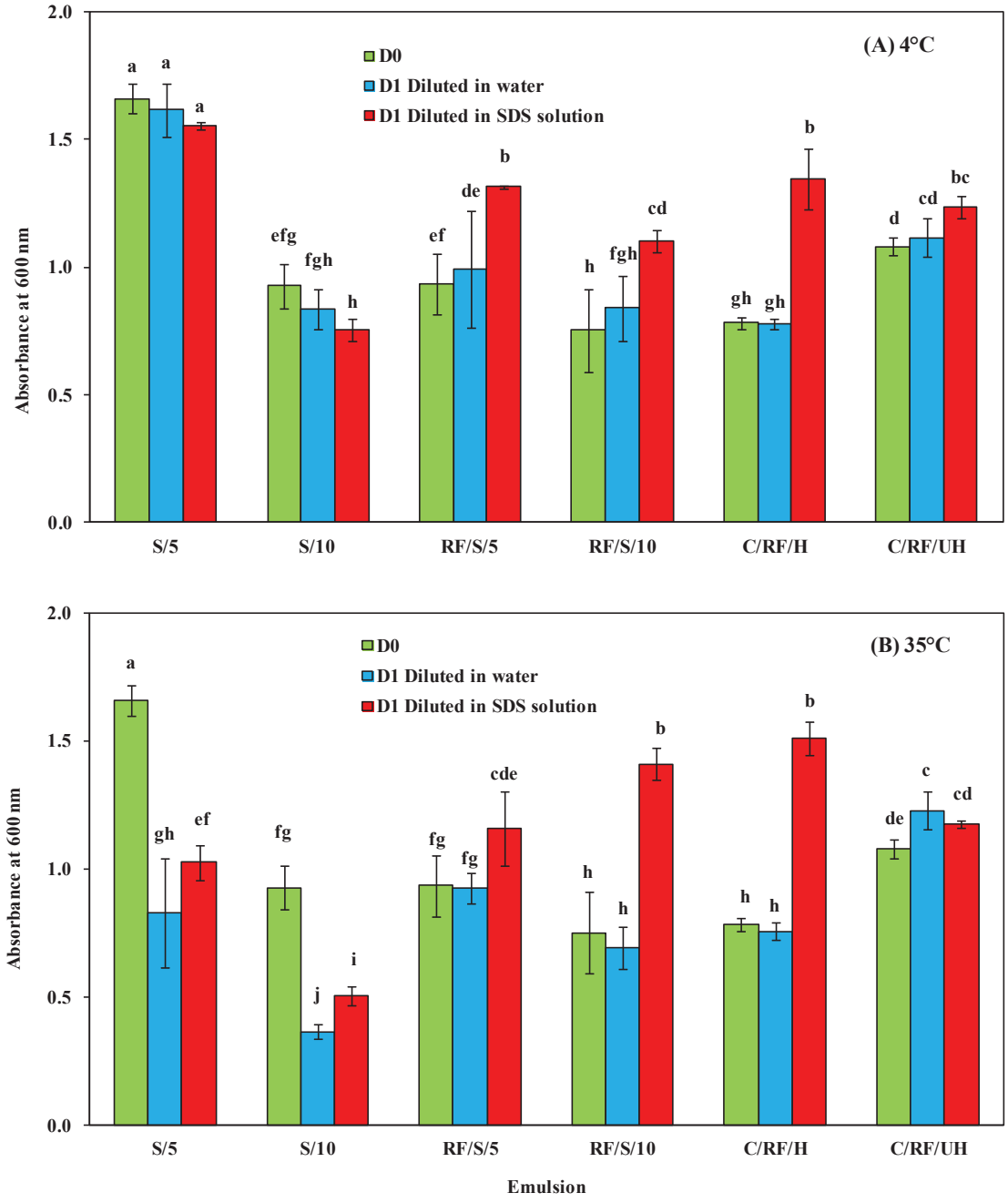

Figure 4.12 Turbidity of emulsions measured after the emulsion preparation with dilution with water (D0) and after one day storage at 4°C (A) and 35°C (B) after dilution with water (D1) and 1.25% SDS solution (D1). S/5 and S/10 mean emulsions made from CSM with 5% and 10% CO, respectively; RF/S/5 and RF/S/10 mean emulsions made mainly from a mixture of RFM and CSM with 5% and 10% CO, respectively; C/RF/UH and C/RF/H represent emulsions made from a mixture of CM/RFM without and with homogenisation, respectively. Data points are mean values ± standard deviation, n = 6.

It was observed that flocculation was less significant in the CSM emulsions because their absorbance values were relatively similar regardless of their dilution with water or SDS solution. However, when the RFM emulsions were diluted with the SDS solution, greater absorbance was observed relative to the respective samples diluted with water. The SDS absorbs at the oil-water interface and it can displace proteins at the interface and dissociate protein-protein interactions (Walker, 2009), thus more discrete fat droplets could be released from flocs, resulting in the higher absorbance values. This leads to a conclusion that the flocculation present in the RFM emulsions, possibly by the hydrophobic interaction between the droplets but the protein bridging can also not be ruled out because the interactions of proteins could be broken down by the additional small molecule surfactants like SDS (Dalgleish, 2006).

4.5 Conclusions

Emulsions were prepared from CSM and RFM as main emulsion bases at two different oil concentrations at 5 and 10 wt%. The comparisons of emulsification and stabilisation capacities of proteins between CSM and RFM confirmed some findings of the previous studies described in Chapter 3 that the proteins in CSM were more effective than ones in RFM in emulsifying coconut oil. Also, homogenisation led to a significant change in the properties of oil droplets naturally present in CM and RFM. When a mixture of CM and RFM was homogenised, the oil droplets became more flocculated after homogenisation compared to the corresponding un-homogenised mixture of CM and RFM.

In terms of the effect of oil concentration, the diameter of droplets did not show any significant differences between the CSM-based emulsions containing two different concentrations of 5 and 10% coconut oil. However, the oil droplet size was observed to increase in the RFM-based emulsion at the higher oil concentration of 10%. This suggests that the ability of proteins in RFM in emulsifying oil droplets is lower than the proteins in CSM and has some limitation. Although the proteins in CSM possess a higher emulsification capacity, they could not render their emulsions with a long-term stability as the CSM-based emulsions also exhibited a clear phase separation after one

day storage at 4°C and 35°C as the emulsions made from RFM did. From the results of SDS-PAGE, the proteins predominantly present in CSM were identified as coconut albumin proteins while in RFM, 11S globulin proteins were main predominant proteins including a relatively small amount of 7S globulins and albumins. After homogenisation of RFM, 11S globulins were mainly observed at the droplet surface. The presence of these globulin proteins is believed to lead to more extensive droplet flocculation in the emulsions based on RFM. This caused the rapid creaming and phase separation of oil droplets. The most possible mechanism involved in the droplet aggregation in these emulsions could be due to hydrophobic interactions as globulins are more hydrophobic than albumins, hence creating more aggregation of droplets in emulsions.

Chapter 5

Characterisation of properties and stability of coconut oil-in-water emulsions prepared using freeze dried coconut skim milk and reduced fat cream powders

5.1 Abstract

This study was carried out to investigate the effect of ratios of proteins derived from two protein powders prepared from coconut skim milk (CSM) and coconut cream (CC) on the size and stability of 10 wt% coconut oil-in-water emulsions. CSM and CC fractions were prepared from coconut milk by centrifugation and both fractions were freeze dried to produce coconut skim milk powder (CSMP) and coconut cream powder (CCP). Emulsions were prepared using these two freeze-dried powders at two different levels of protein concentrations (0.4 and 1.2%) consisting of four different ratios of proteins derived from CCP and CSMP (10:0, 7:3, 3:7 and 0:10). The results of SDS-PAGE show that major types of proteins found in CCP were subunits of cocosin (11S globulins) while albumin proteins were predominant proteins in CSMP. The average volume weighted diameter (d_{43}) of emulsion droplets was measured, in the presence and the absence of dissociating agent (e.g. SDS), to determine the real mean and apparent diameters, respectively. Among the emulsion samples prepared, the smaller real and apparent mean diameters (2.4 μm and 9.4 μm) were observed in emulsions prepared at 1.2% protein derived only from CSMP whereas the larger real and apparent mean diameters (13.3 μm and 68.2 μm) were detected in emulsions prepared at 0.4% protein derived only from CCP. The more proteins added from CSMP than CCP resulted in the formation of smaller emulsion droplets, whereas the more significant droplet flocculation was observed in emulsions as the more proteins from CCP were added into emulsions at a given protein concentration. Most emulsions underwent phase separation during one day storage at both 4°C and 35°C due to the existence of droplet aggregates and also due to droplet coalescence, especially at 35°C. At 4°C, the CSMP protein-stabilised droplets at 1.2% protein were relatively more stable to droplet coalescence compared to the same emulsion at 0.4% protein and the CCP protein-stabilised droplets at 0.4 and 1.2% protein. Overall the results indicate the significant

influence of ratios and concentration of proteins from coconut milk fractions between CSMP and CCP on the formation and stability of emulsions.

5.2 Introduction

During homogenisation of oil in an aqueous solution containing proteins that possess emulsifying properties, the protein molecules migrate and adsorb at the oil-water interface and spread around the surface of oil droplets as a stabilising layer, thus enabling the reduction of interfacial tension between oil and water and stabilising the oil droplets in the water phase without separation (Dalgleish, 2004). The properties of proteins, such as molecular size, electrical net charge, ratio and distribution of hydrophilic to hydrophobic amino acids, conformational structure and the presence of hydrophobic patches on the exterior surface of protein molecules, are determined by the composition and sequence of amino acids (Damodaran, 1994). These properties in turn determine the functional properties of proteins, such as solubility and surface active properties (Damodaran, 1994). This means that in some way the emulsifying capacity of proteins and the stability of emulsions stabilised by proteins depend on the delicate balance of the hydrophilic and hydrophobic amino acid residues of proteins.

A strong correlation between the surface hydrophobicity of proteins and their emulsifying capacity has been shown (Aoki et al., 1981, Kato and Nakai, 1980). The more hydrophobic proteins have a higher binding affinity to the oil and water interface. Therefore, proteins that have the high surface hydrophobicity may be considered as good emulsifiers (Kato and Nakai, 1980, Nakai, 1983, Townsend and Nakai, 1983). However, proteins with high hydrophobicity but with a low electrical net charge can cause droplet flocculation and coalescence in emulsions by hydrophobic interaction. For instance, it was reported that soybean 11S globulins led to a substantial aggregation in emulsions stabilised with these proteins (Floury et al., 2002). According to Kwon et al. (1996), coconut globulins have a high level of hydrophobicity compared to coconut albumins.

From the previous study shown in Chapters 3 and 4, two fractions of coconut milk, i.e. reduced fat coconut milk (RFM) and coconut skim milk (CSM), were utilised to prepare coconut oil-in-water emulsions containing 10% coconut oil. Smaller emulsion droplets could be formed by proteins in the CSM fraction, however when RFM was used, the emulsion droplets in RFM-based emulsions were measured to be greater in size. Despite the fact that CSM is one of the constitutive components of RFM, the emulsion droplets formed in RFM by homogenisation were very different being larger.

This study was aimed to further investigate the protein-rich powders derived from two different fractions, coconut skim milk and coconut cream, of coconut milk for their capacity to emulsify and stabilise coconut oil. To investigate this, coconut skim and coconut cream separated from coconut milk were freeze dried to increase the protein level in both fractions to determine whether the stability of emulsions against phase separation can be improved by increasing the level of protein concentrations and emulsifying only bulk coconut oil unlike the previous experiments for the preparation of emulsions based on coconut cream containing naturally existing emulsified oil droplets. In this study, to eliminate a factor of natural coconut oil droplets, the removal of coconut oil from coconut cream was attempted by a solvent fat extraction. Both coconut skim milk and reduced fat coconut cream were freeze dried and then used to prepare emulsions containing 10% coconut oil at two different protein levels consisting of four different ratios of these two freeze-dried powders.

5.3 Materials and Methods

5.3.1 Materials

The same frozen coconut milk (Sagana delights, The Philippines) and virgin coconut oil (Zanian organic, Thailand) used in the previous experiments of Chapters 3 and 4 were also used in this study of Chapter 5.

5.3.2 Preparation of coconut skim milk powder and coconut cream powder

Frozen coconut milk was thawed, warmed and separated into two fractions, coconut skim milk (CSM) and coconut cream (CC) using the same methods as described in Section 4.3.1. These two fractions were freeze dried to produce coconut skim milk powder and coconut cream powder. Briefly, frozen fresh coconut milk was thawed, heated to 35^{o}C and separated into two phases, coconut skim milk and coconut cream, using a cream separator (Model LWA 205, Westfalia separator AG, Germany) at 12,000 rpm. Coconut skim milk and coconut cream fractions were then frozen at -20^{o}C for 24 hr and freeze-dried (Labconco, Freezone 6, Labconco Corporation, Kansas City, Missouri, USA) for approximately 4 hr or until dry. In case of the freeze-dried cream powder, it was further treated for the removal of coconut oil by solvent extraction with diethyl ether at a ratio of 1:4 (w/v) in order to produce the reduced fat coconut cream powder and increase its relative protein concentration. The removal of coconut oil from the freeze-dried cream powder was done by dispersing in diethyl ether and mixing the mixture with a stirrer bar for 30 min before filtering solvent out using a filter paper. The obtained reduced fat cream powder was air dried for 3 hr followed by drying in an oven at 50°C for 1 hr.

These two powders, coconut skim milk powder (CSMP) and reduced fat coconut cream powder (CCP), containing proteins mainly derived from coconut skim and cream phases (e.g. mainly serum proteins of coconut milk and adsorbed interfacial layer proteins of emulsion oil droplets of coconut milk, respectively) were used to prepare oil-in-water emulsions containing coconut oil.

5.3.3 Emulsion preparation

Coconut oil-in-water emulsions containing 10% coconut oil were prepared at two different levels of protein concentrations (0.4 and 1.2%) and four different ratios of protein fractions derived from CCP and CSMP (100:0, 67:33, 33:67, and 0:100), which are denoted as 10:0, 7:3, 3:7 and 0:10, respectively. The emulsion formulations used to produce a total of 8 different batches are shown in Table 5.1.

Table 5.1 Formulations of emulsions prepared with CCP and CSMP at two different levels of protein concentrations, each consisting of four different ratios of protein fractions derived from CCP and CSMP.

Ingredient (g)	0.4% protein				1.2% protein			
	Ratio of proteins derived from CCP to CSMP							
	10:0	7: 3	3: 7	0:10	10:0	7: 3	3: 7	0:10
CCP	1.1	0.7	0.4	0	3.1	2.1	1.1	0
CSMP	0	0.9	1.8	2.6	0	2.6	5.3	7.9
Coconut oil	10	10	10	10	10	10	10	10
Water	89	88	88	87	87	85	84	82
Total	100	100	100	100	100	100	100	100

The aqueous solutions (0.4% and 1.2% protein) containing CCP and/or CSMP at four different ratios (10:0, 7:3, 3:7 and 0:10) were prepared by dispersing and solubilising the powders in distilled water with a gentle stirring and left overnight at 4^{o}C for full dissolution and hydration. The virgin coconut oil (Zanian organic, Thailand) melted at 65^{o}C was added into the aqueous solutions that were pre-heated at 65^{o}C for 5 min in a water bath. The mixture was then roughly homogenized using a high shear mixer (Silverson L4RT, Silverson Machine ltd, Waterside, England) at 6,000 rpm for 2 min to prepare a coarse emulsion. The coarse emulsions prepared were homogenised four times by a two-stage high pressure homogeniser (APV 2000, Rannie/Gaulin, Albertslund, Denmark) with the first and second stage pressures at 220 and 20 bars, respectively. All emulsion samples were prepared in duplicate.

5.3.4 Sample analyses

5.3.4.1 Proximate analysis

The composition of CCP and CSMP was analysed by the proximate analysis for the determination of moisture, ash, protein and fat contents following the methods as described in Sections 3.3.4.1-3.3.4.4.

5.3.4.2 Particle size diameter and size distribution

The particle size and size distribution of emulsions were measured within 3 hours after the emulsion preparation and also after one day storage at two different temperatures (4^{o}C and 35°C) by a static light scattering technique using a particle size analyser (Mastersizer 2000S, Malvern Instruments Ltd., Malvern, Worcester-shire, UK) following the method as described in Section 4.3.3.2 with some modifications. In this chapter, the particle size of emulsions was measured with and without dilution with 1.25% (w/v) sodium dodecyl sulphate (SDS) solution at a ratio of 1:4 (v/v). The samples mixed with SDS was kept at least 3 hr at 25^{o}C prior to the particle size measurement. SDS is used as a dissociating agent to dissociate flocculated or agglomerated droplets (i.e. clusters of emulsion droplets) into single droplets but does not affect the droplet size of non-aggregated droplets (Gelin et al., 1994, Granger et al., 2003, Sánchez and Patino, 2005, Tangsuphoom and Coupland, 2009, Tomas et al., 1994a). The particles of emulsions measured in the presence and absence of the dissociating medium are referred to as "real droplets or dissociated droplets" and "apparent droplets", respectively (Sánchez and Patino, 2005, Tomas et al., 1994a).

5.3.4.3 Zeta potential of emulsions

The electrical charge (ζ-potential) of the coconut oil droplets in emulsion samples was measured using the method as described in Section 3.3.6.

5.3.4.4 Microscopic examination of emulsions

The characteristics of emulsion droplets were observed following a method as described in Section 4.3.3.4.

5.3.4.5 Emulsion stability against phase separation

The stability of emulsions against phase separation was carried out following a method as described in Section 4.3.3.5.

5.3.4.6 Analysis of protein composition by SDS-PAGE

The types of proteins present in CCP and CSMP and the interfacial proteins of the cream and serum phases of emulsions were characterised by SDS-PAGE (Mini-PROTEAN Tetra cell, Bio-Rad) following a method described by Laemmli (1970) as described in Section 4.3.3.7.

5.3.4.7 Analysis of protein content by a Lowry method

The protein content of washed cream was determined by the Lowry method as described by Waterborg (2009). To 100 µl of samples, 100 µl of 2N NaOH was added. The alkali mixture was hydrolysed at 100 °C for 10 min in a boiling water bath then cooled down to room temperature before adding 1 ml of a freshly prepared complex-forming reagent (see Appendix B.2). The solution was left to stand at room temperature for 10 min. After cooling, 100 µl of a folin reagent was added and mixed with a vortex mixer (Velp Scientica, Italy) and then allowed the mixture to stand at room temperature for at least 30 min (not over 60 min). The absorbance of the solution was observed at 500 nm using a spectrophotometer (Shimadzu, Shimadzu Corporation, Japan). The concentration of protein in samples was determined by using a standard curve created from whey protein isolate (WPI 895, Fonterra, New Zealand) that was determined under the same conditions. All reagents used and a standard curve generated from WPI are shown in the Appendix B.2.

5.3.4.7 Determination of interfacial tension of coconut milk proteins

The capacity of coconut milk proteins from different fractions of CCP and CSMP to reduce the interfacial tension at the coconut oil-water interface was measured by a pendant drop technique using an optical contact angle and surface tension meter (KSV CAM; KSV Instrument Ltd, Helsinki, Finland) with CAM2008 software. Aqueous solutions of proteins from CCP and CSMP at different ratios were prepared by dispersing the CCP and CSMP powders into deionised water from a Millipore filtration unit. For each sample, the aqueous phase containing the dissolved protein powder was placed in the Manual Hamilton 1 ml syringe and coconut oil was placed in a cuvette as

the surrounding continuous phase. A droplet of the protein solution was produced and left hanging as a 'pendant drop' in the coconut oil placed in the cuvette. The drop profile was curve fitted to the Young‐Laplace equation to calculate the interfacial tension. All experiments were conducted at room temperature (20±2°C).

5.3.4.8 Statistical analysis

The preparation of samples was replicated at least two times on the different occasions. All experiments and measurements were carried out at least in duplicate and analysed as described in Section 3.3.10.

5.4 Results and Discussion

5.4.1 Composition of freeze dried powders

The freeze-dried powders of CSMP and CCP were analysed by the proximate analysis for the determination of moisture, protein, fat and ash contents. The carbohydrate content was calculated by subtracting the sum of percentages of moisture, protein, fat and ash from 100. The results are shown in Table 5.2.

Table 5.2 Composition[a] (%) of reduced fat coconut cream powder (CCP) and coconut skim milk powder (CSMP).

Powder	Moisture	Protein	Fat	Ash	CHO[b]
Reduced fat coconut cream powder (CCP)	8.1 ± 0.3	38.1 ± 0.3	21.7 ± 0.4	4.2 ± 0.1	27.9 ± 0.8
Coconut skim milk powder (CSMP)	7.3 ± 0.1	15.2 ± 0.1	2.0 ± 0.1	13.2 ± 0.1	62.4 ± 0.2

[a] Mean values of 6 replicates ± standard deviation.
[b] Carbohydrate (CHO) estimated based on calculation.

Despite the removal of fat by solvent extraction, the CCP appeared to retain a substantial amount of fat, indicating that the fat extraction used in this study was not highly efficient. Nevertheless, the quantity of fat retained in the CCP had no significant contribution to the total fat content (10%) of emulsions being less than 0.68% even when the CCP was only used to make emulsions containing 10% coconut oil. The protein content was also significantly higher in CCP than CSMP, suggesting that a large proportion of proteins in coconut milk were adsorbed at the interface of coconut oil droplets rather than being present in the serum phase of coconut milk (Table 5.2), while the contents of ash (minerals) and carbohydrate were greatly higher in CSMP than CCP.

Table 5.3 shows the percentages of protein, fat, carbohydrate and minerals distributed into the two fractions of cream and serum phases. This was estimated via calculation based on the previous experimental data for the composition of coconut milk and coconut skim milk shown in Table 4.4 in Section 4.4.1.

Table 5.3 The estimated proportions (%) of protein, fat, carbohydrate and mineral of coconut milk distributed between the cream (dispersed oil droplets) and serum phases.

Coconut milk	Protein	Fat	Mineral	Carbohydrate
Serum phase	29	0	86	77
Cream phase	71	100	14	23

The results shown in Table 5.3 show that a substantial amount of carbohydrates was also present in the cream phase. Coconut milk is extracted from the mature coconut meat (endosperm) of coconut nut. In the meat, water-soluble galactomannans and mannans and some water-insoluble galactomannans, alpha-cellulose and hemicelluloses have been reported to be present (Balasubramaniam, 1976). In addition, some water soluble sugars, such as arabinose, glucose and galactose, and some disaccharides were also reported to be present in coconut meat (Chandrasekaran and King, 1967). For the cream and skim milk fractions of coconut milk in this study, the carbohydrates present in the serum phase can be water soluble carbohydrates (galactomannans, mono-

saccharides and disaccharides) and some water insoluble polysaccharides extracted from the coconut meat during the extraction of fresh coconut milk. The carbohydrates in the cream phase may be some simple sugars or di- and oligosaccharides complexed with some proteins present at the interface. This means that the oil droplets in the original fresh coconut milk may be surrounded by a layer made up of proteins in that some proteins (e.g. glycoproteins) may be complexed with some carbohydrates (oligosaccharides or simple sugars) and some salts associated with them rather than the presence of only simple proteins. In some studies by Onsaard et al. (2005) and Onsaard et al. (2006), it was reported that the carbohydrate content between coconut cream powder and coconut skim milk powder derived from coconut milk was different and had no influence on the formation of emulsions containing corn oil.

5.4.2 Size of emulsion particles

Eight different batches of emulsions containing 10% coconut oil were prepared as described earlier at two different levels of protein concentrations (0.4% and 1.2%) consisting of four different ratios of proteins (100:0, 67:33, 33:67 and 0:100) derived from CCP and CSMP. This was to investigate the effects of CCP and CSMP proteins for their ability to form and stabilise an oil-in-water emulsions containing coconut oil. All emulsion samples prepared were analysed for their particle size and size distribution within 3 hr after the emulsion preparation and after one day of storage at two different temperatures (4°C and 35^{o}C). In all cases, the emulsion droplet sizes were measured in the presence and absence of SDS to determine the size of dissociated droplets (real droplets) and apparent droplets, respectively.

In the presence of SDS, the size of dissociated droplets was in a range of 5.33 to 13.25 μm and 2.37 to 5.05 μm at 0.4 % and 1.2 % protein, respectively, and was found to be dependent on the relative concentrations of proteins derived from the two fractions of CCP and CSMP (Figure 5.1A). The results indicated that the size of oil droplets formed was smaller when the protein concentration increased from 0.4% to 1.2%, and that at both protein levels, the oil droplet size also seemed to become smaller as the ratio of proteins derived from CSMP increased over CCP although this pattern was clearly

shown in some samples. The largest droplets were observed in the emulsions stabilised with solely CCP proteins (C10S0) while the smallest droplets formed in the emulsions stabilised with only CSMP proteins (C0S10) (Figure 5.1A). This means that the proteins derived from CSMP have a better capability of forming smaller oil droplets than the proteins from CCP.

In the absence of SDS, the apparent droplets measured were significantly larger than the corresponding dissociated droplets (Figure 5.1B). At 0.4% and 1.2% protein, the apparent droplets were in the size range of 15.2 to 68.2 μm and 9.4 to 37.8 μm in diameter, respectively. The similar patterns of changing the droplet size affected by the different protein concentrations and ratios of CCP and CSMP observed in the presence of SDS could also be observed in the absence of SDS. The apparent droplets were greater at 0.4% protein than at 1.2% protein, but in the emulsions stabilised with only CSMP proteins (C0S10), the difference in the apparent droplet sizes was relatively less significant as 15.2 μm and 9.4 μm at 0.4% and 1.2% protein, respectively, compared to the other emulsion samples. A significantly larger particle size of apparent droplets compared to their corresponding dissociated droplets means that the emulsions droplets were significantly aggregated, that is, the oil droplets were highly flocculated. This was also able to be visualised to a certain extent by a microscopic examination of emulsions that was also conducted in the presence and absence of SDS (Figure 5.5).

After one day storage at 4^{o}C and 35^{o}C, the dissociated and apparent droplets were observed to have undergone some changes in their size to differing extents, depending on the types of emulsions stabilised by the different concentrations and ratios of proteins from CCP and CSMP (Figure 5.2). At a high protein concentration of 1.2%, the dissociated droplets remained relatively stable without a pronounced change in their size after one day storage, regardless of storage temperatures, in comparison to their initial dissociated droplet sizes prior to storage. However, at the lower concentration of 0.4% protein, there was a significant size increase in the dissociated droplets at both 4^{o}C and 35^{o}C after storage, except the one stabilised by only CSMP proteins and stored at 4^{o}C.

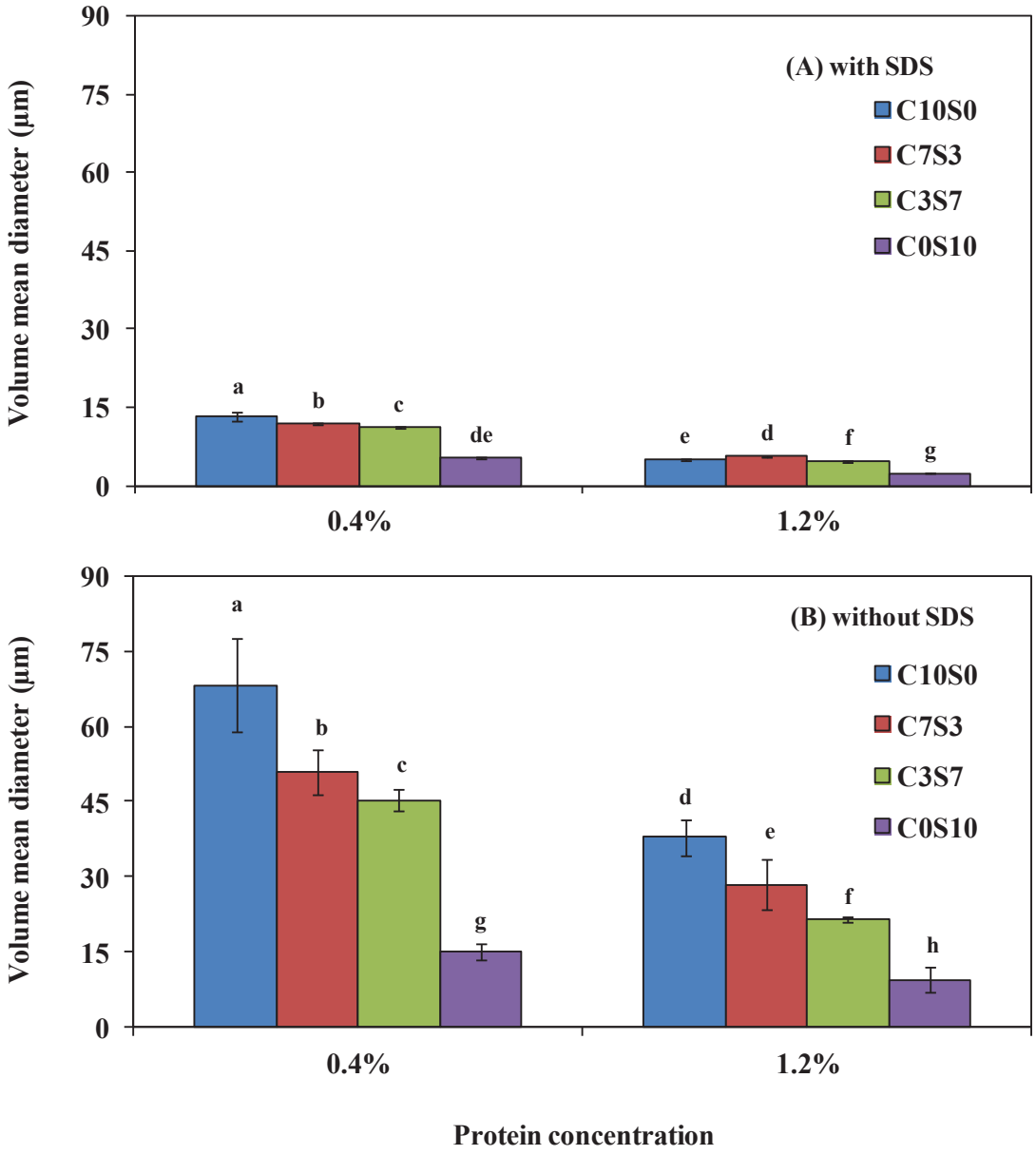

Figure 5.1 Average droplet sizes (d_{43}) of coconut oil-in-water emulsions measured within 3 hr after the emulsion preparation. Emulsions were prepared at two different protein concentrations (0.4% and 1.2%) with four different ratios of protein fractions derived from CCP and CSMP. The particle size was measured in the presence (A) and absence (B) of SDS (1.25%) at a ratio of 1:4 (v/v). Abbreviation: a ratio of proteins derived from CCP and CSMP: C10S0 = 10:0, C7S3 = 7:3, C3S7 = 3:7 and C0S10 = 0:10. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

This size increase in the dissociated droplets resulted from the droplet coalescence as their apparent droplet sizes measured in the presence of SDS were found to be similar in size to the dissociated droplets. The coalescence of droplets occurred in these samples was probably because the concentration of 0.4% protein was not high enough to enable them to form a rigid thick interfacial layer to prevent the aggregation and coalescence of droplets when the droplets came into close contact to each other. The emulsion prepared at 0.4% protein solely from CSMP (C0S10) remained relatively stable at 4^{o}C although this sample also exhibited the significant size change at 35^{o}C due to the droplet coalescence. The stability of this emulsion at 4^{o}C may be due to the effect of oil droplets existing in a solid and static state at low temperature, thus less movement and collision between the droplets.

As expected based on the results of dissociated droplets, the apparent droplets were greater in size than the dissociated droplets in all emulsions (Figure 5.2B). However, the extent of difference in size between the dissociated and apparent droplets was relatively minor at both temperatures for all emulsions prepared at the level of 0.4% protein than at 1.2% protein, except the emulsion stabilised by the proteins from CSMP (C0S10). As described earlier, there was a big difference in size between the dissociated and apparent droplets for the samples prepared with both 0.4% and 1.2% proteins before storage due to their droplet flocculation. The small size difference between the dissociated and apparent droplets observed for the samples prepared with 0.4% proteins after storage at both temperatures was however due to the extensive droplet coalescence. Interestingly, it was noticed that the sizes of apparent droplets measured before and after storage were similar, except for a few samples prepared at 0.4% protein and stored at 35^{o}C. This implies that the droplet coalescence occurred in most emulsions prepared with 0.4% protein after storage at both temperatures whereas the droplets in most emulsions prepared with 1.2% protein had undergone flocculation, not coalescence, after storage at both 4^{o}C and 35^{o}C. This was because the size of dissociated droplets remained the same or only a bit bigger after storage.

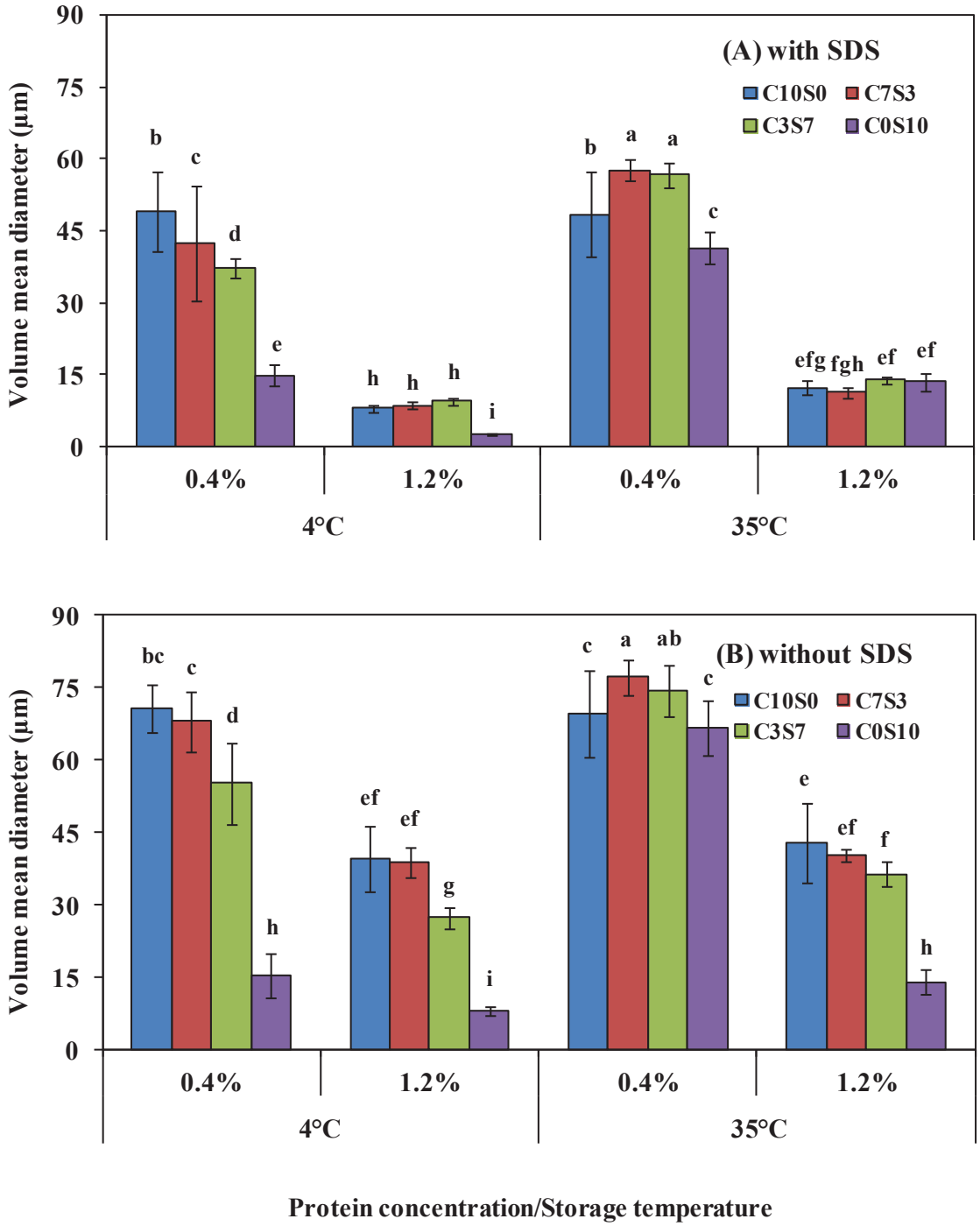

Figure 5.2 Average droplet sizes (d_{43}) of coconut oil-in-water emulsions after one day storage at two different temperatures (4 and 35°C). Emulsions were prepared at two different levels of protein concentrations (0.4% and 1.2%) with four different ratios of the protein fractions derived from CCP and CSMP. The particle size was measured in the presence (A) and absence (B) of SDS (1.25%) at a ratio of 1:4 (v/v). Abbreviation: a ratio of proteins derived from CCP and CSMP: C10S0 = 10:0, C7S3 = 7:3, C3S7 = 3:7 and C0S10 = 0:10. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

5.4.3 Particle size distributions of emulsions

Figures 5.3 and 5.4 show the particle size distributions of emulsions in the presence and absence of SDS that were measured before and after storage at 4^{o}C and 35^{o}C. The size distributions of dissociated and apparent droplets in most emulsions were bimodal, regardless of the measurements in the absence and presence of SDS. In the fresh emulsions before storage, the dissociated droplets (Figures 5.3B and 5.4B) had the particle size distributions with a smaller size range than their corresponding apparent droplets (Figures 5.3A and 5.4A) at both protein levels of 0.4% and 1.2%, indicating the presence of flocculated droplets. After one day storage, the size distributions of dissociated and apparent droplets for some emulsions had significant changes due to the droplet agglomeration and coalescence, thereby resulting in a broader and larger size range of particle distributions. The protein concentration and storage temperature were observed to have a significant influence on the stability of emulsions against the droplet coalescence during storage.

The emulsions prepared at 1.2% protein exhibited the higher stability as there was not much change in the particle size distributions for both apparent and dissociated droplets after storage at both temperatures, except for the dissociated droplets of emulsions at 35^{o}C that showed a decrease in the volume of some small size droplets and the corresponding increase in the larger droplets from the distribution curves. Overall, the results indicate that more stable emulsions against droplet coalescence could be obtained at a high protein concentration (i.e. 1.2%), especially derived from the CSMP fraction, and when the emulsions were stored at low temperature of 4^{o}C.

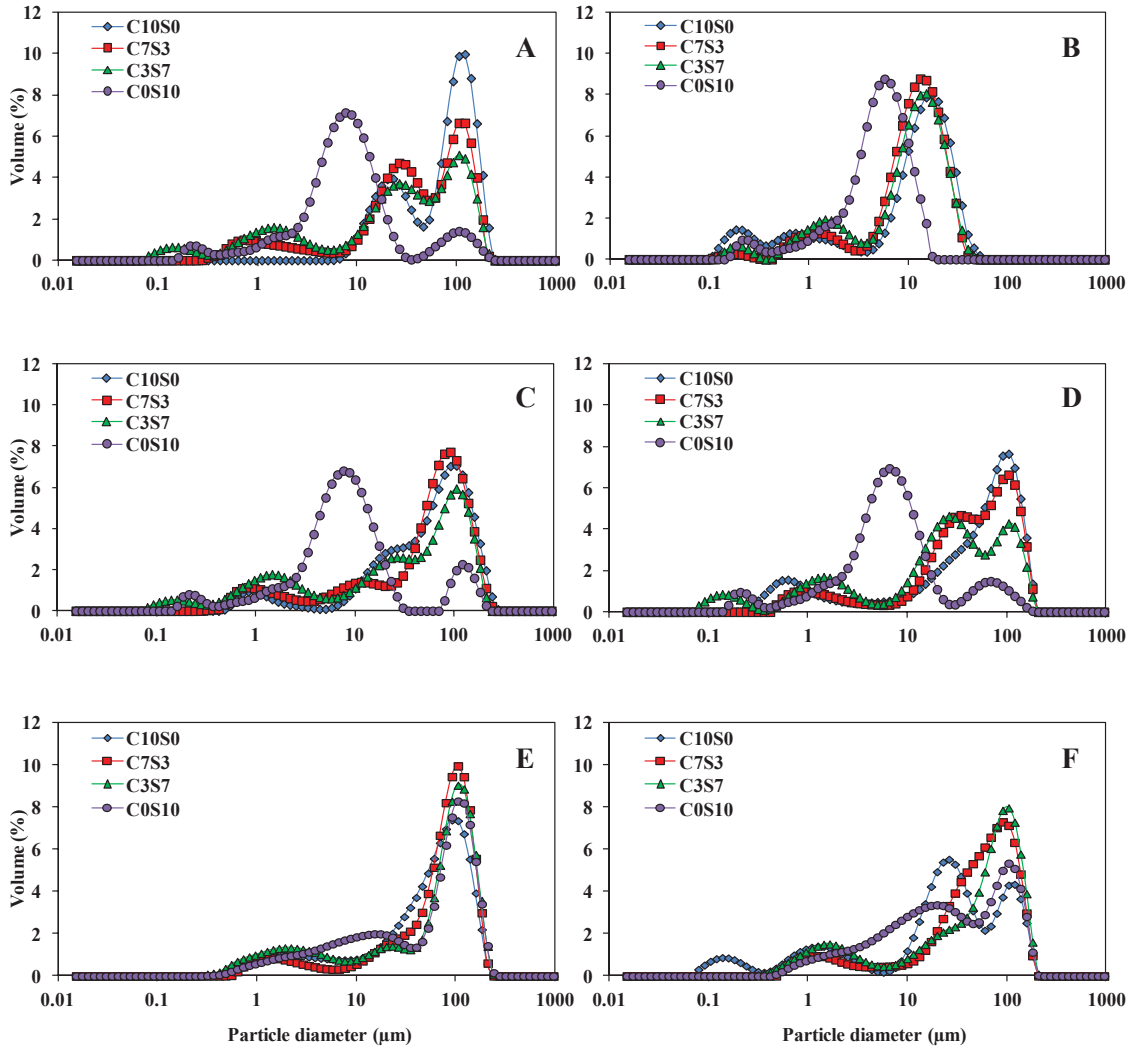

Figure 5.3 Particle size distributions of coconut oil-in-water emulsions containing 0.4% protein measured in the absence (A, C and E) and presence of SDS (B, D and F) after the emulsion preparation (A and B) and after one day storage at 4°C (C and D) and 35°C (E and F). Emulsions were prepared at 0.4% protein concentration with four different ratios of proteins derived from CCP and CSMP. The ratios of proteins derived from CCP and CSMP are C10S0=10:0, C7S3=7:3, C3S7=3:7 and C0S10= 0:10.

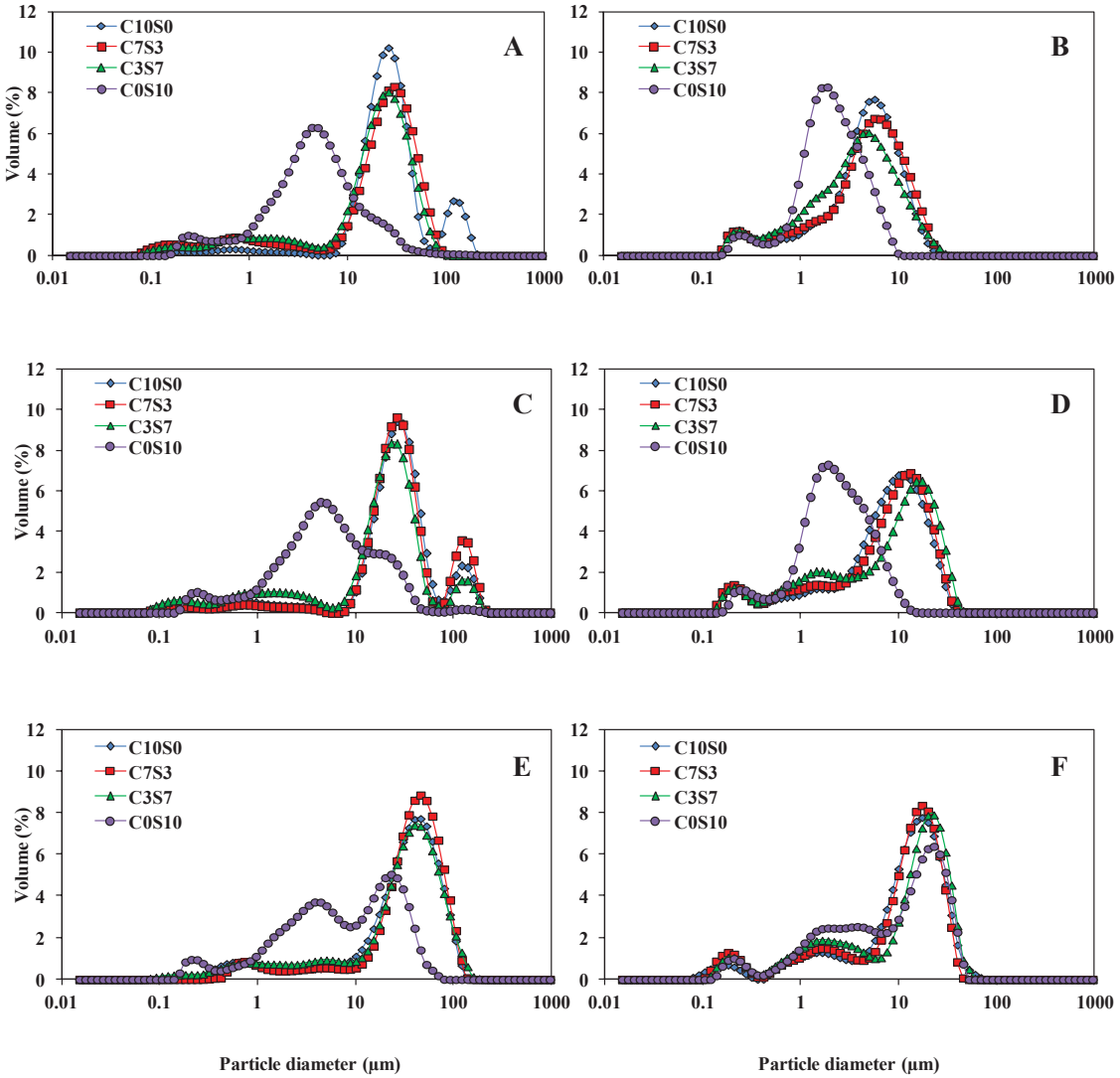

Figure 5.4 Particle size distributions of coconut oil-in-water emulsions containing 1.2% protein measured in the absence (A, C and E) and presence of SDS (B, D and F) after the emulsion preparation (A and B) and after one day storage at 4°C (C and D) and 35°C (E and F). Emulsions were prepared at 1.2% protein concentration with four different ratios of proteins derived from CCP and CSMP. The ratios of proteins derived from CCP and CSMP are C10S0=10:0, C7S3=7:3, C3S7=3:7 and C0S10= 0:10.

5.4.6 Microscopic examination of emulsions

The microscopic images of oil droplets taken from fresh emulsions and after one day storage are shown in Figure 5.5. The emulsion droplets stabilised by proteins derived from different fractions of coconut milk (i.e. cream and serum phase) differed in their size and appearance. Overall, the emulsions stabilised with CCP had a larger droplet size and more pronounced flocculation and aggregation especially at the low protein concentration of 0.4% compared to the emulsions stabilised with CSMP, For the emulsion systems prepared with a mixture of CCP and CSMP at different ratios, the extent of droplet flocculation tended to be more significant with increasing ratios of CCP to CSMP.

As mentioned, the less droplet flocculation was observed in the emulsions stabilised with only CSMP (C0S10). The levels of protein content in emulsions conveyed the effect on the size of droplets. The emulsions stabilised with CSMP protein at 1.2 % protein comprised smaller droplets than the same emulsion stabilised with 0.4 % protein. Few aggregates could be seen in these two emulsions. The droplet flocculation could however be observed in the emulsions at both proteins levels when CCP was used to form and stabilise the emulsion droplets. The more proteins derived from CCP were used, the more droplet aggregates resulted in. The most aggregated droplets were observed in the emulsions containing proteins derived from CCP at different ratios (C10S0, C7S3 and C3S7). In the presence of SDS, the droplets with less flocculation could be observed from all emulsion samples. The smallest single droplets could be seen from the emulsions containing proteins derived only from CSMP (C0S10) at 1.2 % protein.

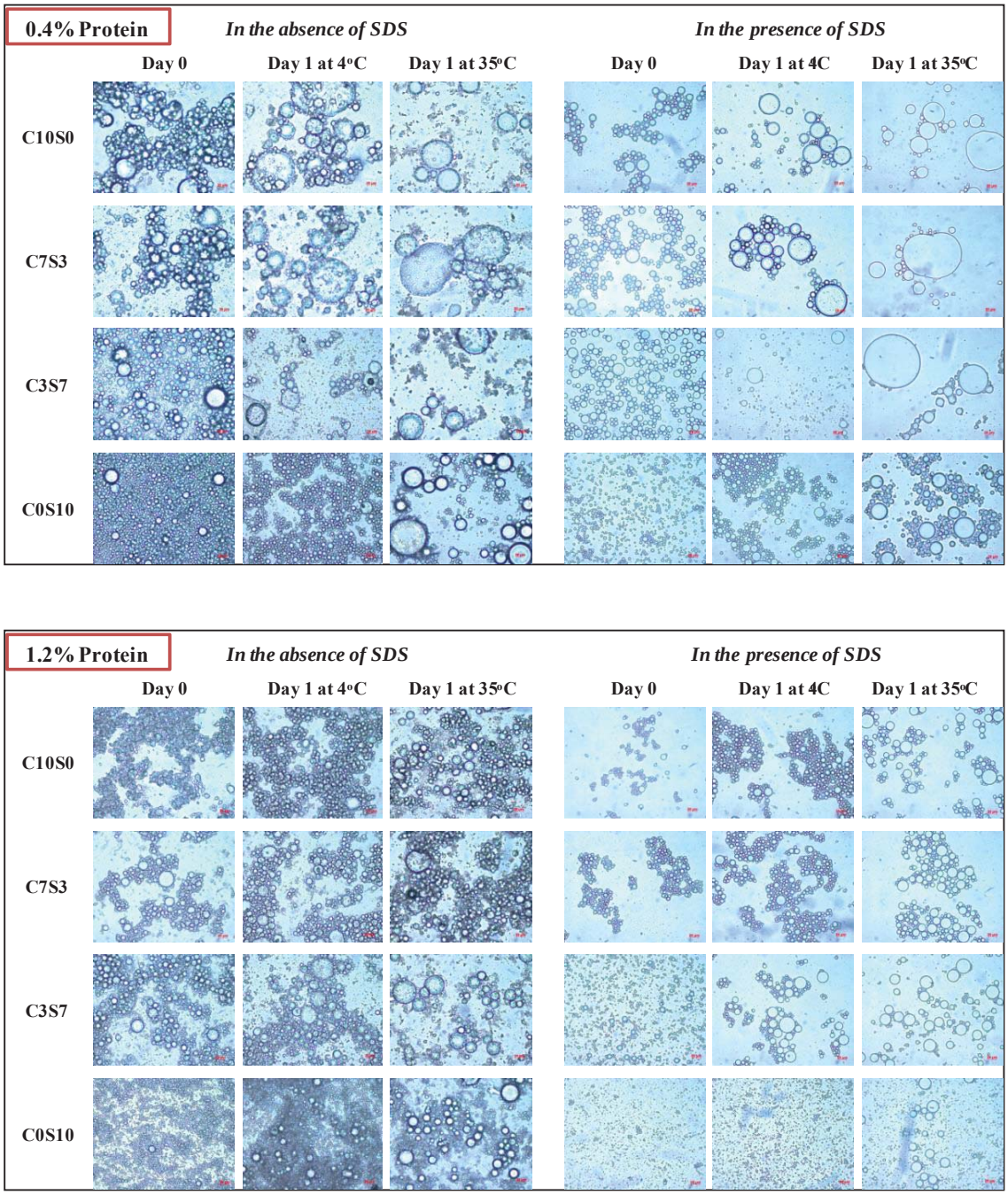

Figure 5.5 Optical micrographs of oil droplets in coconut O/W emulsions (10% oil) prepared with CCP and CSMP at two different levels of protein concentrations (0.4 % and 1.2%) with four different ratios of proteins from CCP and CSMP. Emulsions were stored at two different temperatures (4°C and 35°C). A ratio of proteins derived from CCP and CSMP: C10S0=10:0; C7S3=7:3; C3S7=3:7; and C0S10=0:10. Emulsions were diluted with and without 1.25% SDS solution (1:4, v/v) before observations.

Temperatures of storage also affected changes in the size and an induction of flocculation. At the high temperature (35°C), the droplets showed an increase in their size, due to droplet coalescence. Droplet coalescence occurred in emulsions containing a high ratio of proteins derived from CCP. At low temperature (4°C), the droplets were quite stable in size. At a low protein level, the droplets became a bit greater in size, but no sign of coalescence was observed. Although the emulsions prepared at both protein concentrations and stabilised with the proteins derived from CSMP (C0S10) also showed phase separation due to an effect of gravity, the flocculated droplets in these emulsions were stable against coalescence at low temperature.

5.4.4 Zeta potential of emulsion droplets

The stability of protein-stabilised emulsions against aggregation is primary maintained by an electrostatic repulsion because the interfacial layers of emulsion droplets stabilized by proteins are electrically charged (McClements, 2004b). Theoretically, a zeta potential value of greater than ± 60 mV is required for an excellent stability of emulsions or greater than ± 30 mV is required for a good stability of emulsions against aggregation (Riddick, 1968). In this study, the droplets of emulsions stabilised by coconut proteins at the concentrations of 0.4% and 1.2% protein were negatively charged ranging from -23.1 to -42.5 mV and from -26.7 to -34.5 mV, respectively (Figure 5.6). It is thought that the zeta potential values of coconut protein-stabilised emulsions were not very low and that the oil droplets of emulsions stabilised by coconut milk proteins could remain stable to a certain extent by the electrostatic repulsive force.

The electrical charges between the emulsions prepared with 0.4% and 1.2% protein were not very different and a consistent pattern of changing the zeta potentials attributable to the protein concentration was not obtained in this study. The most negatively charged droplets were found in the emulsions made only with CSMP at 0.4% protein whereas the lowest zeta potential was when only CCP was used at 0.4% protein level. However, the pattern of zeta potentials of oil droplets being more negatively charged with increasing ratio of CSMP to CCP was clearly observed, suggesting the influence of proteins in CSMP on the increase in the zeta potential of emulsion droplets.

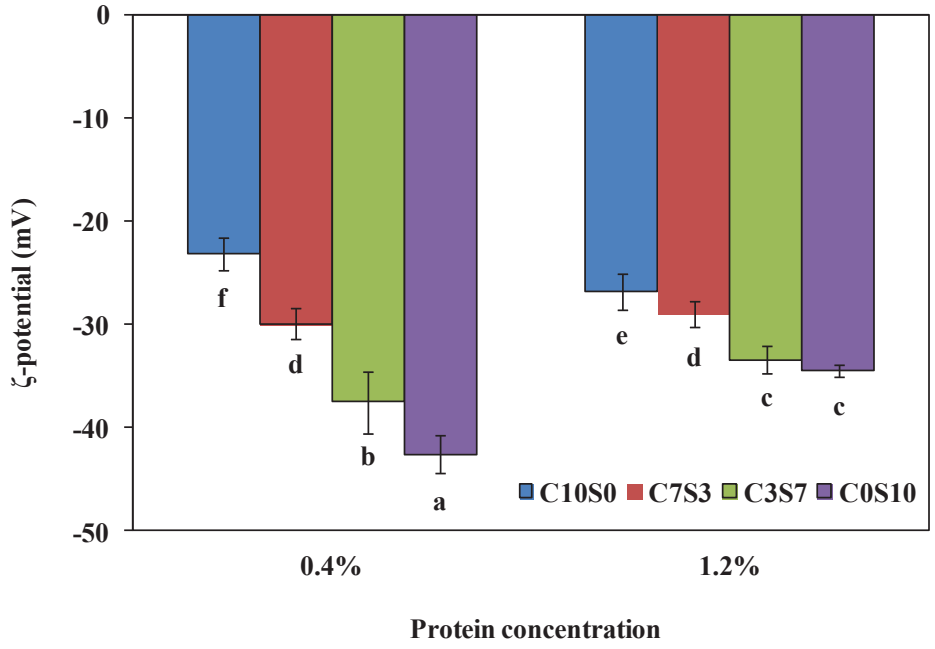

Figure 5.6 Zeta potentials of coconut oil-in-water emulsions prepared at two different protein concentrations (0.4 % and 1.2 %) with four different ratios of proteins derived from CCP and CSMP. The zeta potentials of emulsions were measured in the absence of SDS after the emulsion preparation. Abbreviation: a ratio of proteins derived from CCP and CSMP: C10S0 = 10:0, C7S3 = 7:3, C3S7 = 3:7 and C0S10 = 0:10. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

There are several possible reasons for the higher zeta potentials observed from the emulsions prepared with the higher ratio of CSMP to CCP. Firstly, the type of major proteins present between CSMP and CCP that differs in each other. The CSMP is mainly composed of albumins whereas the CCP contains globulins and some albumins. Globulins have a lower ratio of hydrophilic to hydrophobic amino acid residues than albumins, thus higher in the frequency of non-polar amino acids (Kwon et al., 1996). Therefore, the number of electrically charged amino acids can be thought to be higher in albumins than in globulins, enabling the increased electric charges when the oil droplets are stabilised by the proteins with a high ratio of albumins to globulins. Secondly, the increase in the zeta potentials with increasing ratio of CSMP over CCP could be partly related to the size of oil droplets being decreased as the ratio of CSMP increased. As it was noticed in the other experimental trials (data not shown) that the zeta potential of oil droplets was measured to be affected by the size of droplets to some extent. Thirdly, the other possibility may be due to some differences in the ionic strength and the

presence of some other ionisable components other than proteins between CSMP and CCP. As shown in Table 5.2, the amount of minerals present in CSMP and CCP was quite different which was much higher in CSMP than in CCP, thus the ionic strength of CSMP can be expected to be higher than that of CCP.

5.4.5 Emulsion stability

The stability of coconut oil emulsions was assessed visually by monitoring phase separation and creaming around one hour after the emulsion preparation and also after storage for one day as shown in Figure 5.7. After the emulsion preparation, the most stable emulsion against creaming was the one prepared with CSMP at 1.2 % protein (C0S10). This emulsion showed no pronounced creaming or phase separation unlike the other emulsions exhibited the phase separation or creaming. Interestingly, the emulsion stabilised with 0.4 % CCP protein (C10S0) exhibited creaming to a much less extent (marked as a dotted line in Figure 5.7) with some droplets suspended in the serum phase, thus making the whole emulsion opaque in appearance. This was in spite of the fact that the droplet size of this emulsion was much larger than the other emulsions. This phenomenon was however not seen in the emulsion stabilised with the same protein but at a higher concentration of 1.2% protein. After storage at 4°C and 35°C for one day, almost all emulsions separated into two distinct phases (cream and serum phases) except the emulsion containing 0.4% CCP proteins (C10S0) at 4^{o}C that still maintained the stability without a noticeable change during storage at low temperature. It is not clearly understood the main cause of this observation as to what factors induced the stability of this emulsion without complete phase separation during storage at 4^{o}C even though the mean particle size was larger and the zeta potential of oil droplets was lower in this emulsion than the other emulsions as shown in Figures 5.1-5.5 and Figure 5.6, respectively.

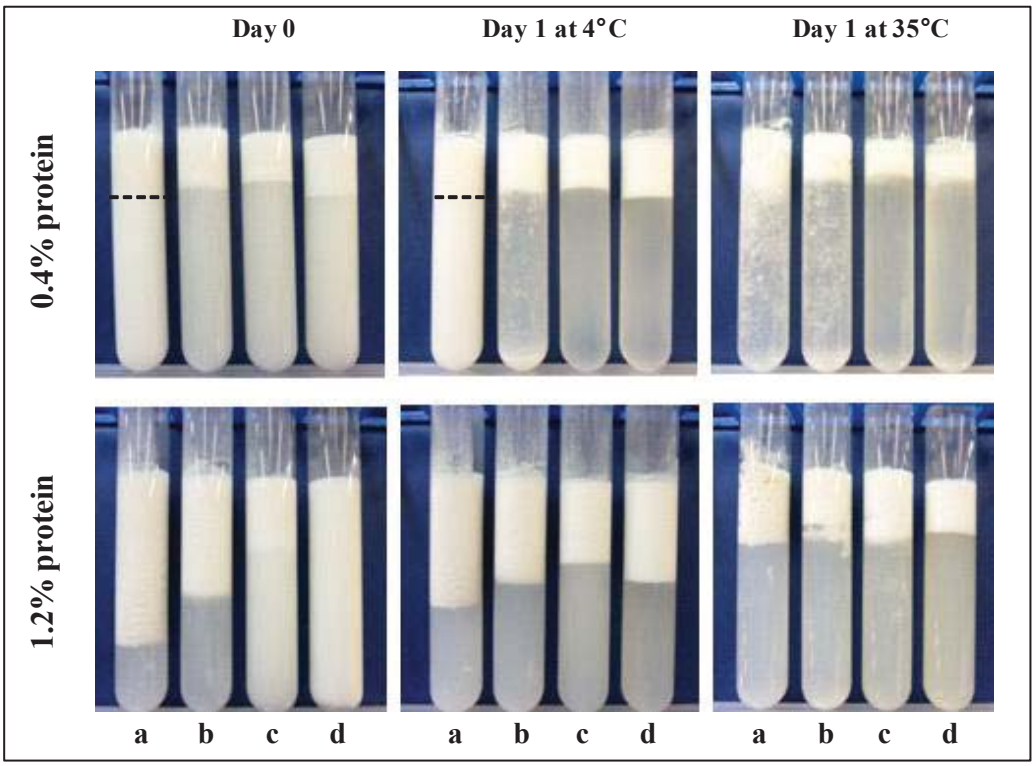

Figure 5.7 Stability of coconut oil-in-water emulsions to creaming and phase separation after one day of storage at 4 and 35°C. Emulsions were prepared at two different levels of protein concentrations (0.4% and 1.2%) with different ratios of proteins derived from CCP and CSMP, a=10:0 (C10S0); b = 7:3 (C7S3); c=3:7 (C3S7); and d=0:10 (C0S10).

5.4.7 Interfacial tension of coconut proteins

The values of interfacial tensions at the coconut oil-water interface were determined by a pendant drop method as a function of the ratios of proteins derived from CCP and CSMP at the concentrations of 0.4 and 1.2 wt%. The results are shown in Figure 5.8. The initial values of interfacial tension at the oil-water interface after an addition of protein mixtures of CCP and CSMP were in the range between 16.5 and 20.7 mNm^{-1} and 12.0 and 21.9 mNm^{-1}, depending on their ratios, at 0.4% and 1.2% protein, respectively. At the initial stage, the lowest interfacial tension was measured from the sample containing only CSMP, whereas the highest interfacial tension was obtained from the sample containing only CCP, both regardless of the protein concentrations.

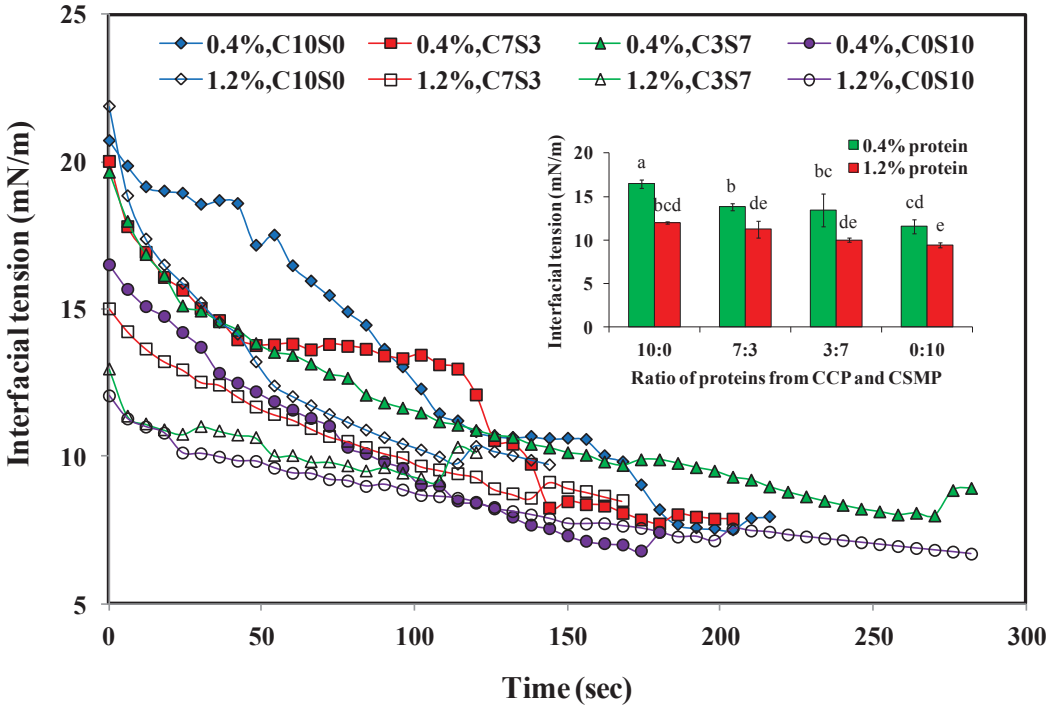

Figure 5.8 Time-dependence of interfacial tensions at the coconut oil-water interface for the mixtures of proteins derived from CCP and CSMP at the ratios of C10S0=10:0; C7S3=7:3; C3S7=3:7; and C0S10=0:10 and two different protein levels (0.4 and 1.2% protein). A small bar graph inserted is for the comparison of interfacial tension between the samples measured at 60 sec. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

After the protein mixtures were added for 60 sec, the order of the protein ability to lower the interfacial tension at the oil-water interfaces was still the same, ranking from the pure CSMP solution to the pure CCP solution, respectively (a graph inserted in Figure 5.8). The lowest and highest interfacial tensions could be still seen from the samples containing only CSMP and CCP, respectively. The higher protein concentration of 1.2% derived from both powders, had the slight higher ability to lower the tension of coconut oil-water interfaces. Also, at both 0.4% and 1.2% protein, the interfacial tension decreased with increasing ratio of CSMP and CCP. However, after 2 min, the interfacial tension reduced was not significantly for the samples containing CSMP (0:10) at two different concentrations as the interfacial tension was measured to be same at 8.4 mNm^{-1}. In summary, the proteins derived from CSMP were more effective for lowering the interfacial tension than the proteins derived from CCP.

5.4.8 Analysis of coconut milk proteins and interfacial proteins by SDS-PAGE

The freeze-dried CSMP and CCP and the liquid coconut skim milk (CSM) and cream collected during their separation from coconut milk were analysed by SDS-PAGE under a reducing condition. The proteins in all samples were resolved into several bands representing the polypeptide subunits of coconut milk proteins and the electrophoresis patterns of proteins are shown in Figure 5.9. Proteins in CSMP were separated into 4 bands above and 1 band under a standard molecular weight marker of 14.4 kDa. Above 14.4 kDa, the subunits were two strong bands with the estimated molecular weights (MWs) of 18 and 26 kDa and two faint bands with the MWs of 55 and 66 kDa (lane 2). These subunits with the MWs of 18, 26 and 55 kDa were quite similar to the subunits of coconut skim milk shown in lane 4 as anticipated and were in accordance with the subunits of coconut albumins reported elsewhere in the literature (Garcia et al., 2005, Kwon et al., 1996). It is noteworthy that the subunits with the MWs of 66 kDa (a) and 22 kDa (doublets) (b and c labelled in lane 2) were minor serum proteins present in coconut skim milk shown also as very faint bands (marked with * in lane 4) which was used to prepare the CSMP. The presence of these bands with relatively high intensity from CSMP was because they were more concentrated after the freeze drying of skim milk. The subunits of polypeptides with the MW of 66 kDa and doublets at 22 kDa were quite similar to the polypeptide subunits of salt-soluble 7S globulin proteins shown in several studies (Morcillo et al., 1998, Morcillo et al., 1997). The 7S globulins are known as a trimeric protein (Adachi et al., 2003). An immunoblot analysis of coconut endosperm proteins showed that it consists of a minor band with the MW of 67 kDa and 2 minor bands with the MWs of around 22 kDa (DeMason and Sekhar, 1990). However, Garcia et al. (2005) reported the MWs of 7S globulins from coconut endosperm at 24, 22 and 16 kDa. Although 7S globulins were present in CSMP, the intensities of their polypeptide bands were very light compared to the polypeptide bands of albumins, suggesting that the amount of 7S globulins present in the CSM fraction was minor.

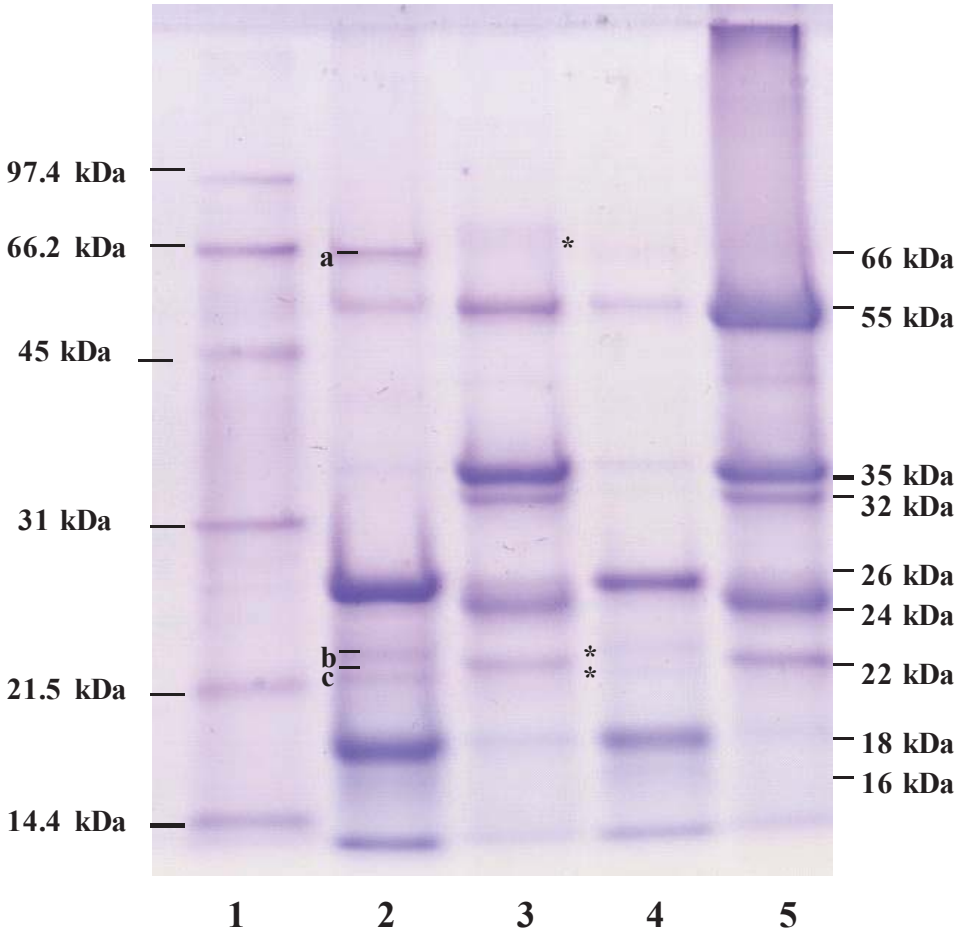

Figure 5.9 SDS-PAGE of subunits of proteins resolved from CCP and CSMP. Standard molecular weight marker (lane 1), freeze dried CSMP (lane 2) and CCP (lane 3) and coconut skim milk (lane 4) and coconut cream (lane 5 fractionated from coconut milk.

For the proteins in CCP, they were migrated into several bands with the MWs of around 55, 35, 32, 24 and 22 kDa (lane 3). There was no strong migrated band of subunits under a MW of 14.4 kDa. These migrated polypeptide subunits were fairly similar to the bands observed from the cream of coconut milk (lane 5). The subunits with the MWs of 35 and 32 kDa were believed to represent the acidic polypeptides of 11S globulins, a hexameric protein, (soybean glycinin) reported in the literature (Adachi et al., 2003, Garcia et al., 2005, Kwon et al., 1996). In this study there were also subunits at the MWs of around 22 and 24 kDa which are believed to be the basic polypeptides as these two subunits are similar to the previously reported 11S globulin's (cocosin's) basic polypeptides at 24 and 21 kDa (Angelia et al., 2010, Garcia et al., 2005).

Therefore it can be concluded that the major proteins in CCP were 11S globulins (acidic polypeptides), also known as cocosin, and the main proteins in CSMP were albumins. Onsaard et al. (2005, 2006) showed that the solubility of proteins extracted from coconut skim milk and coconut cream in water at pH 7 was about 75% and 45%, respectively, confirming the major types of proteins present in the two fractions of CSMP and CCP are different.

In this study, it was observed that the freeze dried CSMP or CCP could not be fully dissolved in water and that some components precipitated at the bottom of glass test tubes when these powders were dispersed in water (Figure 5.10) although CSMP was more soluble than CCP. At the same protein concentration at 1.2%, the pHs of these two solutions containing CCP and CSMP were 6.6 and 6.5, respectively. After centrifugation of the solutions of CSMP and CCP at 4,500 x g for one hour at 4°C, the serum phase was collected and analysed for the content of protein by a Lowry method. The serum phase of the CSMP solution contained 87.1% of the total protein whereas it the serum phase of the CCP solution contained only 3.1% of the total protein, indicating a significant amount of protein in CCP was insoluble in water due to the presence of a large concentration of salt soluble proteins (e.g. 11S globulins).

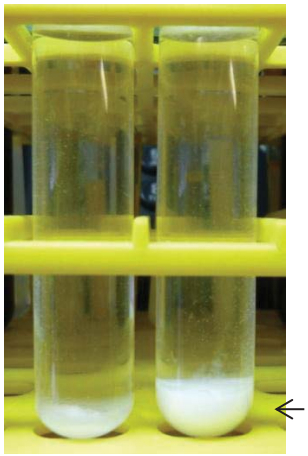

Figure 5.10 Water-insoluble precipitate from an aqueous solution of CSMP (left) or CCP (right) after dissolving them in water.

The serum and precipitate from each of CSMP and CCP solutions were also analysed by SDS-PAGE under a reducing condition. The electrophoresis patterns are shown in Figure 5.11.

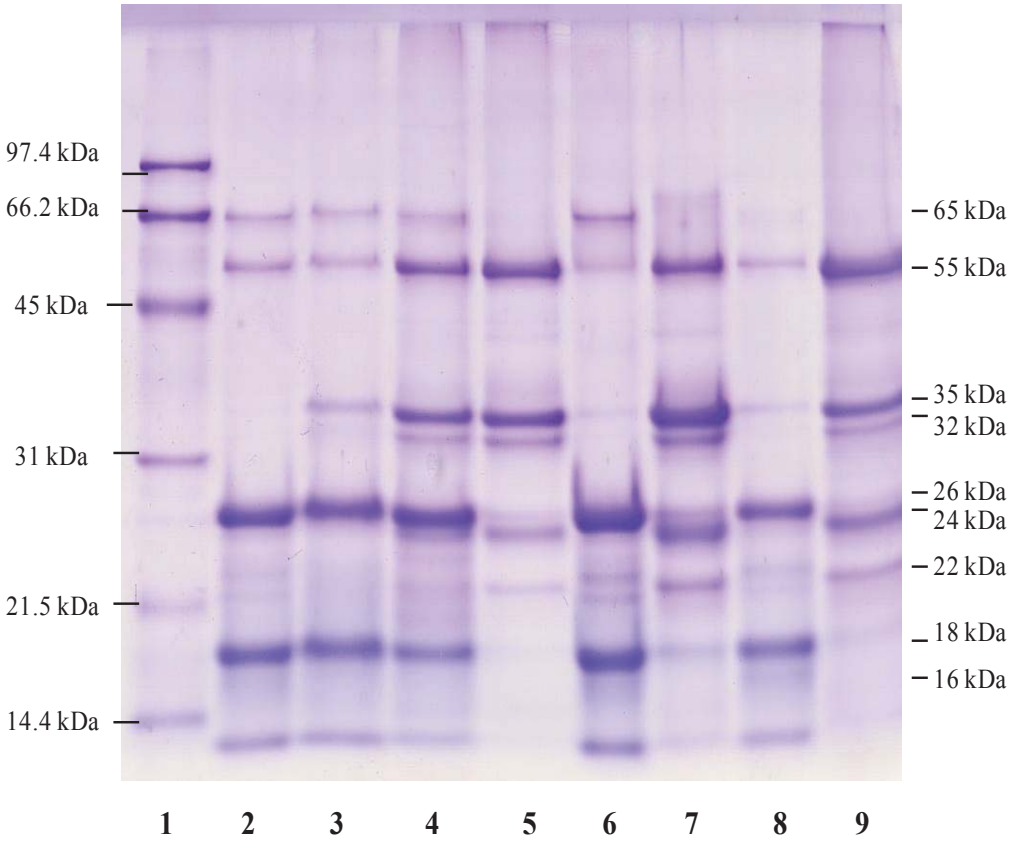

Figure 5.11 SDS-PAGE analysis of proteins in the serum and pellet separated from the solutions of CSMP and CCP. The standard molecular weight marker (lane 1); serum of CSMP solution (lane 2); serum of CCP solution (lane 3); precipitate of CSMP solution (lane 4); precipitate of CCP solution (lane 5); CSMP (lane 6); CCP (lane 7); coconut skim milk (lane 8); and cream of coconut milk (lane 9).

Proteins in the serum phase of CSMP solution were resolved into 4 polypeptide subunits with the MWs of 66, 55, 26 and 18 kDa and also 1 subunit smaller than 14.4 kDa (lane 2). These subunits represented the water soluble albumin proteins (55, 26 and 18 kDa) including 7S coconut globulins (66 kDa). The subunits obtained from the serum of CCP revealed similarly to CSMP but with two minor bands at 35 and 32 kDa (lane 3). The presence of these two extra bands was probably due to the contamination of 11S coconut globulins in the serum phase caused by a low speed centrifugation force used to separate the mixture. The polypeptide subunits of 11S globulin (also referred to as

cocosin) (55, 35, 32, 24 and 22 kDa) could be strongly observed on the gel loaded with CCP (lane 7). On a lane loaded with CSMP precipitate, the subunits of albumins and acidic polypeptides were dominant (lane 4).

The proteins adsorbed at the interface (interfacial proteins) of emulsions were also analysed by SDS-PAGE (Figure 5.12). The emulsions prepared at two different protein concentrations (0.4% and 1.2%) with four different ratios of proteins derived from CCP and CSMP were separated into the serum and cream phases. The washed cream fractions were characterised by SDS-PAGE. The patterns of polypeptide subunits obtained from all emulsion samples were fairly similar at both protein concentrations (lanes 2 to 5). In the emulsions containing proteins derived from only CCP, most bands observed were 11S coconut globulins (lane 2). In the emulsions prepared with only CSMP, most bands were albumins corresponding to 55, 26 and 18 kDa, indicating that these proteins dominated as the interfacial proteins in the emulsions prepared with CSMP (lane 5).

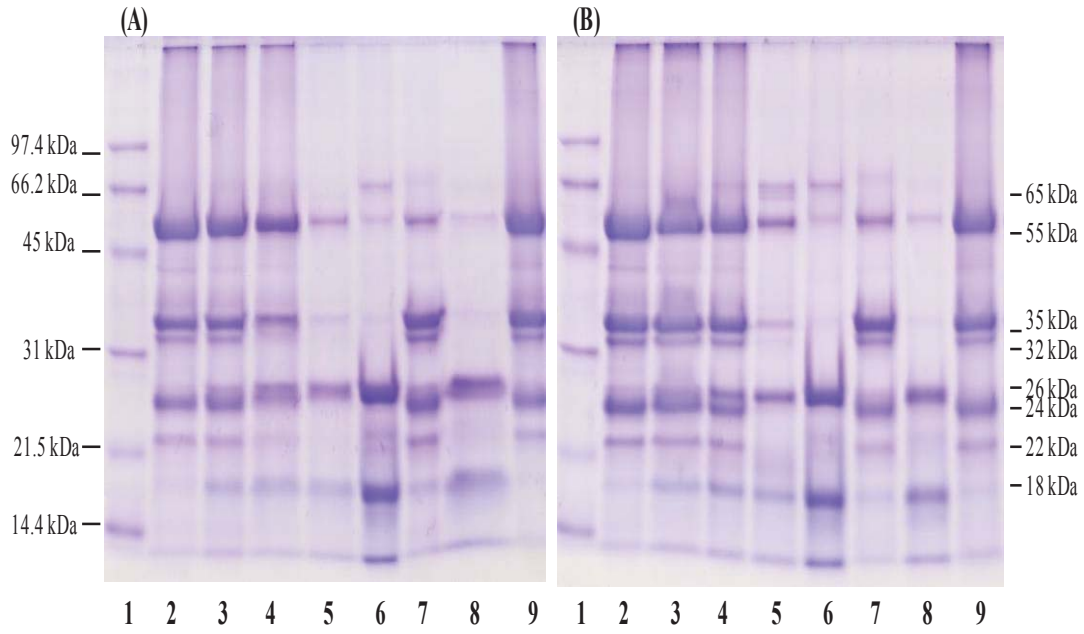

Figure 5.12 SDS-PAGE analysis of the interfacial proteins at the oil-water interface of the emulsions stabilized by proteins from freeze dried CSMP and CCP at two different protein concentrations, 0.4% (A) and 1.2% (B). MW marker (lane 1); Ratio of proteins from CCP and CSMP in emulsions: 10:0 (lane 2); 7:3 (lane 3); 3:7 (lane 4); 0:10 (lane 5); CSMP (lane 6); CCP (lane 7); coconut skim milk (lane 8); and cream of coconut milk (lane 9).

At a high protein concentration, the subunit of 7S globulins with the MW of 66 kDa was more pronounced. In the emulsions containing proteins mixed from CCP and CSMP (lanes 3 and 4), the subunits representing 11S globulins (55, 35, 32, 24 and 24 kDa) dominated at the interface, while some faint bands showing the subunits of albumins (55, 26 and 18 kDa) were also observed. When a higher protein ratio from CCP was used, acidic and basic polypeptides were more pronounced, whereas in the emulsions with a high ratio of CSMP derived proteins, both types of polypeptides were fader. Therefore, when mixed proteins were utilised to stabilise droplets in emulsions, the results showed that 11S coconut globulins were more adsorbed at the oil-water interface in preference to albumins.

5.5 Conclusions

Two freeze-dried powders obtained from two different fractions of coconut milk such as CCP and CSMP were, as shown by SDS-PAGE, coconut 11S globulin-rich and albumin-rich powders, respectively. As the two powders were used to stabilise 10 wt% coconut oil-in-water emulsions, the real and apparent diameters of emulsion droplets were dependent on the concentration and ratio of both fractions in the binary protein mixtures used to emulsify 10% coconut oil. The diameter of emulsion droplets became smaller as the more protein concentration and albumin-rich fraction were used to stabilise the newly formed droplets. The more incorporation of the globulin-rich fraction in the binary protein mixtures led to a substantial increase in droplets size and became the biggest apparent droplets when the emulsion droplets were stabilised solely by the globulin-rich fraction of CCP. With the microscopy observation, the droplets stabilised solely by CSMP rich in coconut albumins at a concentration of 1.2% exhibited the most stable droplets with a number of individual droplets but in association with other droplets after the emulsion was stored at 4°C and 35°C. Although small droplets could be formed by the albumin-rich fraction of CSMP, no stable emulsion against phase separation was observed. Most emulsions showed phase separation, irrespective of concentration and ratios of both fractions in the protein mixture, reflecting the low ability of these proteins to provide stability to emulsions. The findings suggest that the proteins (albumins) in CSMP appear to be relatively more

suitable proteins for form and stabilise small emulsion droplets with less flocculation than the proteins (11S globulins) in CCP.

Chapter 6

Effect of small molecule surfactants on the formation and properties of coconut oil emulsions stabilised by fractionated coconut milk proteins

6.1 Abstract

Coconut milk was separated into reduced fat coconut milk (RFM) and coconut skim milk (CSM). Coconut oil-in-water emulsions were then prepared from these two fractionated milk samples with and without an addition of different types of small molecule surfactants, which were lipid-soluble surfactants (mono- and diglycerides and partially unsaturated mono- and diglyceride) at a total concentration of 0.25% and a water-soluble surfactant (Tween 80) at 0.05% concentration. The main focus was to investigate the effects of surfactants on the properties and stability of emulsions. All emulsions prepared were analysed for the determination of the particle size, zeta potential, type of adsorbed proteins and surface protein load of emulsion oil droplets. The results of SDS-PAGE revealed the interfacial proteins of RFM-based emulsions were composed of 11S and 7S globulins while albumin proteins were predominantly found at the interface of CSM-based emulsions. The surface protein load of these proteins on both emulsion systems was reduced when the emulsions were prepared with the addition of surfactants, regardless of whether it is water or lipid-soluble. Between the CSM-based and RFM-based emulsion systems, the surface protein load was much lower in the former than the latter. The addition of water-soluble surfactant (Tween 80) significantly reduced the particle size of emulsions prepared from CSM, regardless of the presence of lipid-soluble surfactants, compared to the control emulsion, whereas, in the absence of Tween 80, the lipid-soluble surfactants caused a significant particle size increase. The latter phenomenon was however not observed in the case of emulsions prepared from RFM. The addition of only lipid-soluble surfactants also reduced the particle size and its effect was more pronounced when they were present together with Tween 80. The particle sizes of emulsions measured in the presence and absence of a dissociating agent (SDS) indicated that the oil droplets of all RFM-based emulsions were highly flocculated, regardless of the presence of surfactants, whereas the CSM-

based emulsion droplets were more discrete with less droplet flocculation. In all cases, the surface protein load was much lower in the emulsions prepared with CSM than with RFM. The surface protein load of both emulsion systems made from CSM and RFM was decreased by the presence of surfactants regardless of the type of water-soluble or lipid-soluble surfactant. For the stability of emulsions against phase separation, the droplets of RFM-based emulsions added with both Tween 80 and lipid-soluble surfactants were more stable at 4ºC exhibiting less change in their particle size and less phase separation than the CSM but all emulsions had phase separation at high temperature of 35^{o}C after one day. Overall the results indicated that the stability of oil-in-water emulsions containing 10% coconut oil stabilised by coconut milk proteins could be improved by small molecule surfactants.

6.2 Introduction

Surface active macromolecules used in making oil-in-water (O/W) emulsions are normally water-soluble amphiphilic molecules (e.g. caseins and whey proteins from cow's milk) with both hydrophilic and hydrophobic functional groups. Water-soluble emulsifying polymers and/or small molecule surfactants adsorb at the oil and water interface of small oil droplets formed in the aqueous phase during the homogenisation process, thus lowering the interfacial tension and stabilising the droplets from coalescence (Dickinson, 1992a, Dickinson, 1994). Emulsion stability, structures and properties are influenced by a variety of factors, including composition, size and size distribution of emulsion droplets, electrical charge, density of surface layers surrounding emulsion droplets, type and concentration of surface active materials, ratio of oil to aqueous phases, pH and ionic strength (Dickinson, 1992a, Dickinson, 1994).

In the previous studies described in Chapters 3-5, it was shown that the emulsions formulated with coconut milk (CM) or its fractionated milk, such as reduced fat milk (RFM) and coconut skim milk (CSM), revealed some differences in their properties and stability. The emulsions made with CSM possessed the smaller oil droplets than the emulsions made with CM or RFM and was more stable to phase separation although all the emulsions exhibited the short term stability after emulsion preparation. In contrast,

the droplets in emulsions based on the aqueous phase of CM or RFM were larger in their size with droplet aggregation which was suggested to be due to hydrophobic interaction (Floury et al., 2002). As a result, the emulsions creamed very fast, resulting in a quick phase separation. That was because CM and RFM contain predominantly globulin proteins which are hydrophobic than albumin proteins that are the main CSM proteins (Kwon et al., 1996), thus increasing the magnitude of droplet flocculation than the droplets in CSM emulsions. This suggests the emulsions containing droplets stabilised by proteins in CSM were more stable than the emulsions based on CM or RFM.

Emulsions are often produced by homogenising oil/fat with an aqueous solution containing a mixture of different surface active molecules, such as small molecule surfactants, proteins and phospholipids, using a mechanical shear force by a high pressure homogeniser (Nylander et al., 2008). During homogenisation, it has been reported that a competitive adsorption between the added surface active materials at the interface of oil droplets takes place (Chen and Dickinson, 1998, Euston et al., 1995, Furusawa et al., 1982). For instance, in a system containing water-soluble or oil-soluble surfactants together with proteins, the competitive adsorption to the oil and water interface between those surface active molecules occurs (Chen and Dickinson, 1998). In general, the small compounds adsorb first and then can be replaced by the bigger ones (Furusawa et al., 1982). However, this depends on the physicochemical properties of surface active materials, such as size, molecular dimension, conformation, solubility, hydrophilic-lipophilic balance and ionic or non-ionic nature. In terms of the replacement of the pre-adsorbed proteins from the interface by other surface active materials, the water-soluble surfactants are reported more effective than oil-soluble surfactants in replacing proteins from the interfacial layers of droplets in oil-in-water emulsions (Chen and Dickinson, 1993, 1998). The effects of small molecule surfactants on the formation and properties of oil-in-water emulsions made from coconut oil have not been well studied and there is not much information available about this.

Many research studies have been done on dairy ice cream emulsions. When making ice cream, the droplets of ice cream mix emulsion primarily stabilised by milk proteins remain stable to coalescence and phase separation (van Aken, 2003). These protein-stabilised droplets then undergo a destabilisation process when the ice cream emulsions are aged at 4°C in order to increase the sensitivity of those destabilised droplets to partial coalescence, which enables to develop a three-dimensional network in an ice cream structure (Goff, 1997b). During ageing at that low temperature, some of proteins adsorbed at the oil droplet interface become desorbed (Gelin et al., 1994). The extent of protein desorption is magnified by small molecule surfactants added into the ice cream mix emulsion before ageing (Gelin et al., 1996, Pelan et al., 1997b). This suggests that the addition of small molecule surfactants into emulsions leads to the destabilisation of protein-stabilised droplets and promotes partial coalescence of fat globules for the development of ice cream structure when the ice cream mix emulsion is kept at low temperature during aging. There are many factors affecting partial coalescence of fat globules in ice cream making, including type and concentration of oil (fat) and small molecule surfactants. The objective of this study was to investigate the effects of small molecule surfactants which are commonly used in an ice cream production, including mono- and diglycerides, partially unsaturated mono- and diglycerides and Tween 80 (polysorbate 80) on the formation and properties of coconut oil-containing emulsions made from the fractionated coconut milk (RFM and CSM) of coconut milk.

6.3 Materials and Methods

6.3.1 Materials

The commercial frozen coconut milk and virgin coconut oil used in the previous experiments (Chapters 3-5) were also used in this chapter. Two different types of non-ionic small molecule surfactants, such as oil-soluble and water-soluble surfactants, were used. The oil-soluble surfactants used were mono- and diglycerides (GRINDSTED MONO-DI HP 40-M) (MDG) and partially unsaturated mono- and diglycerides (GRINDSTED PS217) (PUMDG). The water-soluble surfactant used was polyoxyethylene (20) sorbitan monooleate (Palsgaard 7463) also known as Tween 80

(T80). These small molecule surfactants were supplied by Danisco Australia Pty Ltd, Australia.

6.3.2 Preparation of coconut milk fractions

Two different coconut milk fractions, reduced fat milk (RFM) and coconut skim milk (CSM), were prepared from frozen coconut milk (CM), as described in Section 4.3.1. The contents of protein and fat analysed by the proximate analysis from RFM and CSM are shown in Table 6.1.

Table 6. 1 Contents of protein and fat (wt%) in RFM and CSM

Material	Protein	Fat
RFM	1.3 ± 0.2	5.4 ± 0.2
CSM	0.5 ± 0.1	0.1 ± 0.1

[a] Mean values of 6 replicates ± standard deviation

6.3.3 Preparation of emulsions

Two different sets of emulsions containing 10% coconut oil were prepared, on the basis of RFM or CSM (Table 6.2).

Table 6. 2 Proportion of three different base materials (RFM, CSM and CO) mixed to make two different sets of emulsions derived from RFM and CSM and the concentrations of oil to protein (O/P) in emulsions.

Emulsion base	Proportion of base ingredient (wt%)			Oil (%)	Protein (%)	O/P Ratios
	RFM	CSM	CO			
RFM	95		5	10	1.2	8
CSM		90	10	10	0.5	20

Abbreviation: RFM = reduced fat milk, CSM = coconut skim milk and CO = bulk coconut oil.

In order to study the effects of small molecule surfactants (SMSs), MDG, PUMDG and T80 were added into the two sets of emulsions based on RFM and CSM at different combinations and concentrations of SMSs, which are shown in Table 6.3. The protein concentration and the ratio of SMSs to protein in emulsions are shown in Table 6.4.

Table 6. 3 Formulations of emulsions containing 10% CO, based on RFM or CSM, with different types of small molecule surfactants (wt%)

No.	Sample code	RFM	CSM	CO	MDG	PUMDG	T80
1	C (control)	✓		✓	-	-	-
2	C/M	✓		✓	0.25	-	-
3	C/P	✓		✓	-	0.25	-
4	C/M/P	✓		✓	0.125	0.125	-
5	C/T	✓		✓	-	-	0.05
6	C/T/M	✓		✓	0.25	-	0.05
7	C/T/P	✓		✓	-	0.25	0.05
8	C/T/M/P	✓		✓	0.125	0.125	0.05
9	S (control)		✓	✓	-	-	-
10	S/M		✓	✓	0.25	-	-
11	S/P		✓	✓	-	0.25	-
12	S/M/P		✓	✓	0.125	0.125	-
13	S/T		✓	✓	-	-	0.05
14	S/T/M		✓	✓	0.25	-	0.05
15	S/T/P		✓	✓	-	0.25	0.05
16	S/T/M/P		✓	✓	0.125	0.125	0.05

Abbreviation: C = emulsions based on reduced fat milk (RFM), S = emulsions based on coconut skim milk (CSM), M = mono- and diglycerides, P = partially unsaturated mono- and diglycerides and T80 = Tween 80

Table 6. 4 Protein concentration and ratio of surfactant to protein in emulsions

No.	Emulsion base	Protein (%)[a]	Sample code	Ratio of surfactants to protein			
				MDG	PUMDG	T80	Total
1	RFM	1.19	C (control)	-	-	-	-
2			C/M	0.21	-	-	0.21
3			C/P	-	0.21	-	0.21
4			C/M/P	0.10	0.10	-	0.21
5			C/T	-	-	0.04	0.04
6			C/T/M	0.21	-	0.04	0.25
7			C/T/P	-	0.21	0.04	0.25
8			C/T/M/P	0.10	0.10	0.04	0.25
9	CSM	0.46	S (control)	-	-	-	-
10			S/M	0.54	-	-	0.54
11			S/P	-	0.54	-	0.54
12			S/M/P	0.27	0.27	-	0.54
13			S/T	-	-	0.11	0.11
14			S/T/M	0.54	-	0.11	0.65
15			S/T/P	-	0.54	0.11	0.65
16			S/T/M/P	0.27	0.27	0.11	0.65

[a] by calculation. Abbreviation: The first letter: C = emulsions based on reduced fat coconut milk (RFM), S = emulsions based on coconut skim milk (CSM); The following letters: C = control (no MDG and PUMDG), M = mono- and diglycerides, P = partially unsaturated mono- and diglycerides and T80= Tween 80

Figure 6.1 shows a flow diagram for the preparation of emulsions which involved two homogenisation steps. Briefly, MDG and/or PUMDG were added and solubilised into the melted CO at 35°C and Tween 80 was added into liquid phases of RFM and CSM. The liquid phase was then mixed with bulk CO, heated at 65°C for 5 min and homogenised using a high shear mixer (Silverson L4RT, Silverson Machine ltd, Waterside, England) at 6,000 rpm for 2 min to make a coarse emulsion. The coarse emulsion was subsequently homogenised with a two-stage high pressure homogeniser

(APV 2000, Rannie/Gaulin, Albertslund, Denmark) at 220/20 bar ($1^{st}/2^{nd}$ stage) with four passing times to make a fine emulsion. All emulsions were prepared at least in duplicate.

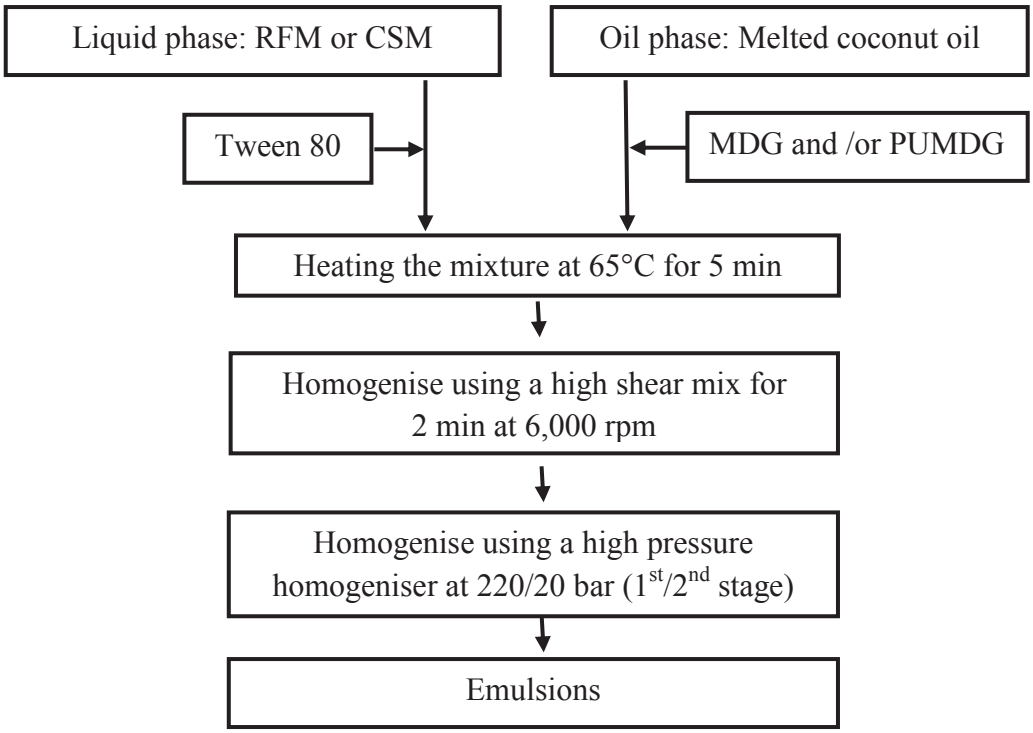

Figure 6.1 A flow diagram for the preparation of emulsions

6.3.4 Sample analyses

6.3.4.1 Analysis of protein and fat in RFM and CSM

RFM and CSM were determined for their contents of protein and fat using the methods described in Sections 3.3.4.1-3.3.4.4.

6.3.4.2 Particle size diameter and size distribution of emulsions

The particle size and size distribution of emulsions were measured after the method described in Section 5.3.4.2.

6.3.4.3 Zeta potential of emulsions

The electrical charge (ζ-potential) of coconut oil droplets in emulsions was measured after a procedure as described in Section 3.3.6.

6.3.4.4 Microscopic examination of emulsions

Emulsions were observed after the preparation and one day after storage at 4°C and 35°C as described in Section 4.3.3.4.

6.3.4.5 Emulsion stability against phase separation

The stability of emulsions against phase separation was analysed using the method as described in Section 4.3.3.5.

6.3.4.6 Analysis of interfacial proteins by SDS-PAGE

The composition of proteins adsorbed at the interface of emulsion droplets were analysed by SDS-PAGE as described in Section 4.3.3.7.

6.3.4.7 Surface protein load of emulsions

The amount of surface protein load was analysed from amount of protein of washed cream of emulsions (analysed by the Lowry method (see Section 5.3.4.7)) following an equation as described in Section 4.3.3.8.

6.3.4.8 Statistical analysis

All experiments and measurements were carried out at least in duplicate and analysed as described in Section 3.3.10.

6.4 Results and Discussion

6.4.1 Emulsion particle size

The average volume weight diameter (d_{43}) of emulsion particles was analysed with and without mixing with a dissociating medium (1.25% SDS solution) to investigate the presence and occurrence of droplet flocculation or coalescence after emulsion preparation (Figure 6.2) and also before and after storage for one day at two different temperatures (4^{o}C and 35^{o}C) (Figure 6.3). Concerning about the effect of individual surfactants, when Tween 80 was added to the emulsions made with CSM and RFM, the mean diameters of apparent droplets were reduced from 7.73 μm to 3.91 μm and from 34.74 μm to 14.69 μm, respectively. For the emulsions based on CSM, Tween 80 aided reducing both the apparent and real droplet diameters two times smaller than the control emulsions without added Tween 80. In contrast, the addition of oil-soluble surfactants (MDG and/or PUMDG) only without Tween 80 led to a noticeable increase in the droplet size of the emulsions based on CSM from 7.73 μm to around 15-38 μm rather than a reduction in their droplet size but such an increase did not occur when Tween 80 was added together in combination with them. In contrast, the oil-soluble surfactants helped reducing the size of droplets in the emulsions made with RFM. The former case indicates that the oil soluble surfactants (MDG and PUMDG) hindered the emulsifying efficiency of proteins in CSM to form and stabilise the oil droplets. This may be due to the fact that the emulsions made with CSM contained a low level of protein (0.5%) compared to the emulsion made with RFM at 1.2% protein level. This implies that even though CSM was efficient in forming the small droplet size (real droplets) at the lower concentration, the amount of proteins adsorbed at the interface could be much lower, thus probably resulting in a very thin film formed around the droplets. As a consequence, the layer of emulsion droplets might not be strong enough to resist to a change by a change introduced when the oil-soluble surfactants were added in the oil phase. The level of proteins in both emulsion systems could be insufficient to cover the surface of oil droplets. According to a work reported by Onssard et al. (2006), the amount of coconut proteins that was enough to cover the homogenised oil droplets of an emulsion containing 10% corn oil was 0.2% proteins.

It should also be noted that the droplet sizes measured in the presence and absence of SDS did not shown a significant difference for all the emulsions made with CSM, indicating the oil droplets were present discretely without flocculation. However, the dissociated droplets from the emulsions made with RFM became much smaller in their size (Figure 6.2B), indicating the droplet destabilisation in some manners such as flocculation because the aggregates could be dissociated with SDS (Demetriades and McClements, 2000, Goff, 1997b). Also, it should be highlighted that the apparent mean droplet diameter was much smaller in the control emulsion prepared from CSM without added Tween 80 than in the emulsion prepared from RFM, which was about 7.7 μm in the former and 34.7 μm in the latter but their dissociated mean droplet sizes were smaller in the latter case but not very different which were 7.2 and 4.57 μm, respectively. Although it is not clear, the reason for the high degree of droplet flocculation only observed from the emulsions prepared from RFM may be due to the hydrophobic interaction caused by the main proteins in RFM which covered the surface of oil droplets.

Interestingly, the changing patterns of the particle size observed in the emulsions made with CSM that were affected by the two different species of water and oil soluble surfactants could not be seen in the emulsions made with RFM. Firstly, the addition of oil soluble surfactants helped reducing the droplet size (apparent droplets). Secondly, the extent of the size reduction (apparent droplets) became more prominent when both species of surfactants were used together, probably resulting from a synergistic effect. Thirdly, the size of droplets was much smaller when it was measured in the presence of SDS and there was however no difference in the size of dissociated droplets between all emulsions made with RFM, indicating as already described above, the presence of droplet flocculation, regardless of the addition of surfactants. It should also be noted that the dissociated (real) droplet size was not significantly different between the two emulsion systems made with CSM and RFM when they were prepared with the addition of Tween 80, irrespective of the addition of oil soluble surfactants as also indicated in the above.

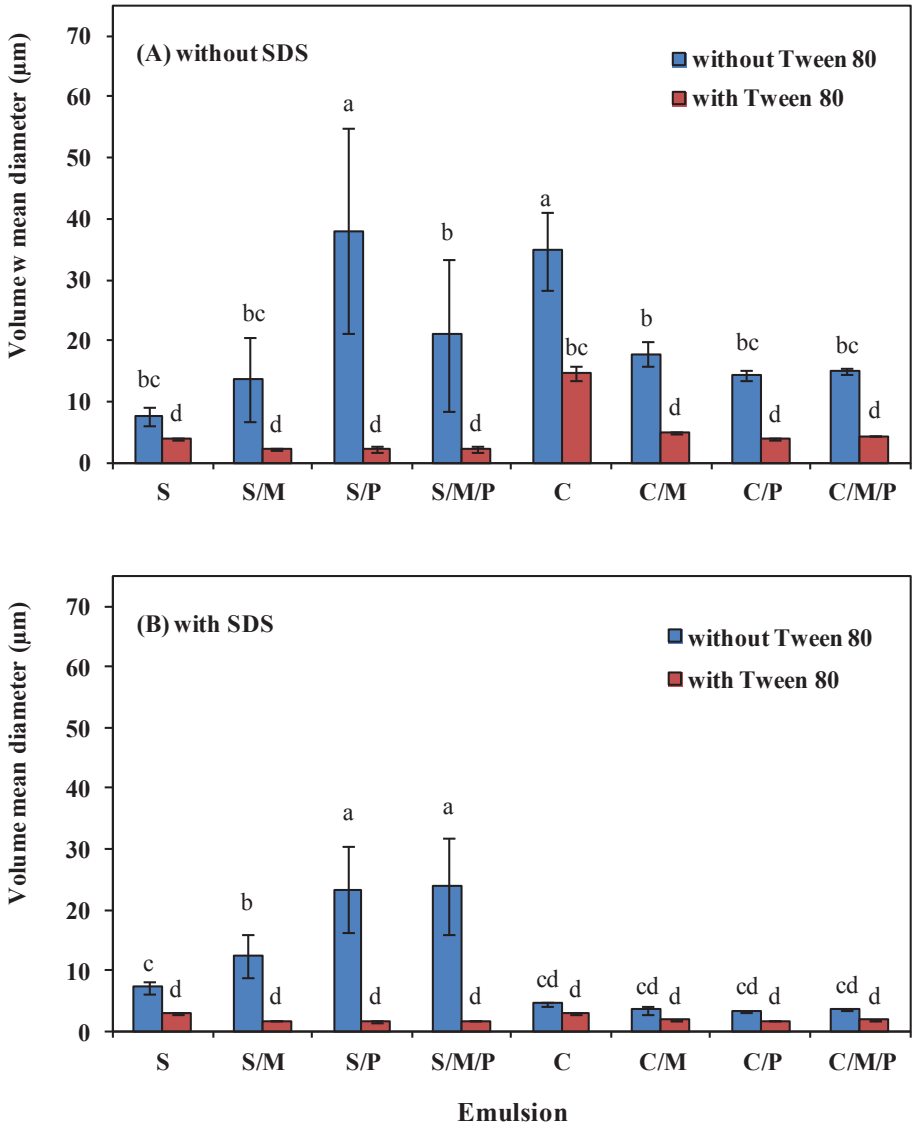

Figure 6.2 Mean particle diameters of emulsions prepared from CSM and RFM with the addition of different types of surfactants that were measured on the same day after the preparation in the absence of SDS (A) and in the presence of SDS (B) after mixing with 1.25 wt% SDS solution before analysis. The first letters: S and C indicate the emulsions made with CSM and RFM, respectively and the subsequent letters represent the surfactants added with M = mono- and diglycerides; and P = partially unsaturated MDG. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

The effects of storage of emulsions at different temperatures 4°C and 35°C are shown in Figure 6.3. Overall the results indicated that in the presence of only lipid surfactants without Tween 80, the apparent and dissociated droplets of both emulsion systems prepared from CSM and RFM tended to increase in size after the emulsions were stored

at high temperature (35°C). However, the droplet size increment was more pronounced for the groups of emulsions in the absence of Tween 80 than in the absence of Tween 80 and also for the emulsions prepared with RFM than with CSM. At low temperature (4°C), the particle size in both emulsion systems based on CSM and RFM did not change much in size. The size of droplets in both emulsions was relatively stable, regardless of the presence of Tween 80.

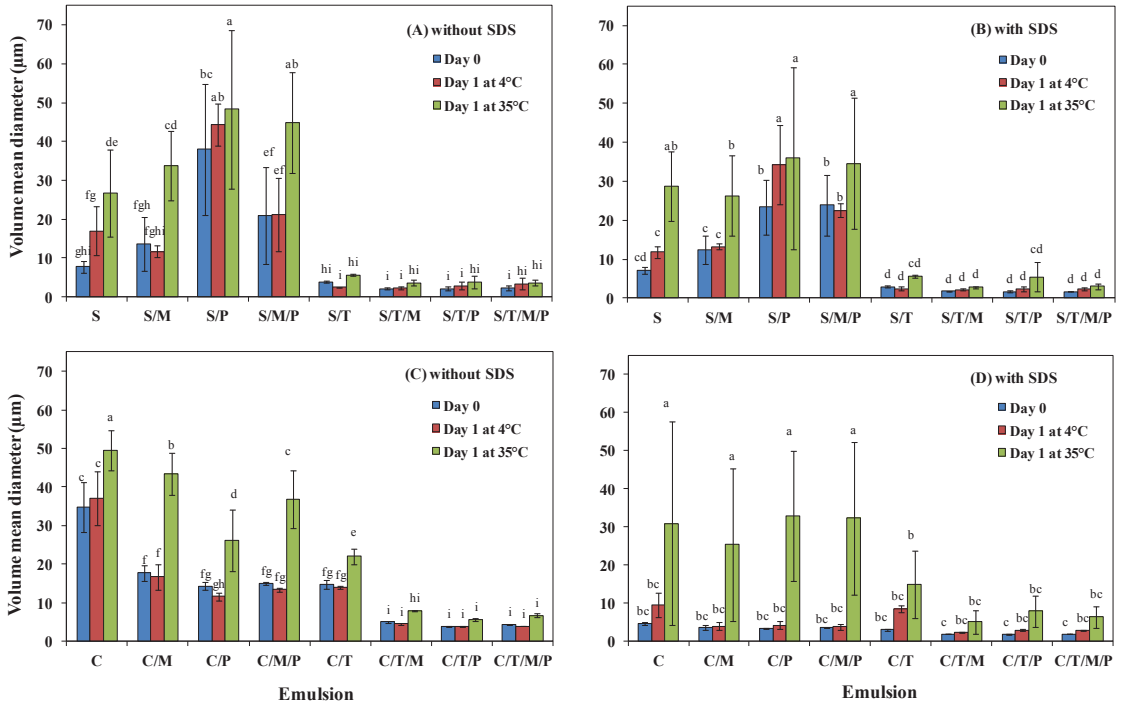

Figure 6.3 Comparisons on the mean particle diameters of emulsions prepared from CSM (A and B) and RFM (C and D) with different surfactants that were measured after the preparation and after one day storage at two different temperatures (4 °C and 35°C) in the absence of SDS (A and C) and in the presence of SDS (B and D) after mixing with 1.25 wt% SDS solution before analysis. The first letters: S and C indicate the emulsions made with CSM and RFM, respectively and the subsequent letters represent the surfactants added with M = mono- and diglycerides; and P = partially unsaturated MDG. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

The apparent and dissociated droplets measured in the absence and presence of SDS, respectively was not different for the emulsions prepared from CSM, suggesting that droplet flocculation did not exist in these emulsions and that if there was an increase in

the particle size resulting from storage at different temperatures, it was due to the droplet coalescence. For the emulsions prepared with RFM, the emulsions stored at 4°C showed that they contained the flocculated droplets similar to the fresh emulsions whereas the increased droplet size observed at 35°C was found to be caused by the droplet coalescence because the droplets could not be dissociated by SDS, thus the droplets were similar in size between the absence and presence of SDS. The results indicate that in the presence of both water-soluble and oil-soluble surfactants, the droplets in both emulsion systems showed relatively high droplet stability with little flocculation during storage for one day at both 4°C and 35°C compared to the emulsions containing only lipid-based surfactants without Tween 80.

In several recent studies, the protein bridging and/or hydrophobic interaction was suggested to be a mechanism causing the droplet flocculation in the emulsions stabilised by 11S globulins which is also found in coconut milk. It is because the aggregates could be dissociated with the deflocculating agent (Demetriades and McClements, 2000, Floury et al., 2002, Puppo et al., 2011, Puppo et al., 2005). This could be the possible reasons for the high degree of the droplet flocculation observed in the emulsion droplets based on RFM. The emulsions prepared from RFM is believed to be covered by a relatively thick interfacial layer with a large amount of coconut milk proteins compared to the emulsion droplets based on CSM which is thought to be surrounded by a relatively high amount of Tween 80. This was indicated by the measured amount of adsorbed proteins at the interface which is shown in Table 6.5 in the following section.

6.4.2 Surface electrical charges of emulsions

The initial droplet surface charges of unhomogenised oil droplets naturally present in RFM and CSM were measured. The zeta (ζ) potential of each of these samples was about -23.3±1 mV and -7.6±0.5 mV, respectively. The surface charges of droplets in homogenised emulsions based on RFM and CSM that were prepared with or without containing small molecule surfactants were analysed (Figure 6.4). It was found that the homogenised droplets possessed more negative charges than the initial charges of droplets in those two unhomogenised emulsions. The electrical charge was changed

from -7.6±0.5 mV to -43.0±1 mV for the emulsions based on CSM and from -23.3±1 mV to -28.9±1.1 mV for the emulsions based on RFM. The levels of these zeta potentials were however not different from the respective emulsions with added small molecule surfactants. These results indicate that homogenisation caused the droplets to be more negatively charged.

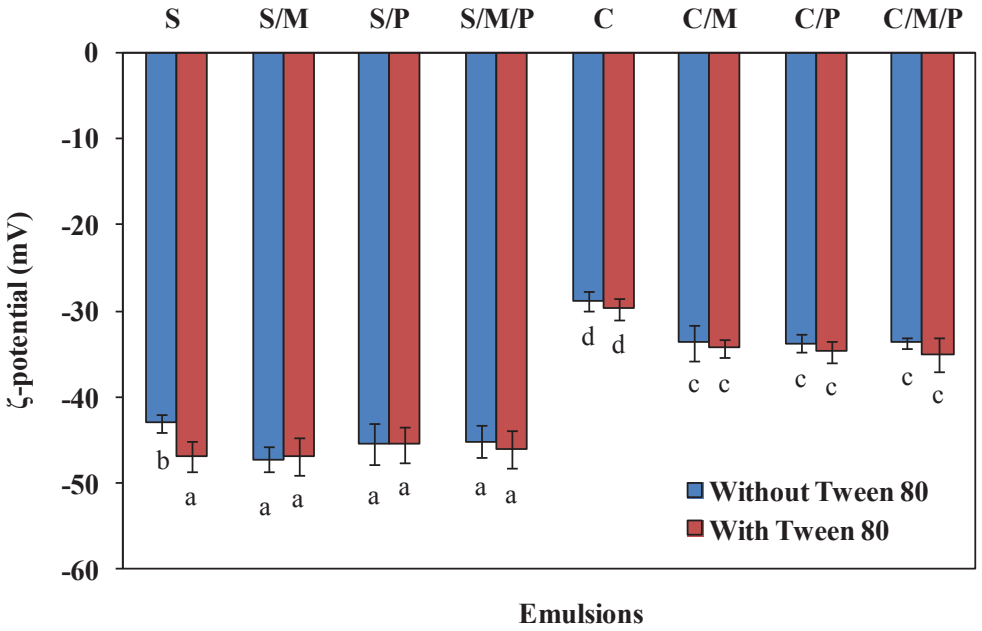

Figure 6.4 Zeta potential of emulsions prepared from CSM and RFM with different surfactants that were measured after the preparation before storage. The first letters: S and C indicate the emulsions made with CSM and RFM, respectively and the subsequent letters represent the surfactants added with M = mono- and diglycerides; and P = partially unsaturated MDG. Mean values ± standard deviation (n = 6) with the same letter are not significantly different (P > 0.05).

In the absence of small molecular surfactants, it can be thought that the only surface active molecules are proteins. Therefore, the higher negative zeta potentials observed after homogenisation and also in all the emulsions prepared with CSM than in the emulsions based on RFM could be attributed to the several possible reasons as follows: a) change and difference in the composition of proteins adsorbed at the interface, b) difference in the ionic strength between the emulsions with different ratios of ingredient bases and different types of stabilisers, c) decrease in the droplet size after homogenisation and the formation of smaller droplet size in the emulsions based on

CSM than the emulsion made with RFM. Among these factors, the more predominant factor for the higher negative charges of droplets in the CSM-based emulsions than in the RFM-based emulsions could be possibly due to the adsorption of water-soluble coconut milk proteins present in CSM which are main CSM proteins and can be more negatively charged than the other proteins, such as hydrophobic or salt-soluble proteins, that are present more in RFM than in CSM. Also, the smaller size of droplets formed in the CSM-based emulsions could be attributed to the high in their zeta potentials compared with the RFM-based emulsions. Also, the relatively minor change in the droplet charge after homogenisation of RFM from -23.3±1 mV to -28.9±1.1 mV indicates that a relatively less change in the protein composition at the droplet surfaces in the emulsions based on RFM. The zeta potential levels between -29 mV and -33 mV observed from the RFM-based emulsions with or without added surfactants were close to the zeta potential value of -38.0 mV at pH 8 in 10 wt% corn oil emulsions emulsified with the proteins extracted from coconut cream as reported by Onsaard et al. (2006). The addition of either water-soluble or oil-soluble emulsifiers led to small changes in droplet charges in both emulsion systems. In the presence of Tween 80, the droplet charge in both emulsion systems became slightly decreased. Similarly, the presence of oil-soluble surfactants led to a reduction of zeta-potential slightly.

6.4.3 Surface protein loads on emulsion droplets

The amount of proteins loaded on the surface of oil droplets was analysed from the washed creams of all emulsion samples (Table 6.5). It was found that the surface protein load in the emulsions based on RFM was found to be much higher than the emulsions based on CSM in all cases regardless of the addition of small molecule surfactants. The addition of surfactants to both emulsions caused a decrease in the surface protein load. Emulsion droplets with the highest interfacial protein concentration of 14.8% was observed from the droplets in the RFM emulsion without containing any surfactants in which the surface protein load was 6.02 mg/m^2.

The addition of Tween 80 substantially lowered the surface protein load in this RFM-based emulsion from 6.02 to 1.99 mg/m^2. Also, the addition of MDG and/or PUMDG

Table 6. 5 Amount of interfacial protein (wt%) and surface protein load (mg m^{-2}) of emulsion droplets containing 10% coconut oil, based on RFM or CSM, with different types of small molecule surfactants.

Surfactant	wt% of interfacial protein		Surface protein concentration (mg m^{-2})	
	RFM	CSM	RFM	CSM
No surfactant	14.76 ± 3.4^{a}	0.37 ± 0.1^{a}	6.02 ± 1.4^{a}	0.38 ± 0.1^{b}
M	12.96 ± 3.1^{ab}	0.28 ± 0.1^{cd}	2.84 ± 0.7^{b}	0.18 ± 0.1^{c}
P	12.97 ± 2.2^{ab}	0.32 ± 0.1^{bc}	2.66 ± 0.5^{b}	0.45 ± 0.1^{a}
M+P	8.79 ± 1.2^{c}	0.26 ± 0.1^{d}	1.73 ± 0.2^{cd}	0.02 ± 0.1^{f}
T	8.89 ± 0.7^{c}	0.28 ± 0.1^{cd}	1.99 ± 0.2^{bc}	0.16 ± 0.1^{cd}
T+M	12.31 ± 1.0^{ab}	0.33 ± 0.1^{ab}	1.34 ± 0.1^{cd}	0.14 ± 0.1^{de}
T+P	11.07 ± 1.3^{bc}	0.33 ± 0.1^{ab}	0.94 ± 0.1^{d}	0.13 ± 0.1^{e}
T+M+P	11.85 ± 0.6^{abc}	0.33 ± 0.1^{ab}	1.18 ± 0.1^{cd}	0.14 ± 0.1^{de}

Abbreviation: M = mono- and diglycerides, P = partially unsaturated mono- and diglycerides and T = Tween 80. Mean values are means of three replication ± standard deviation (n = 6) and values in the same column with the same letter are not significantly different ($P > 0.05$).

significantly reduced the surface protein load but its effect was more significant when both types of lipid-soluble surfactant were added together with Tween 80. For the surface protein load of emulsions based on CSM, a significant reduction in the surface protein load was also observed with the addition of either water-soluble or lipid-soluble surfactants or both of them but without no pronounced different effects between the different types of surfactants. The ratios of oil to protein in two different emulsion systems formulated with RFM and CSM were 8 and 20, respectively. Based on the rough estimation via calculation, almost all proteins present in the emulsions formulated with RFM appeared to be bound to the interface of oil droplets while the percentage of adsorbed proteins from the CSM-based emulsions was very small being about less than 10% of the total protein present in the CSM emulsions. Even taking into account some possible errors that might have been associated with the measurement of surface protein load, the obtained data seemed to clearly indicate a significant difference in the surface protein loads between the two emulsion systems. Regarding the analysis of surface

protein load, it should also be mentioned that the content of adsorbed protein was probably measured from partially hydrolysed washed creams rather than from the proteins completely extracted from washed creams and also the amount of water entrapped in the drained washed creams after removing water using filter papers could be different between the samples. This might have affected the measurement to a certain extent.

6.4.4 SDS-PAGE of emulsions

The two liquid bases, CSM and RFM, used to prepare all emulsions were analysed for protein composition by SDS-PAGE in the previous experiments as shown in Section 4.4.2. The same gel image is presented again in Figure 6.5. As described in Section 4.4.2, a migration pattern of proteins shows that the protein components of CSM are composed of two major subunits with the estimated molecular weights (MWs) of 18 and 26 kDa and some minor subunits with the MWs of 14, 16, 22, 35 and 55 kDa. Among these subunits, the bands at the MWs of 18, 26 and 55 kDa seemed to correspond to the polypeptide bands of coconut albumins that have been reported in the literature (Angelia et al., 2010, Garcia et al., 2005, Kwon et al., 1996, Tangsuphoom and Coupland, 2009). These proteins are water-soluble proteins solubilised in the serum phase of CM (Kwon et al., 1996). The protein components of RFM which were supposed to be identical to the proteins of CM were resolved into several bands of polypeptide subunits comprising of all polypeptide subunits presented in CSM with additional several bands with the MWs of 24, 32 and 35 kDa. These extra three subunits and one minor polypeptide of 22 kDa were reported to be the constituent polypeptide subunits of 11S globulins (cocosin) consisting of the acidic polypeptide subunits (32 and 35 kDa) and the basic polypeptide subunits (22 and 24 kDa) (Angelia et al., 2010, Garcia et al., 2005, Kwon et al., 1996, Tangsuphoom and Coupland, 2009). It should be noted that the protein bands corresponding to the MWs of 22, 24, 32, 35 and 55 KDa found in CM and RFM were not present in the electrophoresis pattern of proteins from CSM or the band intensity of some of those proteins were very faint (circled on the gel lane for CM in Figure 6.5). These proteins are the subunits of coconut 11S globulins which are salt soluble proteins. The results suggest that these proteins are the native membrane proteins found mainly to

be present on the surface of natural oil droplets in coconut milk, not in the serum phase of coconut milk. The minor band at 55 kDa detected from CSM is albumin which is water soluble as described already in the above.

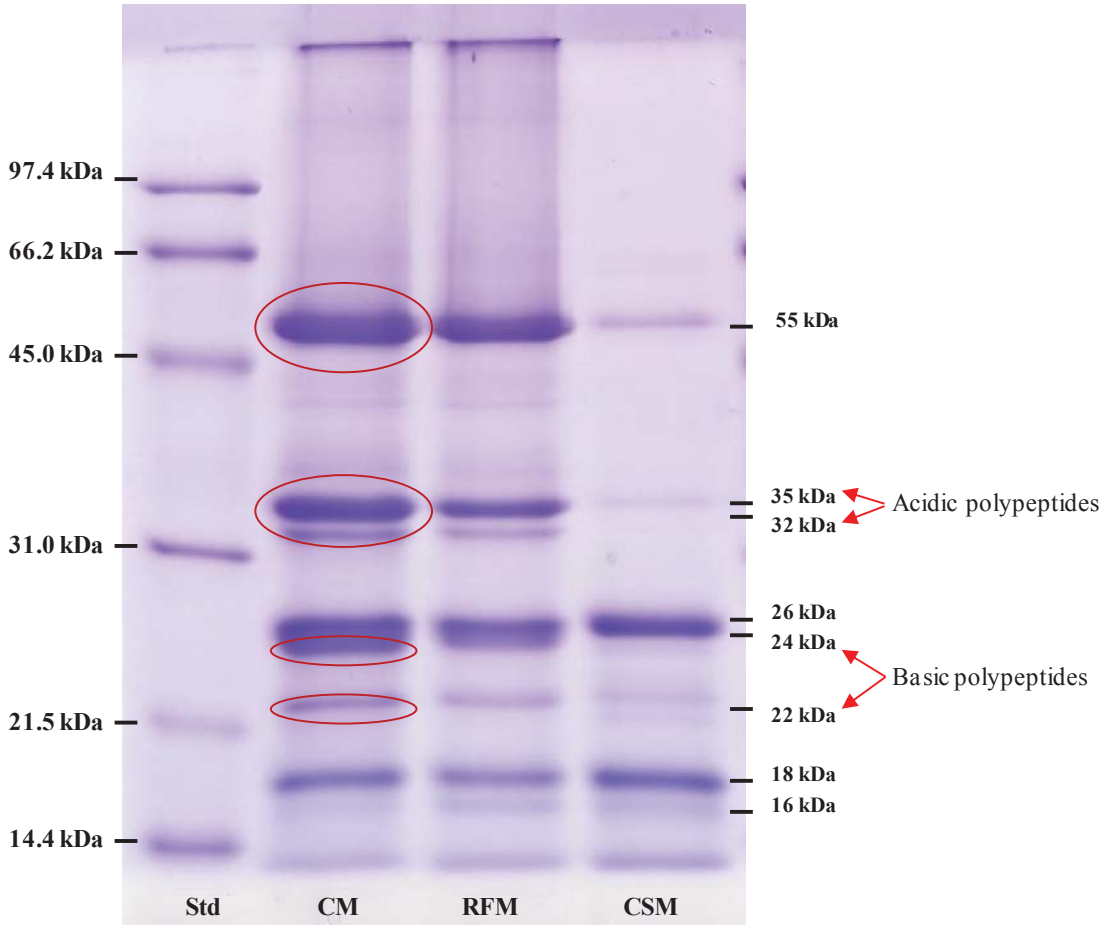

Figure 6.5 The migration pattern of the polypeptide subunits of proteins in coconut milk (CM), reduced fat milk (RFM) and coconut skim milk (CSM) after the separation on the 12% gel by SDS-PAGE. Std means standard molecular weight marker.

After several emulsions were prepared from CSM and RFM formulated with different types of small molecule surfactants, the cream phase from each emulsion was separated by centrifugation and washed with phosphate buffer. The washed cream obtained from each emulsion was analysed for its interfacial protein composition by SDS-PAGE. The protein bands characterised from the interfacial layers of emulsions containing different surfactants which were formulated based on RFM and CSM are shown, respectively, in

Figures 6.6A and 6.6B. After an electrophoresis with a reducing condition, several polypeptide bands with the MWs ranging from 55 to 16 kDa could be observed. The cream phase obtained from all emulsions made with RFM showed fairly similar patterns of polypeptides with the MWs of 55, 35, 32, 24, 22, and 16 kDa (Figure 6.6). These bands were consistent with polypeptides bands found in the original RFM, indicating the presence of coconut globulins of RFM of which 11 globulin polypeptide subunits were originally present at the interface and some other polypeptide subunits of 7S globulins later migrated into the oil-water interfacial layers after homogenisation.

The addition of the oil-soluble surfactants, i.e., MDG and/or PUMDG, led to no difference in the polypeptide subunits of interfacial proteins obtained, but the presence of the water-soluble surfactant, i.e., Tween 80, led to a difference in the polypeptide subunits of interfacial proteins in an emulsion added only with Tween 80. The subunits dissolved from this emulsion showed a relatively very faint band at 16 kDa. This phenomenon might be probably due to the displacement of 7S globulins by Tween 80 which was reported to be good at displacing proteins from the interface, resulting in the fader of the polypeptide band (Goff et al., 1987). However, this phenomenon was not seen in other emulsions containing Tween 80 together with MDG and/or PUMDG.

Figure 6.6B shows the migration pattern of interfacial polypeptide subunits of interfacial proteins obtained from the emulsions made with CSM. The resolved subunits in all treatments regardless of surfactants showed a similar pattern of bands comprising of the subunits with the MWs of around 55, 35, 32, 26, 24, and 18 kDa. The bands at 55, 26 and 18 kDa were polypeptides subunits similar to the subunits of albumins as described previously (Garcia et al., 2005, Kwon et al., 1996). As it was shown from the above results, the acidic polypeptides and basic polypeptides usually rendered doublet subunits at 35 and 32 kDa, and 24 and 22 kDa, respectively. This indicates the contamination of the coconut globulins in the CSM fraction due to the presence of a small amount of natural coconut oil droplets, as shown in Section 4.4.1, Table 4.4, during the separation process of CSM from CM.

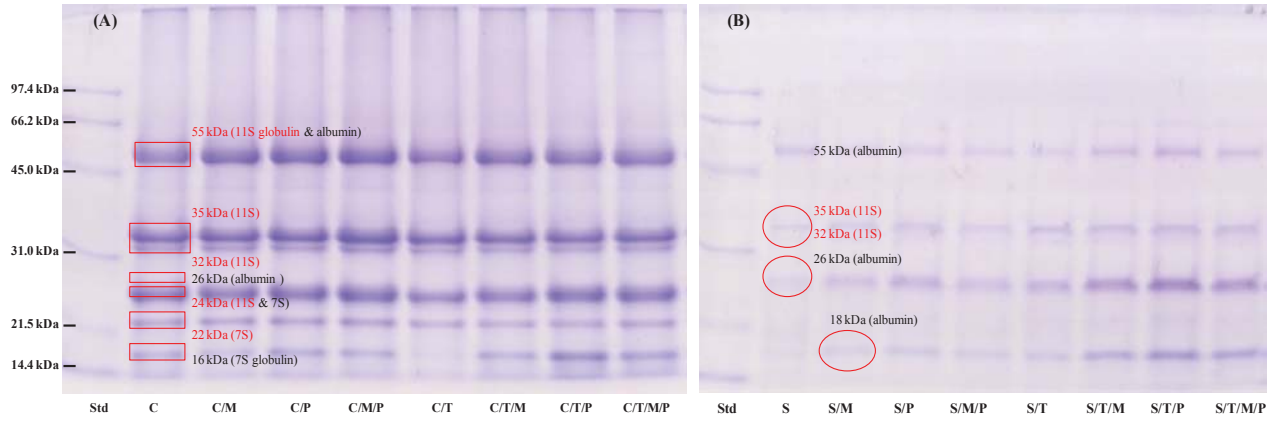

Figure 6.6 SDS-PAGE of polypeptide subunits of interfacial proteins obtained from the emulsions made with RFM (A) and CSM (B). The first letters: C and S indicate the emulsions made with RFM and CSM, respectively and the subsequent letters represent the surfactants added with M = mono- and diglycerides, P = partially unsaturated MDG and T = Tween 80 and Std means standard molecular weight marker.

It is worth noting that the intensity of protein bands observed from the CSM-based emulsions was much weaker than from the RFM-based emulsion samples, suggesting the amount of interfacial proteins made from the emulsions made with CSM was much smaller. This could be resulting from the fact that the concentration of a total protein in the emulsions made with CSM was lower than those made with RFM. Also, it could be partly attributed to the surface to volume ratio of oil droplets which was larger in the former than the latter because their oil droplet size was smaller.

6.4.5 Micrographs of emulsions particles

Emulsion samples were observed for their physical characteristics of emulsion droplets under a light microscope and the droplet images of each emulsion taken are shown in Figures 6.7. The microscopic images show one major noticeable effect of Tween 80 on the properties of emulsions but its effect was quite different between the two emulsions systems made with RFM and CSM.

For the RFM-based emulsions, regardless of the addition or type of lipid-soluble surfactants, the oil droplets in these emulsions were observed to be more clumped and densely aggregated (flocculated) when the water-soluble surfactant (Tween 80) was not added, in comparison to the emulsions with added Tween 80. The particle size of these emulsions with no added Tween 80 was measured to be larger as shown in Figure 6.2. This could thus be confirmed by the presence of more pronounced clusters of droplet aggregates. After storage of these emulsion samples at 4°C and 35°C, the phenomenon (aggregation of droplets) was seen to have become more substantial after one day storage but it was more significant at 35°C than 4°C. Although it is not sure, the observed less flocculation from the emulsions containing Tween 80 could be possibly due to the steric hindrance acting on droplets induced by Tween 80 adsorbed at the interface.

For the CSM-based emulsions prepared without added Tween 80, the droplets were found to be much larger than the emulsions containing Tween 80, except for the control sample with no added Tween 80 and lipid-soluble surfactants at day 0. In these CSM-

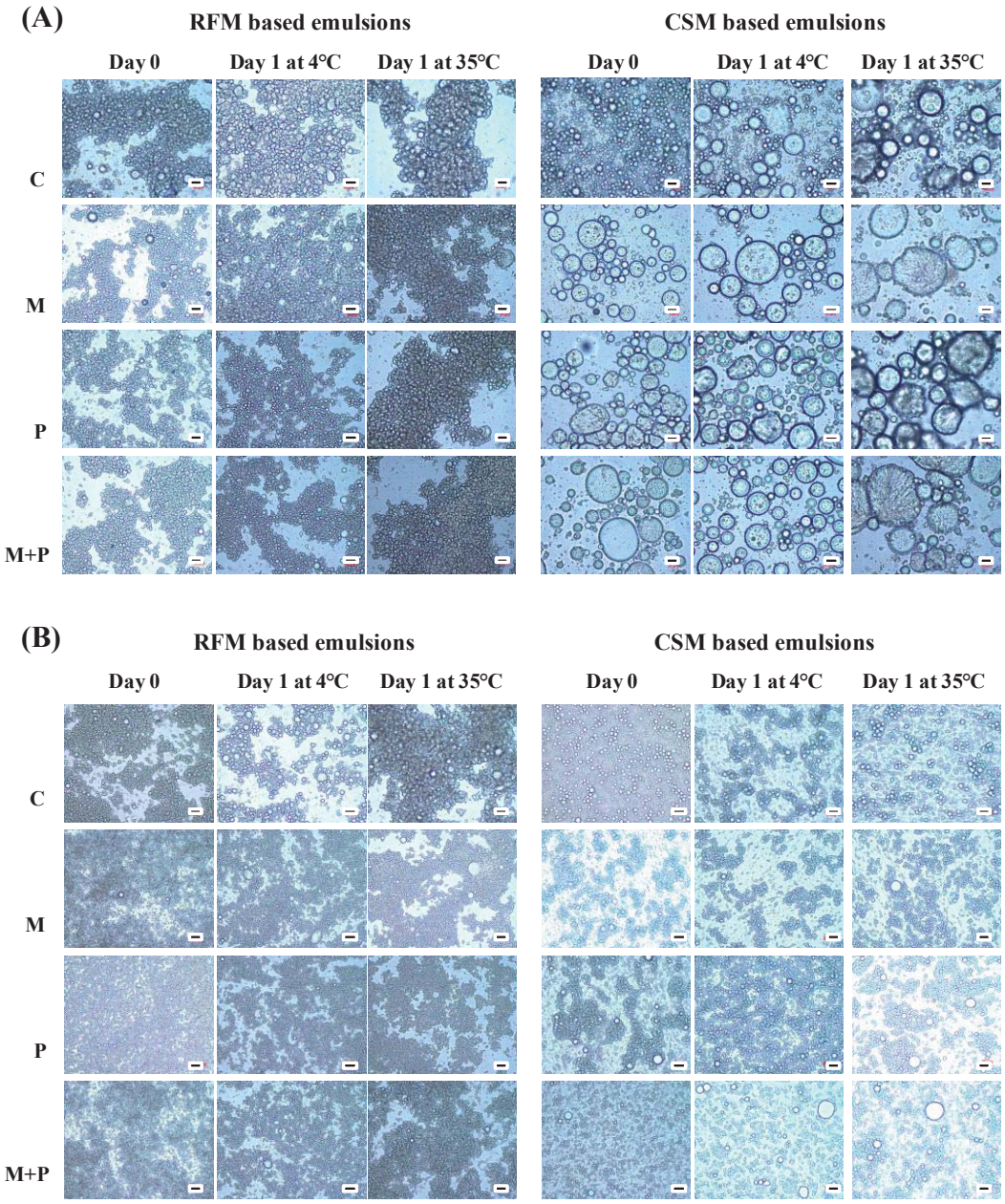

Figure 6.7 Optical micrographs of droplets in emulsions containing different lipid soluble small molecule surfactants without Tween 80 (A) and with Tween 80 (B) at 400X magnification on the preparation day (Day 0) and one day (Day 1) after storage at 4°C and 35°C. Abbreviation: C = control (no lipid soluble surfactants), M = mono- and diglycerides added, and P = partially unsaturated mono- and diglycerides added. The scale bar is 15 µm.

based emulsions, the droplets became bigger in size under a microscope especially at both low and high temperature. In the presence of Tween 80, the large droplets were not observed and the small droplets existed discretely without aggregation unlike the RFM-based emulsions showed the highly aggregated droplets even Tween 80 was

added to it. These emulsions were not also changed too much after storage at both temperatures.

The microscopic observations of the CSM-based emulsions with and without Tween 80 were also in agreement with the particle size data shown in Figure 6.2 that showed that the droplet flocculation was not present in these emulsions and the large particle sizes observed in the samples containing the lipid-soluble surfactants only without added Tween 80 were due to the presence of large droplets. This might be caused by the inhibitory effects of lipid-soluble surfactants on the formation of stable small droplets. Also, from the observation of the control sample without containing any surfactants (both Tween 80 and lipid-soluble surfactants), the droplets stabilised only with proteins in CSM undergo the droplet coalescence after storage, whereas the droplets stabilised by proteins in RFM were more stable to coalescence but sensitive to flocculation.

The droplets size decreased dramatically in both emulsion systems as the water-soluble surfactant, i.e., Tween 80, was added. This may be because water-soluble surfactants could be better adsorbed and reduced in the interfacial tension than oil-soluble surfactants (Krog, 1977, Pelan et al., 1997b). In an emulsion based on CSM, the droplets were observed to have less flocculation and spread evenly on the slide. The evidence of flocculation could be observed in the emulsions based on RFM and the extent of droplet flocculation increased when stored at high temperature. In the presence of both water-soluble and lipid-soluble surfactants, the difference in the droplet characteristics of emulsions in both systems made with RFM or CSM could not be observed and differentiated between the emulsions containing different types of surfactants under the light microscope.

6.4.6 Stability of emulsions to phase separation

Most emulsions were found to be unstable undergoing creaming or two distinct phase separation on the preparation day and after storage at two different temperatures (Figure 6.8). The different storage temperatures resulted in some differences in the characteristics of the emulsion stability. Most emulsions were more stable at low

temperature (4°C) than at high temperature (35°C). At 35°C no stable emulsion was observed, regardless of the addition and type of surfactants added as all the emulsions had complete clear phase separation into the cream and serum phases.

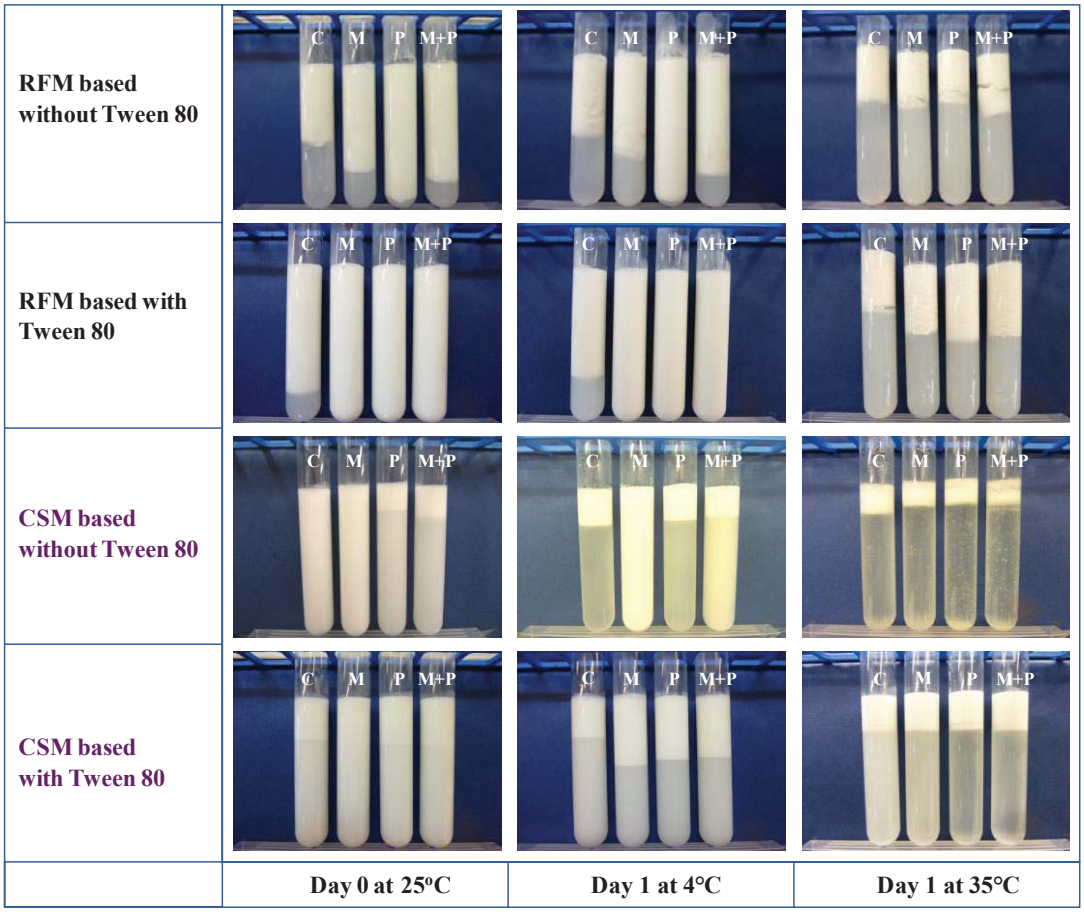

Figure 6.8 Phase separation of emulsions containing different surfactants after the preparation (Day 0) and one day after storage (Day 1) at 4°C and 35°C. Abbreviation: C = control (no lipid soluble surfactant), M = mono- and diglycerides and P = partially unsaturated mono- and diglycerides

In the absence of surfactants, no emulsions showed their stability and separated into two phases. The phase separation was observed from both emulsions made with RFM and CSM because in those emulsions the proteins were only a type of surface active molecules and those proteins in RFM and CSM were reported to have less ability to prevent the newly formed droplets from aggregations, thus a quick separation of emulsions into two phases (Onsaard et al., 2005, Onsaard et al., 2006). In those emulsions without small molecule surfactants, flocculation may be due to hydrophobic

interaction especially in the emulsions made with RFM which usually occurs in an emulsion stabilised by soy proteins which are the similar type of proteins found in coconut milk (Floury et al., 2002). In the presence of a single or both types of small molecule surfactants (PUMDG or MDG) together with Tween80, little separation of an emulsion based on RFM or CSM was observed at 4°C. In the presence of both types of small molecule surfactants, the most stable emulsions were emulsions made with RFM stored at low temperature. This finding informs that the stability of emulsions made from coconut milk, RFM or CSM can be extended and achieved by the addition of two different small molecule surfactants especially when the emulsions are stored at 4^{o}C.

6.5 Conclusions

The types of added small molecule surfactant, lipid-soluble surfactants (MDG and PUMDG) and a water-soluble surfactant (Tween 80), led to the difference in droplet formation and emulsion stability. Without any added surfactants, the droplets of emulsions based on CSM were smaller than the droplets of the RFM-based emulsions. The presence of water-soluble surfactant (Tween 80) led to a substantial reduction in the droplets size in both CSM-based and RFM-based emulsions. A number of small droplets with less flocculation could be observed under microscope in the emulsions based on CSM. Unlike the water-soluble surfactant, the additional lipid-soluble surfactants in emulsions resulted in an increase in the droplet size of CSM-based emulsions but resulted in a reduction in the droplet diameter of RFM-based emulsions. The presence of both types of surfactants makes the emulsion droplets become further decreased in size in the emulsions prepared from RFM but in contrast, this caused an increase in the droplet size of CSM-based emulsions. The competitive displacement of added surfactants was observed to a certain extent, as shown by the reduction in the surface protein load in all cases, regardless of the types of surfactants. The significant reduction in the surface protein load was observed in the emulsions added with both water-soluble and oil-soluble surfactants. The SDS-PAGE suggests that Tween 80 may have displaced proteins from the interface as it showed the absence of a fragment of some polypeptide subunits on the gel. Overall, the results provide a valuable insight into the stability of coconut milk emulsions can be manipulated and improved by the use of small molecule surfactants adequately.

Chapter 7

Properties of ice cream mixes and ice creams made from coconut skim milk at different ratios of coconut oil and sunflower oil and different concentrations of coconut skim milk proteins

7.1 Abstract

Ice cream mixes and ice creams containing 10% coconut oil based on coconut skim milk (CSM) with two different protein concentrations (0.3 and 1.1%) and four different ratios of oil blends of coconut oil (CO) and sunflower oil (SO) at 100:0, 75:25, 50:50 and 0:100 were prepared. The influence of solid fat content and CSM protein concentration was thus investigated as to how they affected the formation and properties of ice cream mixes and the corresponding ice creams. The four different ratios of oil blends corresponded to a solid fat content (SFC) at 4°C of about 90, 68, 45 and 0%, respectively. Another two ice creams were also prepared from coconut milk (CM) and dairy milk (DM) and analysed as reference samples under the same conditions. The differences in the solid fat and protein concentration led to some changes in the ice cream mixes which could be visually observed from the mixes before and after ageing overnight at 4°C. The stiffer aged mixes with less spreadable and more viscous body were observed from the CSM-based ice cream mixes containing higher protein content with 90% and 68% SFC and the CM-based ice creams (90% SFC) after ageing. Those aged emulsions exhibited little phase separation compared to the other samples. Through a microscopic examination and a measurement of flocculation index, the high magnitude of flocculated fat clumps was observed to occur in aged ice cream emulsions containing 68% SFC at both protein levels as well as in their corresponding melted ice creams. This underlines the role of liquid fat which is required to join the crystallised fat globules together. With an increase in the viscosity, storage modulus (G') and an ability of ice cream to resist melting, the aggregated fat clumps were estimated to be present in melted ice creams containing 68% or 45% SFC and DM-based ice cream as well. Ice creams containing 68% SFC exhibited a similar overrun percentage as ice cream based on DM or CM but with the slower melting rate and higher ability to retain the shape of the ice cream samples after melting at 20°C for 90 min.

7.2 Introduction

Homogenisation of ice cream mix is one of the important steps in the ice cream manufacturing process to emulsify liquid bulk oil (if added) and decrease the size of milk fat globules in order to form a uniformly stable emulsion (Marshall et al., 2003, Wilbey, 2003). The oil/fat droplets of ice cream mix after homogenisation is normally below 2 μm in size (Marshall et al., 2003, McClements, 2004a). The homogenised ice cream mix is then aged at a low temperature (e.g. 4°C) for at least 4 hr or up to 24 hr to allow the fat droplets to partially crystallise before freezing (Marshall et al., 2003). After ageing, the ice cream mix is simultaneously whipped and frozen. During the whipping and freezing process, air cells are incorporated in the ice cream mix by agitation and the crystalline fat droplets undergo fat destabilisation known as partial coalescence (Davies et al., 2000, Goff, 1997b, Marshall et al., 2003). The partial coalescence of partly crystallised fat droplets is one of the most important factors that determine the properties and structure of ice cream (Goff, 1997a, Goff, 1997b). It is responsible for the development of a network that stabilises air cells incorporated in the mix, as a result, the formation of a stable ice cream structure (Goff, 1997a, Goff, 1997b, Méndez-Velasco and Goff, 2011).

Partial coalescence of fat droplets involves the formation of two or more fat droplets joined partially through the penetration of fat crystals protruding from one partly crystalline fat droplet to another droplet (Herrera, 2002, van Boekel and Walstra, 1981b). The protruding fat crystals that pierce the neighbouring fat globule are covered by the liquid fat available in the fat droplets and form the semisolid junction (neck) enclosing the crystals joining the two droplets (Fredrick et al., 2010). The partial coalescence of fat droplets is essential during an ice cream manufacturing process for the purpose of creating a desired structure and entrapping air cells in ice cream (Goff, 1997a, Goff et al., 1999b). The degree of partial coalescence that occurs during the ice cream production should be optimised for the desired quality and textural properties of ice cream. The partially coalesced fat droplets aid the whippability of ice cream mix whereas the discrete fat droplets hinder this ability, hence affecting the overrun of ice cream (Dalgleish, 2006, Dickinson, 1992b). The extensive partial coalescence also

leads to a reduction in the whippability of ice cream mix with the formation of smaller air bubbles (Méndez-Velasco and Goff, 2011).

One of the most important factors that determines the partial coalescence of fat droplets is an optimal level of solid fat content (SFC) (e.g., ratio of liquid to solid fat) in oil droplets (Marshall et al., 2003). To create an optimal partial coalescence, fat droplets in the aged ice cream mix should contain about one-half to two-thirds of crystalline (solid) fat at the time of aeration and freezing (Marshall et al., 2003). If the solid fat content is too high, the rigidity of the solidified fat disrupts those fat droplets to form a proper fat structure (Fredrick et al., 2010, Rousseau, 2002). If too much liquid oil is present in the fat droplets during the freezing and whipping process, the liquid fat may become coalesced into a bigger oil droplet and spread onto the air-water interface, causing the rupture of air cells (Eisner et al., 2007, Marshall et al., 2003, Méndez-Velasco and Goff, 2012b).

When coconut milk is used as a base material for making ice cream, the coconut oil which is a highly saturated oil may be almost fully crystallised during the ice cream ageing process (Marshall et al., 2003, Reena et al., 2009). During the freezing and aeration step, there may not be enough liquid oil available in the coconut fat droplets to wet the protruding fat crystals, hence disrupting the formation of partial coalescence between the collided droplets (Rousseau, 2002). The SFC of oil can be changed by blending two or more different types of oils with different meting properties, thus an optimal level of SFC desired can be achieved by adequately modulating the ratio of oil blends. It has been shown, for example, that the SFC of coconut oil at 20°C decreased from 33.1% to 0% when coconut oil was blended with rice bran oil (Reena et al., 2009).

Similarly, mixing coconut oil with some vegetable oils, such as sunflower oil, with different melting and crystallisation temperatures may be used to achieve an optimal level of solid fat content in coconut fat droplets. However, no information is available in the literature as to how the blending of coconut oil with highly unsaturated oils alters the properties of fresh and aged ice cream mix emulsions and also how it affects the

properties of ice creams. Sunflower oil is rich in unsaturated fatty acids which can retain its liquid state down to subzero temperature (Méndez-Velasco and Goff, 2012b). The use of oil blends consisting of coconut oil and sunflower oil may enable the ice cream mix emulsions containing coconut oil with some liquid fat to form partly crystalline fat droplets after ageing and induce the formation of an optimal partial coalescence during the subsequent freezing stage. The objective of this study was to investigate the effects of different solid fat contents of oil blends consisting of coconut oil and sunflower oil at various ratios on the properties of ice cream mix emulsions and the corresponding ice creams made from coconut skim milk.

7.3 Materials and Methods

7.3.1. Materials

Coconut skim milk (CSM) and coconut skim milk powder (CSMP) were prepared following the methods described in Section 4.3.1 and Section 5.3.2, respectively. The fat sources in making ice cream were virgin coconut oil and sunflower oil. Virgin coconut oil (Zanian organic, Thailand) was purchased from a local store and was melted in a water bath at 50°C before use. Sunflower oil (Pams, Auckland, New Zealand) and standard dairy milk and cream (Fonterra, New Zealand) were purchased from local supermarkets and used without any further treatment.

7.3.2 Preparation of ice cream mix emulsions

Nine different ice cream mixes were prepared from the same base formulation consisting of 10% fat, 9% liquid glucose syrup (42 DE) (Avon Glucose Syrup A2151, Penford, Auckland, New Zealand), 0.25% partially unsaturated mono- and diglycerides (PUMDG) (GRINDSTED PS217, Danisco Australia Pty Ltd, Australia), 0.05% Tween 80 (T) (Palsgaard 7463, Danisco Australia Pty Ltd, Australia) and 0.15% stabilisers consisting of a 50:50 wt% blend of guar gum (GRINDSTED GUAR 250) and locust bean gum (GRINDSTED LBG 246) (Danisco Australia Pty Ltd, Australia).

Several mixes were formulated to have about 38.0% total solids by adjusting the amount of granulated sugar in the range between 12.0% and 16.6%. The final balance of the mixes was done with water. The amounts of ingredients used to formulate each of the nine different ice cream mix emulsions are shown in Table 7.1. It should be noted that although the amounts of sugar added appeared to be different between some formulations but the total amount of carbohydrates (i.e. predominantly sucrose) present in all samples was maintained to be similar through the formulations, as shown in Table 7.3, to minimise the effect of differing amounts of sugar on the freezing points and other attributes (e.g. alteration in shearing and fat agglomeration).

Table 7.1 Nine different formulations of ice cream mixes used for making ice creams containing two different levels of protein content and 10% fat with different ratios of coconut oil and sunflower oil.

Ingredients (g)	Formulation								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Coconut milk								63.1	
Coconut skim milk	64	64	64	64	63.41	63.41	63.41		
Coconut skim milk powder					5.3	5.3	5.3		
Coconut oil	9.94	7.44	4.94		9.84	7.34	4.84		
Sunflower oil		2.5	5	9.94		2.5	5		
Cow's milk									18
Dairy cream									25.43
Water								11.88	31.23
Sugar	16.61	16.61	16.61	16.61	12	12	12	15.57	15.89
Glucose syrup	9	9	9	9	9	9	9	9	9
PUMDG	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Tween 80	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Stabilisers	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Total (g)	100	100	100	100	100	100	100	100	100

Since the effect of oil blend was one of the objectives to investigate in this study, the fat sources of ice cream mixes were tailored to contain the different amounts of solid fat (at

4ºC) in the range between 0% and 90% by blending two different oils of coconut oil (CO) and sunflower oil (SO) at different ratios. The first four ice cream mix emulsions (Formulations 1-4) were made from CSM as an aqueous base with oil blends consisting of CO and SO at the ratios of 100:0, 75:25, 50:50 and 0:100. These samples are hereafter denoted as 100, 75, 50 and 0, respectively. The next three mixes (Formulations 5-7) were the same as those formulations 1-3 except for an incorporation of additional extra CSMP to raise the protein concentration from 0.3% to 1.1%. These ice cream mix emulsions are denoted as 100P, 75P, and 50P, respectively. In addition, another two ice cream mix emulsions at the protein level of 1.1% were also prepared directly from coconut milk (CM) (Formulation 8) and cow's milk (DM) (Formulation 9) as reference samples but their fat content of 10% was solely from coconut oil and dairy milk fat, respectively. To maintain the same level of 10% fat content, Formulation 8 was added with water and Formulation 9 was added with water and dairy cream. The percentage of oil derived from each of different fractions in ice cream formulations is shown in Table 7.2.

Table 7.2 Percentage of oil derived from different ingredients in the formulations of ice cream mixes containing 10% oil.

Formulation	Sample code	Aqueous phase base	Fat (wt%) from					
			CO	SO	CSM	CSMP	CM	Dairy milk and Cream
1	100	CSM	9.94		0.06			
2	75	CSM	7.44	2.50	0.06			
3	50	CSM	4.94	5.00	0.06			
4	0	CSM		9.94	0.06			
5	100P	CSM	9.84		0.06	0.10		
6	75P	CSM	7.34	2.50	0.06	0.10		
7	50P	CSM	4.84	5.00	0.06	0.10		
8	CM	CM					10.00	
9	DM	DM						10.00

Abbreviation: CO = coconut oil; and SO = sunflower oil; CSM = coconut skim milk; CSMP = coconut skim milk powder; CM = frozen coconut milk; DM = cow's milk.

Table 7.3 illustrates the final composition of ice cream mixes. As described above, the addition of CSMP in the mixes led to a substantial increase in the protein concentration of ice cream mixes from 0.3% to 1.1%. The amounts of solid fat content expected to be present in the ice cream mixes at 4°C at the ratios of two different oil blends of 100:0, 75:25, 50:50 and 0:100 were approximately 90%, 68%, 45% and 0%, respectively. This was estimated via calculation as indicated in Table 7.3. The solid fat content of dairy milk fat was varyingly reported to be in the range of 48-52% at 5ºC (Kaufmann et al., 2012, Lucas et al., 2005, Pelan et al., 1997a, Wright et al., 2011).

Table 7.3 Composition and solid fat content of ice cream mix formulations estimated on the basis of the composition of each ingredient used in ice cream mixes.

Composition (wt%)	Formulation								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Fat	10	10	10	10	10	10	10	10	10
Protein	**0.32**	**0.32**	**0.32**	**0.32**	**1.12**	**1.12**	**1.12**	**1.12**	**1.12**
Carbohydrates	27	27	27	27	26	26	26	26	27
Solid non fat	28	28	28	28	28	28	28	28	28
Total solids	38	38	38	38	38	38	38	38	38
Solid fat content*	**90**	**68**	**45**	**0.2**	**90**	**68**	**45**	**90**	**50**

* Estimated based on the relative amounts of solid fat content at 5°C of sunflower oil, coconut oil and milk fat which have about 0.2% (Saberi et al., 2012), 90% (Marshall et al., 2003, Reena et al., 2009) and 50% (Kaufmann et al., 2012, Lucas et al., 2005, Pelan et al., 1997a, Wright et al., 2011), respectively.

7.3.3 Preparation of ice creams

The process of making frozen ice creams is shown in Figure 7.1. Briefly, dry ingredients, such as granulated sugar, CSMP, PUMDG and stabilisers, were weighed and mixed well. All dry ingredients were then blended with the pre-heated mixture (65°C) of liquid ingredients (CSM, CO, SO, glucose syrup, Tween 80, dairy milk, cream, CM and water) to form the ice cream premix using a high shear mixer (Silverson L4RT, Silverson Machine Ltd., Waterside, England) at 6,000 rpm for 2 minutes. The

resulting premix was heated to 75°C for 15 minutes and then homogenised with a two-stage high pressure homogeniser (APV 2000, Rannie/Gaulin, Albertslund, Denmark) at 22 MPa (220 bar) and 2 MPa (20 bar) for the first and second stages, respectively, by passing four times. The homogenised mix was subsequently cooled to below 10°C and aged in a refrigerator at 4°C overnight. Each aged mix was then frozen in a 1.5 L-batch ice cream machine (Model 11194, La Turbine à Glace, Magimix) for a total of 30 minutes for freezing and whipping. Ice creams were then transferred from a freezing bowl, at a temperature of about -5°C which was monitored during the whipping and freezing process, into plastic containers and then immediately hardened in a freezer at -20°C. The production of ice cream samples was carried out in duplicate.

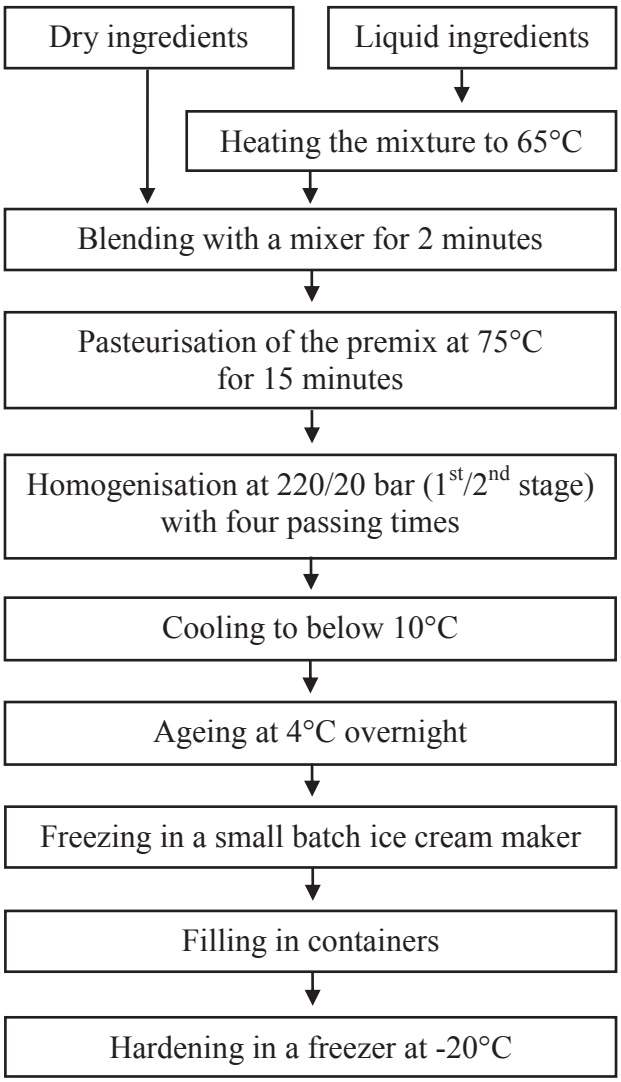

Figure 7.1 Flow diagram of the main steps involved in the production of ice cream.

7.3.4 Sample analyses

Samples of fresh and aged ice cream mixes collected during the preparation of ice creams and the ice cream samples after hardening were analysed as described below. All analyses were carried out at least in duplicate.

7.3.4.1 Particle size and size distribution

The mean particle diameters and particle size distributions of emulsion droplets in fresh and aged ice cream mixes and ice creams were measured as a volume weighted mean diameter (d_{43}) following the method described in Section 5.3.4.2.

7.3.4.2 Microscopic examination of emulsion droplets

Appearance of emulsion oil droplets in fresh and aged ice cream mixes were observed using a light optical microscopy before and after dilution with 1.25 wt% SDS solution at 1:5 ratio as described in Section 4.3.3.4.

7.3.4.3 Emulsion stability to phase separation

The stability of ice cream mix emulsions against phase separation was monitored and measured as described in Section 3.3.8.

7.3.4.4 Rheological properties

Fresh and aged ice cream mix emulsions (4°C) and molten ice cream emulsions (after melting in a refrigerator at 4°C for 2 hr) were measured for their rheological properties by a controlled stress rheometer (Rheometer AR-550, TA Instruments, New Castle, Delaware, USA) using a cone with 40 mm diameter and 2 degree angle and plate geometry with 105 μm gap for all measurement. During the measurement all emulsion samples were covered with a measuring plate for the whole measurement. The temperature of all samples between the cone and plate for the measurements of flow curve and frequency sweep test was controlled at a temperature of 4 ± 0.1°C by a circulating water bath and between 4°C and 30°C for the temperature sweep test. All

samples after loading on the Peltier platform were allowed at rest for 5 min on the platform to allow temperature equilibration and induce stress to relax before the measurement of each test. Three measurements were carried out for each test for each replicate sample and the average of those six measurements was reported (n = 6).

The flow behaviour was determined using a steady flow curve mode with a cone and plate geometry as mentioned above. The test was carried out in the range of shear rates between 0.5 and 500 s^{-1}. Apparent viscosity as a function of shear rate was reported. The small deformation oscillatory tests were performed to measure the viscoelastic parameters such as storage modulus (G′), loss modulus (G″) and loss tangent (tan δ) by conducting the following tests.

7.3.4.4.1 Stress sweep test

This test was conducted at log 0.06 to 5.0 Pa in order to determine a linear viscoelastic region (LVR). The other viscoelastic properties, such as storage modulus (G′), loss modulus (G″) and loss tangent (tan δ), were also recorded.

7.3.4.4.2 Frequency sweep test

This test was carried out in a frequency range between log 0.01 and 10 Hz with a constant stress (0.1 Pa) selected from a stress sweep test. The values of storage modulus (G′), loss modulus (G″) and loss tangent (tan δ) were reported.

7.3.4.4.3 Temperature sweep test

This test was carried out at increasing temperatures ranging from 4°C to 30°C with an increase rate of temperature at 1°C per minute. This test was carried out with a constant frequency at 1 Hz and a constant stress (0.1 Pa) selected from a stress sweep test. The values of storage modulus (G′), loss modulus (G″) and loss tangent (tan δ) were reported.

7.3.4.5 Overrun of ice cream

Overrun of ice cream samples was determined by measuring and comparing the weight of a known volume of the frozen ice cream sample to the weight of the same volume of the unfrozen ice cream mix (Segall and Goff, 2002). Overrun percentage was calculated as the following equation:

$$\textbf{Overrun (\%)} = \frac{\text{weight of unit volume of the mix - weight of unit volume of the frozen sample}}{\text{weight of unit volume of the frozen sample}} \text{ x } 100$$

7.3.4.6 Fat destabilisation (agglomeration) index of ice cream

The degree of fat destabilisation in ice cream samples was determined by a method based on a spectrophotometric technique (Keeney and Josephson, 1958) using the procedure reported by Segall and Goff (2002). Samples (1 g) of aged ice cream mixes and their corresponding ice cream samples after thawing in a refrigerator at 4°C for 2 hr were diluted with distilled water in a 500 ml flask at 1:500. The diluted solutions were measured for their absorbance by a UV-visible spectrophotometer (Shimadzu, Shimadzu Corporation, Japan) at 540 nm against distilled water as a blank. The principle of this measurement is based on the difference in turbidity between the aged ice cream mix and the melted ice cream which gives an indication of the extent of fat destabilisation and agglomeration in the ice cream. The fat destabilisation (agglomeration) index was calculated by using the following equation:

$$\text{Fat destabilisation index (\%)} = \frac{\text{Absorbance in mix - Absorbance in ice cream}}{\text{Absorbance in mix}} \text{ x } 100$$

7.3.4.7 Ice cream meltdown

Meltdown tests were carried out on hardened ice creams that had been stored at -20ºC to determine and compare the melting rate of the different ice cream samples produced in this study. The samples of ice creams with the same dimension were removed from the containers and placed on a 1 cm stainless steel wire mesh screen in a temperature-controlled incubator (Contherm, polar 1000C, Contherm Scientific Ltd., Hutt city, New

Zealand) set at 20 ± 1°C. The weight of melted ice cream was recorded at every 10 min interval over 90 minutes and plotted as a function of melting times.

7.3.4.8 Solvent extractable fat content of ice cream

The content of free fat in ice cream was determined by a solvent extraction method following the procedure of Bolliger et al. (2000a, 2000b, 2000c). Heptane (BDH, VWR International, Geldenaaksebaan, Leuven) was used as a solvent in order to partially extract fat (free fat) from molten ice cream samples. Prior to the test, the samples were stored at −20°C. About 10 g of the frozen ice cream was weighed into a 50 ml glass tube. Samples were thawed for 2 hr at 4°C. After thawing, 30 ml of heptane was added to each sample. The tubes containing a mixture of melted ice cream sample and hexane were gently inverted 30 times by hand. The tubes were then allowed to stand vertically for 60 min in order to attain the separation of the solvent phase from the aqueous layer. The solvent phase was then transferred into a pre-weighed aluminum foil dish (Confoil, New Zealand) using a 25 ml glass pipette. The extraction was repeated two more times by adding 30 ml of heptane into the aqueous phase and standing the mixture for 60 min. The aluminum dishes containing the extracted fat and solvent were kept in a fume hood to remove the solvent via evaporation until it was visually determined as dry and then dried in an oven at 105°C for 1 hr. The dried dishes were transferred to cool down in a desiccator for 30 min and then weighed. The amount of solvent extractable fat was calculated as the percentage of total fat content by weight in a sample.

7.3.4.9 Statistical analysis

All experiments and measurements were carried out at least in duplicate and analysed as described in Section 3.3.10.

7.4 Results and Discussion

7.4.1 Visual observation of ice cream mixes

The samples of fresh and aged ice cream mixes collected during the preparation of ice creams were visually observed for a difference in their flow properties in response to the formulations and treatment of ice cream mixes before and after ageing. This was done by pouring an equal amount of samples (10 g) into a glass petri dish and monitoring the spreadability of samples after 1 min as shown in Figure 7.2.

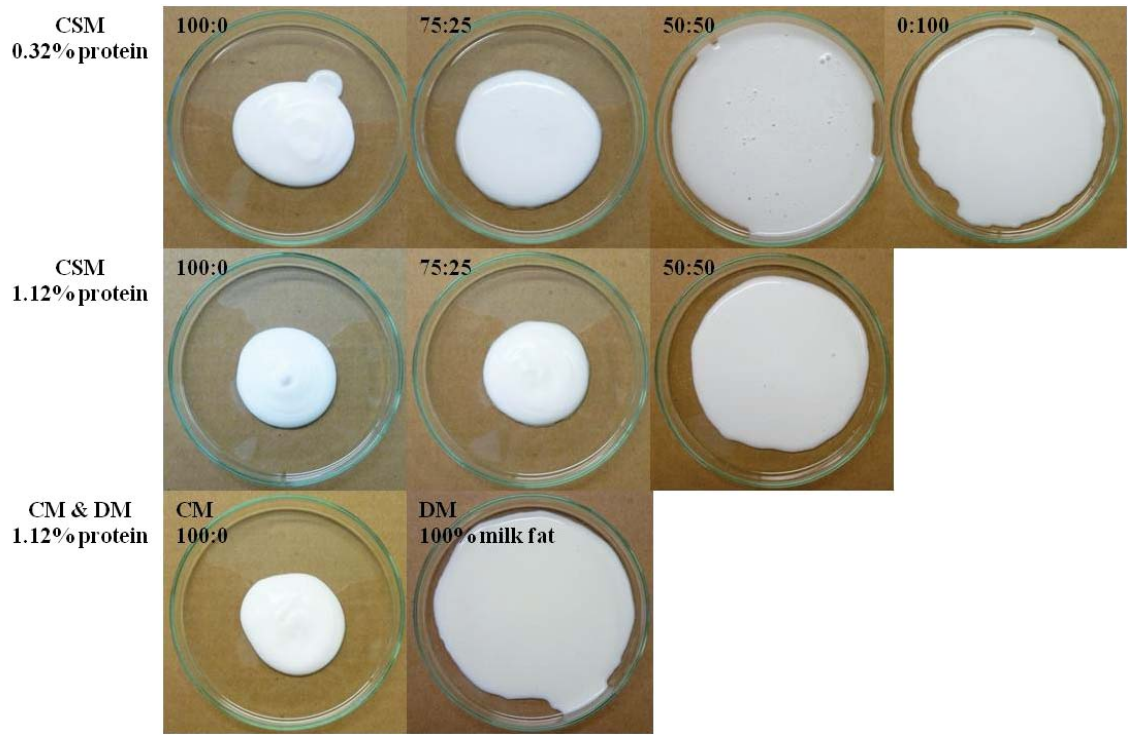

Figure 7.2 Flowability of aged ice cream mixes (10% oil and 0.32% or 1.12% protein) with different ratios of CO to SO oil (100:0, 75:25, 50:50 and 0:100) at 4°C. CSM, CM and DM represent coconut skim milk, coconut milk and dairy cow's milk, respectively, used as the main ingredient base for the formulation of ice cream mixes.

All fresh ice cream mixes after the preparation were in a liquid state and spread all over inside the surface of the petri dish (pictures not shown). After ageing overnight, the flowability of some aged ice cream mixes was observed to be substantially reduced, especially for the ice cream mixes with a high ratio of CO (75% or 100%). These samples were viscous and were more resistant to flow and spread after ageing.

However, the increase in the relative proportion of unsaturated oil (i.e. SO) over saturated oil (i.e. CO) did not change the flow properties of ice cream mixes with a high ratio of SO (50% and 100% SO) before and after ageing and enabled the aged ice cream mixes to flow readily. As a result, the spreadability and flowability of these samples were shown to be higher. This means that the higher the amount of unsaturated SO, the less viscous was the ice cream mix. The DM ice cream mix containing milk fat was also observed to be visually low in the viscosity, thus high in its flowability.

The pronounced reduction in the flowability of some of the ice cream mixes, after ageing, with a high ratio of CO (i.e. 100% or 75%), indicates a significant increase in their viscosity. This may be due to the presence of a rigid network and structure that might have been formed during the ageing process resulting from the solidification or crystallisation of fat in the oil phase, to a greater extent, in these samples by the high ratio of CO (El-Rahman et al., 1997) and the aggregation of solid fat droplets leading to the flocculated network structure (deMan and Beers, 1987, El-Rahman et al., 1997, Goff, 1997b, Hinrichs and Kessler, 1997).

With the estimated content of solid fat at different ratios of CO and SO at 4°C (Table 7.3), the solid fat content of the two samples with a ratio of 100% or 75% CO was about 90% and 68%, respectively, compared to the other samples with about 45% or 0% solid fat content. This indicates that the higher amount in solid fat content was responsible for the increased viscosity and the reduced flowability. According to Narine and Marangoni (1999), when the volume fraction of solid fat increased, the elastic property of materials such as milk fat and high saturated fatty acid oil (e.g. palm oil) increased. In a study reported by El-Rahmen et al. (1997), the viscosity of aged ice cream mixes made from dairy milk fat with varying ratios of low and high melting milk fat fractions was higher when the ratio of high melting fat fraction increased. The contrasting results were reported by Liew et al. (2001b) that ice cream mixes with varying ratios of anhydrous milk fat and palm kernel oil showed a reduction in viscosity when the aged ice cream mixes (6 hr ageing) contained more solid fat content.

The results of the visual observations were also in agreement with the instrumental measurements of viscosity by a rheometer, which are described in more detail in Section 7.4.6. The critical stress determined was below 0.15 Pa for the aged ice cream mixes with a high ratio of SO (50% or 100%) or with dairy milk fat, whereas it was greater than 1.0 Pa for the aged mixes with a high ratio of CO (75% or 100%) (Figure 7.10). The small critical stress may indicate that the fat globules in the aged mix with a high ratio of SO or with dairy milk fat were held together by the weak van der Waals forces (Liew et al., 2001a, van den Tempel, 1961). On the other hand, the large critical stress in samples containing the high ratio of CO may indicate the more resistant force of the fat globules due to the presence of the higher amount of solid fat content in samples (Liang et al., 2008, Marangoni and Hartel, 1998). The another possibility of the large stress may be due to the presence of an aggregated form in those samples because when crystalline fat aggregates are formed in emulsions, the droplets become bigger resulting in the formation of irregular shape clumps which contributes to an increase in the viscosity of the oil-in-water emulsions (Goff, 1997b, van Boekel and Walstra, 1981b).

Regarding the effect of addition of CSMP into the ice cream mixes which raised the level of protein content from 0.3% to 1.1%, it resulted in a more visually stiffer structure in the aged ice cream mixes compared to the corresponding samples without added CSMP. The aged ice cream mix made from CM containing 100% CO at 1.1% protein level also exhibited a high viscous and stiff appearance similar to the CSM sample with 100% CO. In summary, the flowability of ice cream mix after ageing was significantly decreased when the ratio of CO in the oil droplets and the protein concentration were higher due to its contribution to the viscosity increase after ageing resulting from the formation of clumps of fat agglomerations.

7.4.2 Particle size distribution of ice cream emulsions

Particle size distributions (PSDs) of emulsion droplets in aged ice cream mixes and molten ice creams are shown in Figure 7.3 and Figure 7.4, respectively. In the absence of SDS (dissociative agent), the size distributions of apparent emulsion droplets of all aged ice cream mixes were bimodal or multimodal, reflecting that there were several defined populations of different particle sizes (Figure 7.3). In the presence of SDS, the PSDs of almost all aged mix emulsions still remained multimodal with a defined major population, except for the DM-based sample which was monomodal, but the PSDs were shifted to the smaller particle sizes with a narrower distribution span. This suggests that SDS had an effect on the dissociation of flocculated droplets, thus enabling the dispersion of flocculated droplets as single discrete droplets. The effects of SDS on the disintegration of flocculated droplets have been also shown in some studies on the emulsions stabilised by milk proteins (Demetriades and McClements, 2000, Lizarraga et al., 2008, Segall and Goff, 1999, Tomas et al., 1994a) or by soy proteins (Diftis and Kiosseoglou, 2004, Floury et al., 2002), and also on the ice cream emulsions (Gelin et al., 1994, Goff, 1997a, Goff, 1997b, Méndez-Velasco and Goff, 2011, 2012a, b).

The PSDs of droplets in molten ice cream emulsions formulated with CSM or CM were also multimodal, irrespective of the size measurements in the presence and absence of SDS (Figure 7.4). The difference and change in the PSDs measured between with and without SDS for these molten ice cream samples, except for the samples containing 100% and 75% CO, were not as significant as those observed from the aged ice cream mixes. In the presence of SDS, a major peak representing the major population of particles was shifted towards smaller sizes, except for some samples. The loss in the big particles due to the presence of SDS implies the breakdown of big particles which were expected to be fat clusters (flocs) occurring after the subsequent freezing and whipping of the aged mixes. For the DM-based molten ice cream emulsions, the biomodal distribution of the particle size with one small peak in a larger size region was changed to the monomodal distribution with disappearance of the small peak when analysed in the presence of SDS, suggesting the presence of flocculated droplets and their dissociation by SDS.

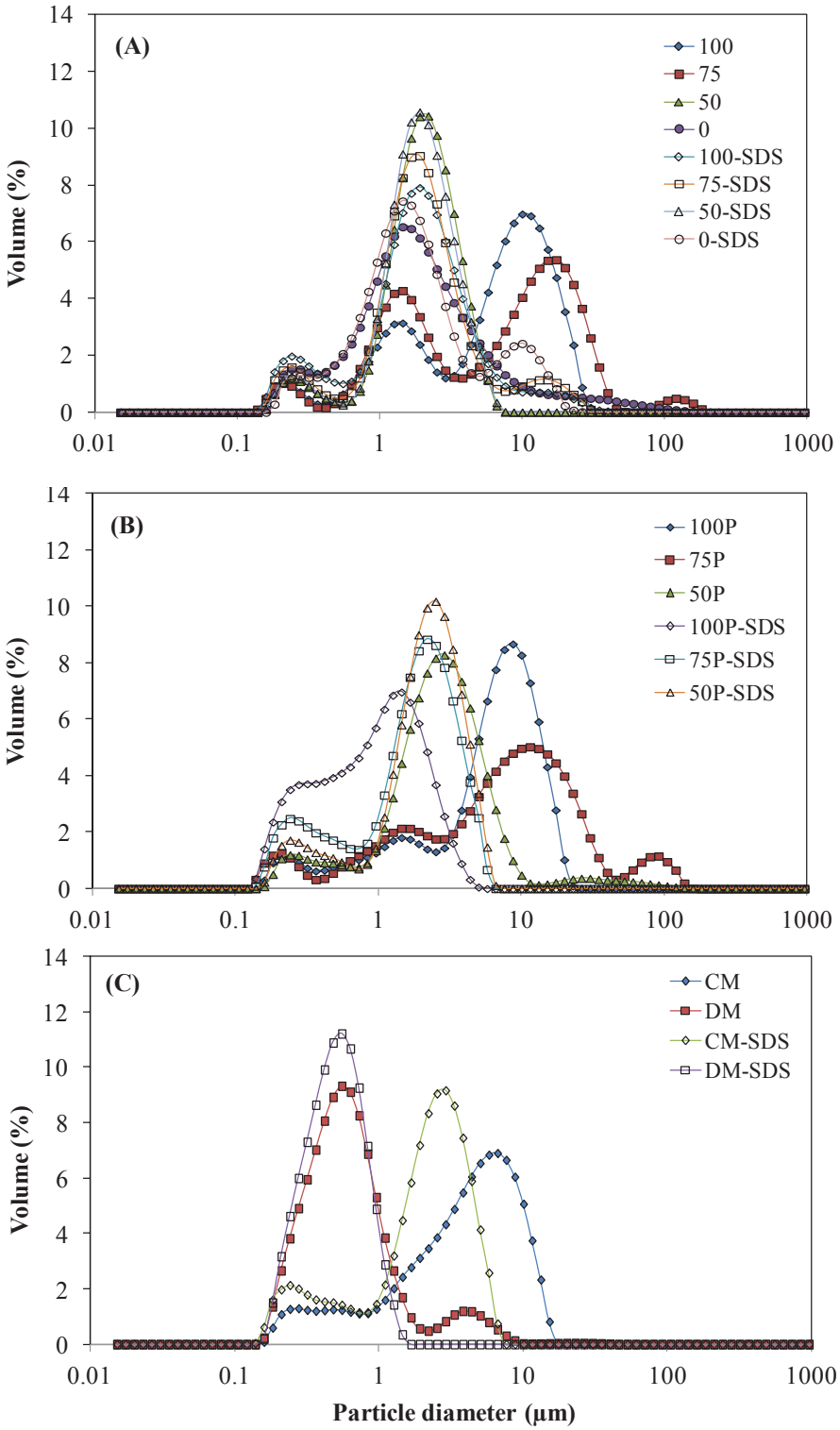

Figure 7.3 Particle size distributions of aged ice cream mix emulsions measured directly from samples (filled symbol) and after dilution with 1.0% SDS solution (open symbol). Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; the letter P in legends means CSMP was added into ice cream mixes; CM means emulsions made from coconut milk; and DM means emulsions made from dairy milk.

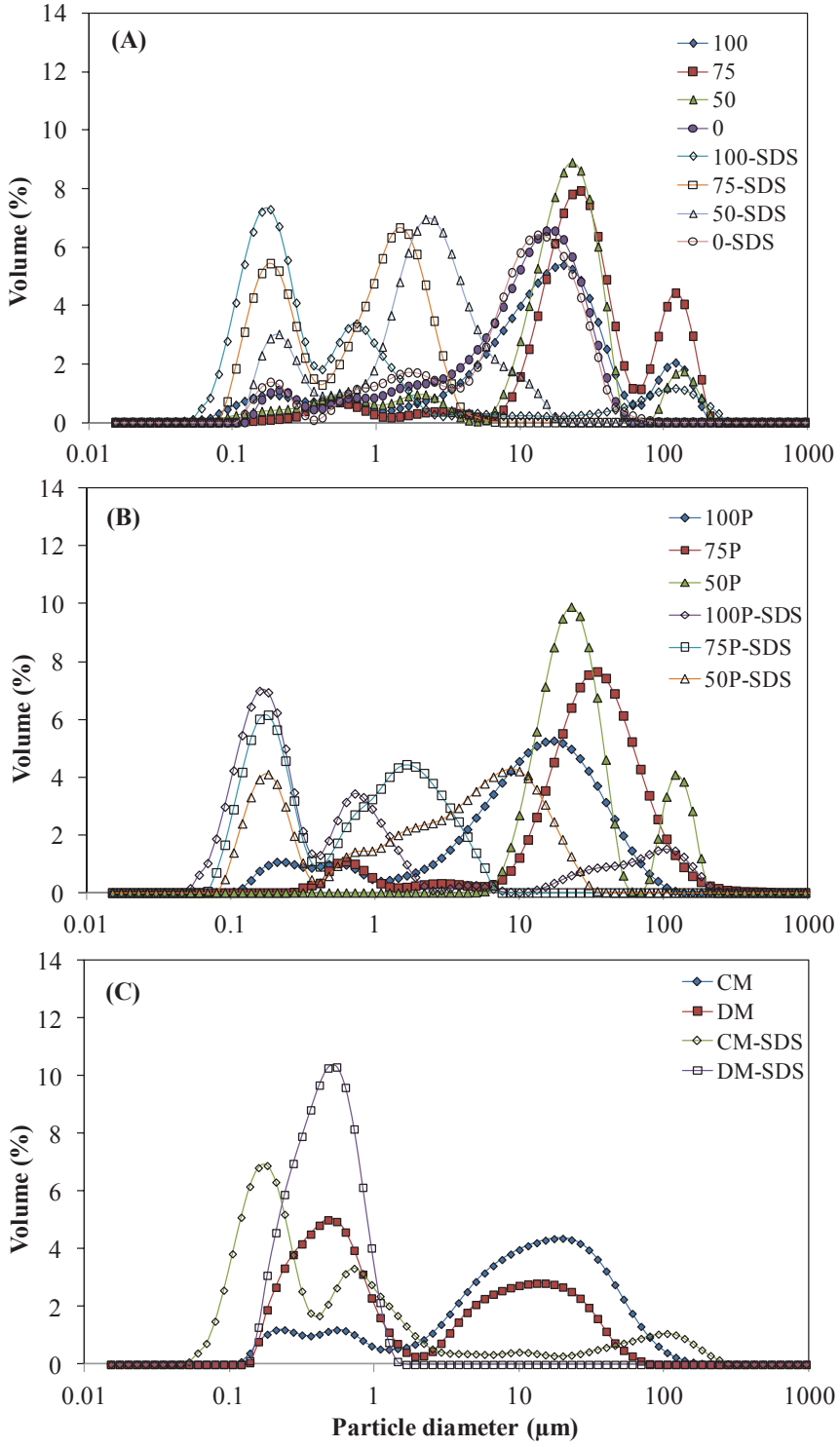

Figure 7.4 Particle size distributions of molten ice cream emulsions measured directly from samples (filled symbol) and after dilution in 1.0% SDS solution (open symbol). Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; the letter P in legends means CSMP was added into ice cream mixes; CM means emulsions made from coconut milk; and DM means emulsions made from dairy milk.

7.4.3 Particle size of ice cream emulsions

Mean particle sizes of emulsion droplets in aged ice cream mixes and molten ice creams were analysed with and without dilution with 1.25% SDS solution for the determination of the mean diameter of dissociated droplets (real droplets) and apparent droplets, respectively. The average apparent and dissociated droplet sizes for all samples are shown as a volume mean diameter (d_{43}) in Table 7.4.

Table 7.4 Volume mean particle diameter (d_{43}, μm) of apparent and dissociated droplets in aged ice cream mixes and molten ice creams analysed in the absence and presence of 1% SDS.

Formulation	Ice cream mix		Molten ice cream	
	Without SDS	With SDS	Without SDS	With SDS
100	7.23 ± 0.23^{c}	2.09 ± 1.03^{c}	21.26 ± 3.44^{c}	13.58 ± 5.85^{a}
75	19.42 ± 9.21^{a}	2.24 ± 0.67^{bc}	37.75 ± 5.56^{a}	0.96 ± 0.09^{d}
50	2.03 ± 0.30^{ef}	1.74 ± 0.23^{c}	23.94 ± 8.01^{c}	2.89 ± 0.40^{cd}
0	3.70 ± 1.31^{e}	2.95 ± 0.24^{a}	11.71 ± 0.61^{e}	11.47 ± 2.15^{a}
100P	6.53 ± 0.09^{cd}	2.77 ± 1.56^{ab}	17.11 ± 1.36^{d}	13.79 ± 8.60^{a}
75P	11.41 ± 3.46^{b}	2.68 ± 0.80^{ab}	38.50 ± 3.13^{a}	1.04 ± 0.07^{d}
50P	3.48 ± 1.47^{ef}	2.01 ± 0.15^{c}	32.14 ± 9.76^{b}	4.14 ± 0.83^{c}
CM	4.14 ± 0.33^{de}	2.80 ± 0.66^{ab}	16.55 ± 1.16^{d}	8.45 ± 4.87^{b}
DM	0.77 ± 0.18^{f}	0.49 ± 0.19^{d}	8.72 ± 2.07^{e}	0.46 ± 0.01^{d}

Mean values (n=6) in the same column with different letters indicate significant difference ($P < 0.05$). Abbreviation: 100, 75, 50 and 0 represent the ratio of CO:SO in oil droplets at 100:0, 75:25, 50:50 and 0:100, respectively; P represents CSMP was added into ice cream mixes; CM and DM mean ice cream mixes made from coconut milk and dairy milk, respectively.

In the absence of SDS, the average size of apparent droplets measured was in a range of 3.48-19.42 μm for the aged ice cream mixes while it was 11.71-38.50 μm for the molten ice creams, excluding the size of DM based samples. This indicates that the apparent droplet size of aged ice cream mix emulsions was increased after the freezing and whipping process. In the presence of SDS, the dissociated droplets were measured to be

smaller in size compared with the apparent droplets. The dissociated droplets for the aged ice cream mix samples were in a range of 1.74-2.95 μm whereas it was in a range of 0.96-13.79 μm for the molten ice cream samples.

Size of emulsion droplets in the ice cream mixes and molten ice creams made from DM was found to be significantly smaller than the other samples. The size of average apparent and dissociated droplets was 0.77 μm and 0.49 μm, respectively, for the aged mix samples and 8.72 μm and 0.46 μm, respectively, for the molten DM ice cream samples. This indicates that dairy milk proteins (caseins and whey proteins) have better emulsifying ability and stability of oil droplets than the coconut milk proteins. In the literature, it was reported that the apparent particle size of fat globules of conventional dairy ice creams is normally around 0.5-1.0 μm with a maximum of about 2.0 μm (Goff, 1997a, Marshall et al., 2003). The relatively large apparent droplets observed in this study could be due to the concentration of milk proteins (i.e. 1.1%) formulated in the ice cream mix that was lower than approximately 4% protein contained in the conventional dairy ice cream formulation. The big particle size of oil droplets for coconut milk-based samples obtained in this study reflects that the ability of coconut milk proteins to form and stabilise oil-in-water droplets is low.

Ratio of CO and SO in oil droplets was also found to affect the size of apparent and dissociated oil droplets for aged ice cream mixes and the molten ice creams, but its effect on the dissociated droplets of aged ice cream mixes was not very pronounced. For the aged ice cream mixes and molten ice creams (Formulations 75, 50, 75P and 50P) containing a ratio of 75% or 50% CO, the dissociated droplet size was observed to be significantly smaller than the apparent droplet size for most of the samples ($P < 0.05$). This suggests that SDS had an effect on the droplet clusters by dissociating them into discrete droplets, reflecting the presence of flocculated droplets and droplet clusters in the aged mixes and molten ice creams. As the flocculated droplets could be dissociated by SDS, the hydrophobic interaction is suggested to be involved in the formation of those aggregates (Floury et al., 2002).

Although the apparent droplet size of aged mixes between samples was significantly different, its difference in the droplet size after dissociation ($P > 0.05$) was not seen very significant between all samples. In contrast, it was found that there was a significant difference in the size of dissociated droplets between some of the molten ice cream samples. It is interesting to note that ice creams (Formulations 100, 0, 100P and CM) containing 100% CO or 100% SO showed that they had a much larger real droplet size (dissociated droplets) than the other samples (Formulations 75, 50, 75P, 50P and DM) containing oil blends of CO and SO or dairy milk fat.

After churning of aged ice cream mixes, the size of apparent droplets was observed to be significantly increased ($P < 0.05$), indicating that some kind of aggregation of fat droplets, i.e., partial coalescence (fat clumping) and/or flocculation (fat cluster), may have been introduced into the ice cream structures. In the presence of SDS, the size of droplets (dissociated) for some of these molten ice cream samples (Formulations 75, 50, 75P, 50P) was found to be significantly smaller than their respective apparent droplet sizes but did not differ substantially from the droplet size (dissociated) of their respective aged mix emulsions. These results indicate that the oil droplets in these molten ice creams (formulations 75, 50, 75P, 50P) were highly flocculated and that significant droplet aggregation and coalescence did not occur during the freezing and whipping of the aged ice cream mixes. The same phenomenon was also observed from the ice cream samples prepared with DM. As the droplets in these emulsions consisted of a mixture of crystalline solid fat (68% in 75 and 75P and 45% in 50 and 50P) and liquid oil, those semi-solid droplets may be likely to have undergone shear-induced partial coalescence, resulting in the larger aggregated fat droplets (Davies et al., 2000, 2001, Fredrick et al., 2010, Vanapalli et al., 2002). The same phenomenon was also observed from the ice cream samples prepared with DM.

In the case of ice creams comprising of the high solid fat content (90%) (Formulations 100, 100P and CM), though upon shearing fat crystals in one droplets collided with the neighbouring droplets, the high content of solidified fat and the lack of liquid oil to wet the piercing crystals would have made those droplets be less likely to undergo partial coalescence (Davies et al., 2000, Vanapalli et al., 2002). It is interesting to note that the

real droplet diameters in these melted ice creams were quite large, the increase in droplet size after shear may have been due to the transformation of the solidified droplets from spherical droplets to elongated platelet-like droplets caused by the applied shear forces (Méndez-Velasco and Goff, 2012a).

The extent of emulsion droplet flocculation has been described, in terms of a flocculation index (FI), by the ratio of apparent droplet size (d_{43}) to dissociated droplet size (Puppo et al. 2008, 2005). The greater of the FI values, the greater extent of flocculation is present in the emulsion samples (Puppo et al., 2008, Puppo et al., 2005). Table 7.5 shows the FI values calculated for the aged and molten ice cream emulsions. The results confirm the explanation given in the above on the droplet flocculation observed in some of the samples. For the aged ice cream mix emulsions, the ratio of CO and SO had a significant influence on the droplet flocculation ($P < 0.05$). The extent of droplet flocculation was observed to be higher at a ratio of 75% CO followed by 100% CO. At a ratio of 50% CO or lower, the droplet flocculation was very minor.

The high extent of flocculated droplets was observed in the aged mix containing 75% CO which had about 68% solid fat content. According to Narine and Marangoni (1999), stronger interactions between fat crystal network in droplets were observed as droplets contain a higher solid fat volume fraction. Therefore, strong droplet interactions could be observed in emulsions containing solid fat such as emulsions with formulations 100 and 75. At the ageing temperature, the droplets of emulsions containing 100% CO is believed to consist mainly of solidified droplets as their solid fat content estimated was 90%. Upon the collision of droplets, those droplets may stay close together by the interaction forces between fat crystals but has no linkage between droplets due to the integrity of droplets and the lack in the liquid oil. In the emulsions (Formulations 75 and 75P) containing the oil blend of 75% CO with 68% solid fat content, about two third of the droplets would have been solid whereas the rest was still in the liquid form. As the droplets in these emulsions collided each other, the liquid fat acts as a cement to link two or more droplets together, hence leading to more aggregation of droplets (Vanapalli et al., 2002). The forces that link between droplets containing about 68% solid fat content may be quite stronger than interactions between droplets in the

formulation (100) containing 100% CO. It is thought that due to this reason, the separation of droplet aggregates would not occur readily when the emulsion was agitated during the particle size measurement, therefore, resulting in the larger apparent droplets observed in these samples than the emulsions containing 100% CO.

The addition of CSMP showed no significant influence on the droplet flocculation of aged ice cream mix emulsions by the FI values when compared to the samples without added CSMP. When the aged ice cream mixes were frozen and churned into ice creams, the most dramatic increase in the FI values that reflects the droplets with a high extent of aggregation was observed for the ice creams containing 75% CO with its FI values of 37-40, irrespective of the addition of CSMP, followed by the samples containing 50% CO with their FI values of 7.7-8.6. It was also noted that the DM ice cream also showed a very high FI value of 19.

Table 7.5 Flocculation index (FI) of aged ice cream mix and molten ice cream emulsions

Formulation	Aged ice cream mix	Molten ice cream
100	4.65 ± 2.09^{b}	2.08 ± 1.40^{d}
75	12.11 ± 8.03^{a}	39.49 ± 6.47^{a}
50	1.13 ± 0.17^{c}	8.60 ± 3.67^{c}
0	1.30 ± 0.59^{c}	1.05 ± 0.18^{d}
100P	3.89 ± 2.40^{b}	2.34 ± 2.08^{d}
75P	5.08 ± 2.70^{b}	37.24 ± 4.81^{a}
50P	1.49 ± 0.41^{c}	7.74 ± 2.04^{c}
CM	1.58 ± 0.46^{c}	3.18 ± 3.11^{d}
DM	1.56 ± 0.32^{c}	19.21 ± 4.91^{b}

Mean values (n = 6) within each column with different letters indicate significant difference ($P < 0.05$). Abbreviation: 100, 75, 50 and 0 represent the ratio of CO:SO in oil droplets at 100:0, 75:25, 50:50 and 0:100, respectively; P represents CSMP was added into ice cream mixes; CM and DM mean ice cream mixes made from coconut milk and dairy milk, respectively.

The contrast phenomena observed were from two ice cream samples with 100% CO and 100% SO. The ice cream samples containing 100% CO had a relatively low FI value of 2.1-2.4 and the ice cream containing 100% SO had a FI value of 1.1 which was almost close to 1. The latter case of droplets in the ice cream containing 100% SO indicates that droplet flocculation was not present but there was droplet coalescence after freezing and aeration as the dissociated droplet size of the corresponding aged mix was much smaller.

In summary, the inclusion of SO at a certain proportion into coconut ice cream mixes could lead to a greater extent of droplet flocculation or fat clumping (partial coalescence), depending on its level added. The oil ratios of 75% or 50% CO in oil droplets resulted in the high extent of droplet clumping. At a ratio of 100% CO, the droplet flocculation was less extensive and droplets have been changed in their size. The use of 100% SO led to the significant droplet coalescence that took place after freezing and churning. In other words, after churning of the aged mixes containing 100% SO, bigger fat aggregates were introduced in the ice creams due to the droplet calescence. For the DM-based ice cream emulsions, it was found that the fat droplets in this sample were highly aggregated and flocculated without coalescence which may be caused by partial coalescence.

7.4.4 Microscopic examination of aged ice cream mix

The microscopic observation of emulsion droplets in aged ice cream mixes was carried out at ambient temperature by using a light microscope. The images of aged ice cream emulsions analysed before and after dilution with 1.25% SDS solution at 1:4 ratio are shown in Figure 7.5. The main characteristic and appearance of oil droplets detected from almost all aged ice cream mixes were the presence of droplet clusters (flocculation). This was more visually able to be observed in the presence of SDS. In the presence of SDS, a number of small or big chunks of droplet clusters (flocculated droplets) were seen from most aged mix samples, except for the aged ice cream mix based on DM. This implies that the electrostatic repulsion between droplets provided by SDS was not strong enough to overcome the attractive forces acting on droplets in these ice cream mix systems, probably when the samples were in a static state and are

not agitated. This is because the particle size of these samples measured in the presence of SDS was smaller than 3 µm (Table 7.4). In contrast to the presence of droplet clusters observed in the CSM or CM-based mixes, the dairy based ice cream mix made from DM showed discrete droplets and had no pronounced droplet flocculation.

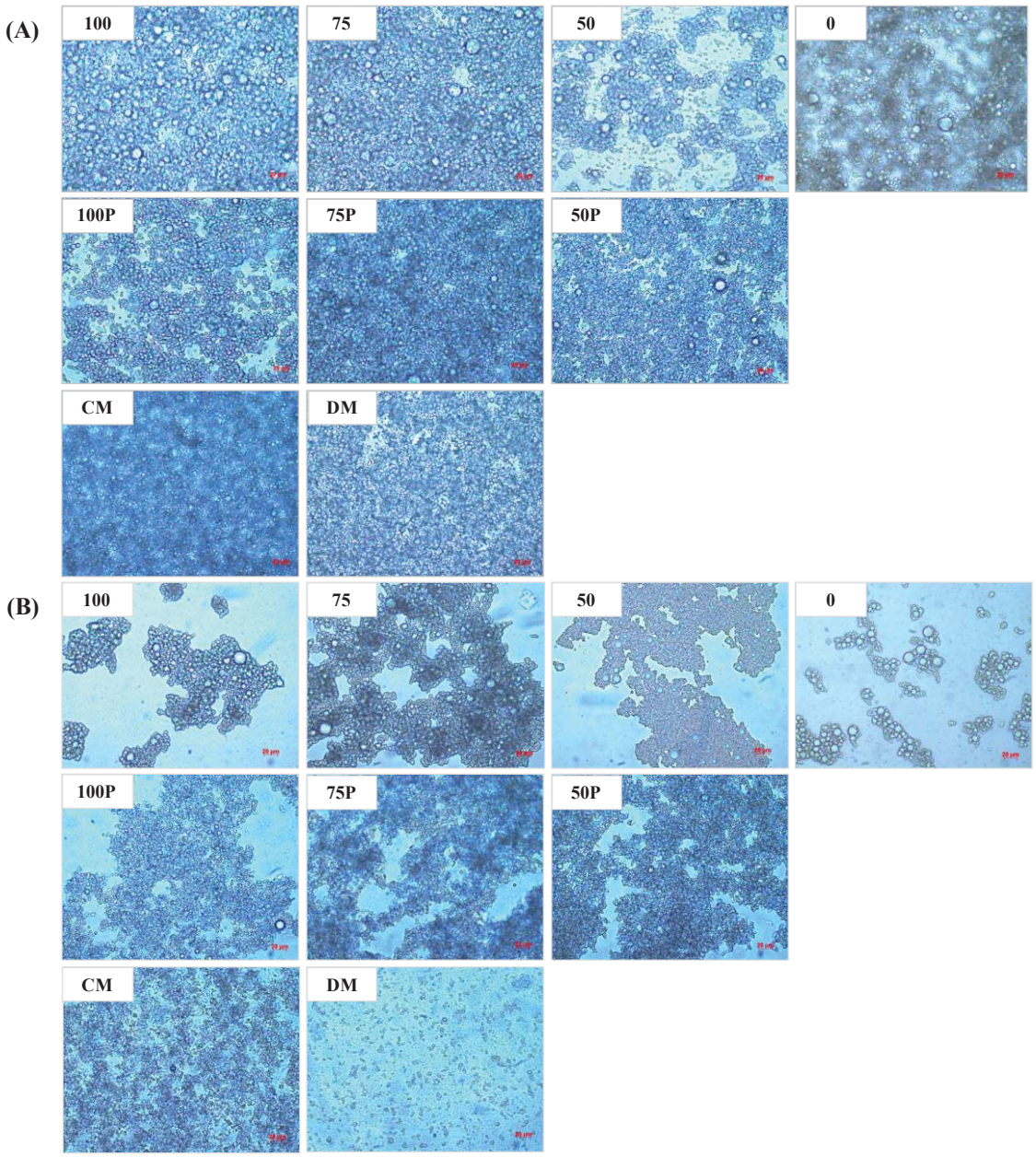

Figure 7.5 Micrographs of emulsion oil droplets in aged ice cream mixes observed under a light microscope at a 400 x magnification without (A) and with (B) dilution in 1% SDS solution. The scale bar represents 20 µm.

The interactions of emulsion droplets by an observation of particle micrographs of the aged mix emulsions and also by the measurement of their particle size in the absence and presence of SDS that showed the droplet flocculation reflect some kinds of destabilisation mechanisms of droplets resulting from various factors as follows: a) the bridging flocculation by proteins due to low concentration of proteins (Demetriades and McClements, 2000, Lizarraga et al., 2008, Tomas et al., 1994a), and b) bridging through the covalent bonds like peptide and/or disulfide bonds (S=S) or non-covalent bonds like hydrogen bonds, hydrophobic or electrostatic interactions which are normally reported to be evidenced in emulsions stabilised with globular proteins (e.g. soy protein) (Floury et al., 2002, Kim et al., 2002a, b, Renkema and van Vliet, 2002, Tangsuphoom and Coupland, 2008a, Tangsuphoom and Coupland, 2009). It should also be noted that the droplet flocculation observed may not be associated with the protein bridging flocculation because sufficient emulsifiers was incorporated into ice cream formulations (Dalgleish, 2004).

7.4.5 Stability of ice cream mixes to phase separation

The stability of fresh ice cream mixes to phase separation was determined during 4 days of storage at 4^{o}C. The results expressed as the percentage of a height of serum phase (i.e. emulsion droplet depleted layer) separated from a total height of samples as a function of time are shown in Figure 7.6. The plots of percentage of serum separation showed that among the different samples, three ice cream mix emulsions (CM, 100P and 75P) prepared from CM or CSM with added CSMP containing 100% or 75% CO had no or a little serum phase separation over time during storage of 4 days (Figure 7.6). On the other hand, the other ice cream emulsions exhibited a relatively significant increase in the serum separation over time. Overall the results indicate that the separation of serum phase decreased as the ratio of CO to SO in the oil droplets increased and the level of protein concentration was higher between 0.3% and 1.1%. The former suggests the solid fat content affects the stability of ice cream emulsions against creaming or phase separation. As a result, the ice cream emulsions prepared with CSM containing 100% or 50% SO without added CSMP rendered the high instability with a rapid increase in the phase separation. This could also be associated with the viscosity of these samples which were lower than the other samples (Figure

7.8). A similar result of rapid phase separation was also observed in the DM ice cream mix containing 1.1% proteins (Figure 7.8). This was also attributable to not only the solid fat content of the milk fat which is about 50% at 5°C (Kaufmann et al., 2012, Lucas et al., 2005, Pelan et al., 1997a, Wright et al., 2011) but also its viscosity that was relatively lower than the other samples made from CM or CSM with or without added CSMP.

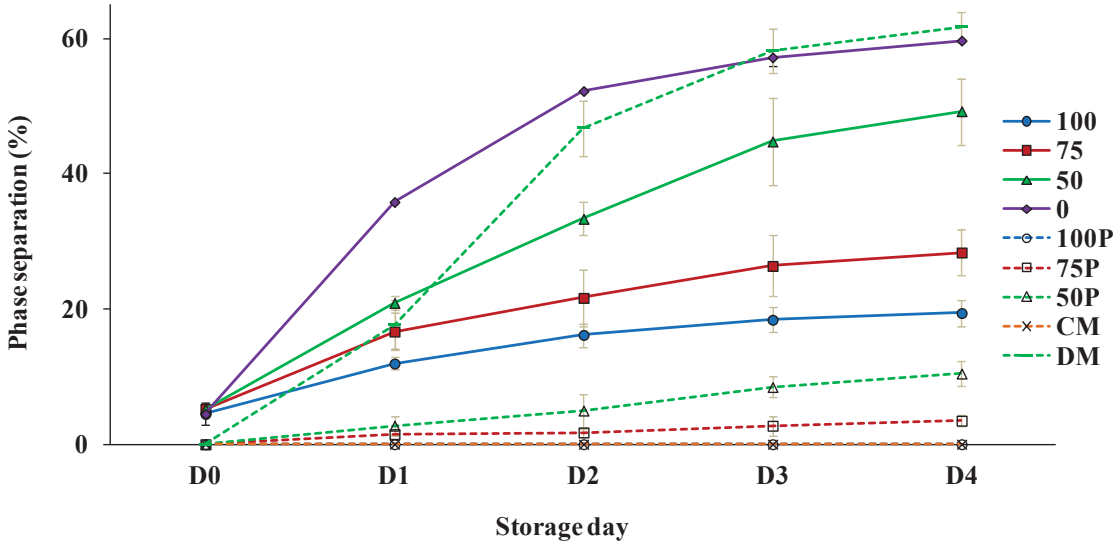

Figure 7.6 Phase separation (%) of ice cream mix emulsions (10% oil) containing different ratios of CO to SO (100:0, 75:25, 50:50 and 0:100) at two different protein concentrations (0.32% and 1.12%) during storage of 4 days at 4°C. Letter P in legends represents the ice cream mixes (1.12% protein) with added CSMP. CM and DM mean ice cream mixes made from coconut milk and cow's milk, respectively.

Pictures of ice cream mix emulsions taken after the preparation (day 0) and after an overnight ageing at 4°C (day 1) are shown in Figure 7.7. On the production date (day 0), all freshly prepared CSM ice cream mix emulsions (e.g. 100, 75 and 50) without added CSMP had a very little phase separation, regardless of the difference in the ratio of CO and SO, whereas phase separation was not observed in the other ice cream mixes made from the CM, DM or CSM with added CSMP (e.g. 100P, 75P and 50P). After one day storage at 4°C (i.e. ageing overnight), the mix emulsions of CM and the CSM-based ones which were added with CSMP containing 100% or 75% CO (100P and 75P) still

remained stable without phase separation. However, separation of the serum phase after ageing was observed from other CSM-based ice cream mixes with no added CSMP and the DM ice cream emulsion. The extent of phase separation increased with increasing ratio of SO with the highest in the mix containing 100% SO as shown in Figure 7.7C. As described earlier, the possible explanation for an increased instability of the mixes may be due to the increasing ratio of SO that led to a decrease in their viscosities which in turn could not retard the creaming rate of those ice cream mix emulsions (Figure 7.8). For the emulsions of ice cream mix prepared with DM, phase separation also occurred after an overnight ageing similar to the mix containing 100% SO. This may also be related to the relatively low viscosity of those aged mixes as shown in Figures 7.2 and 7.8. In conventional dairy ice cream mixes, the concentration of milk proteins in the formulation is about 4% which is large enough for stabilising the emulsion droplets and increasing the mix viscosity (Goff, 1997a, Segall and Goff, 1999). In this study, the DM based ice cream mixes had only 1.1% milk proteins, which might not have been high enough to stabilise droplets or provide ice cream mix emulsions with proper viscosity.

At 4^{o}C, 90% of CO become solid (Marshall et al., 2003, Reena et al., 2009) whereas SO is still in a liquid state (Calligaris et al., 2004, Méndez-Velasco and Goff, 2012b). This solidified CO may possibly form a floc network like an open packing structure of flocs in emulsions which restricts the movement of other dispersed droplets, resulting in a more increase in the creaming stability (less phase separation) of emulsion systems than the one containing a higher content of liquid oil (McClements, 2004a). Although solidified fat may form open structures but not liquid droplets, those liquid droplets could roll around each other during moving upwards and rearranged themselves into free spaces of the pack, resulting in the close packing structures of flocs (McClements, 2004a). Therefore, the more pronounced compact cream layer was observed in the emulsions containing SO at 25% onwards (Formulations 75, 50 and 0) (Figure 7.7).

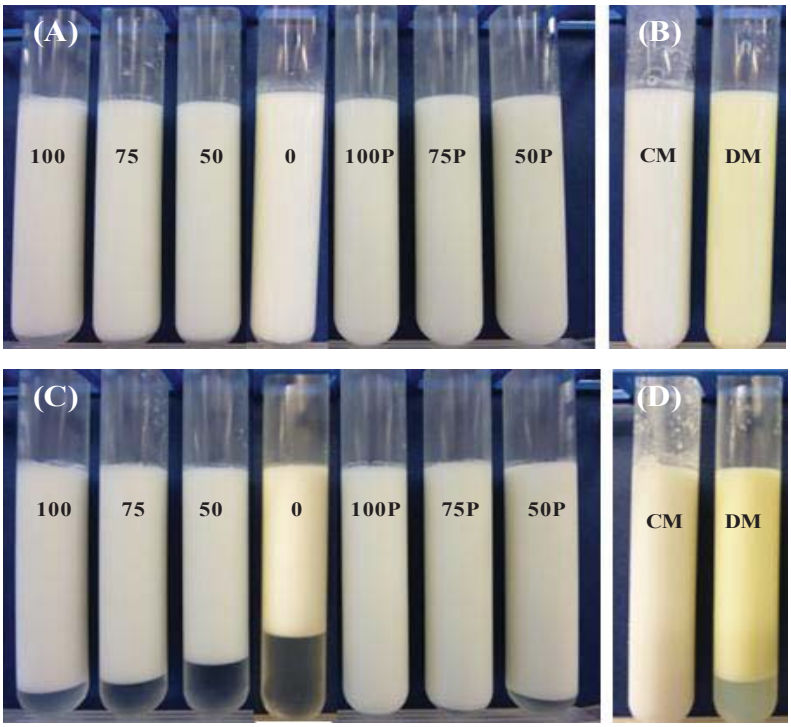

Figure 7.7 Serum phase separation of ice cream mix emulsions (10% oil) containing different ratios of CO and SO at two different protein concentrations (0.32% and 1.12%) before ageing (A and B) and after ageing (C and D) at 4°C overnight. 100, 75, 50 and 0 represent the relative percentage (%) of CO over SO in oil droplets; P represents CSMP was added into the ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and cow's milk, respectively.

As described earlier, the amount of protein content was found to influence the stability of ice cream mix emulsions against the serum phase separation. As shown in Figure 7.7C, the stability of ice cream mix emulsions was higher in the samples with a high protein concentration (100P, 75P, 50P), thus reducing the degree of phase separation, compared to the samples with a low protein concentration (100, 75, 50). A similar phenomenon was observed in the CM ice cream mix emulsion containing the high concentration of protein (1.1%). This observation could be attributed to the effect of viscosity combined with the particle size of oil droplets. The low spreadability of these samples (100P, 75P and CM) shown in Figure 7.2 indicates that these samples were higher in their viscosity probably due to the formation of a viscous gel network, thus retarding the movement of dispersed phase (oil droplets). It has been shown that the viscosity of ice cream mixes could be increased with increasing concentration of soy

protein isolate from 0% to 4% (Friedeck et al., 2003). The two dominant proteins, 11S and 7S globulins, of soy protein isolate are reported to be responsible to form a gel like structure in emulsions, hence increasing the viscosity of emulsions (Renkema et al., 2001). The addition of extra coconut skim milk proteins resulted in the emulsions with relatively high stability possibly by creating the elastic structures as evidenced by the observed higher G' (Granger et al., 2004b) (see Section 7.4.6.2 for more details), resulting in an increase in the emulsions viscosity. This was able to be confirmed by the analysis of rheological properties of samples which is described in the following section.

7.4.6 Rheological properties of ice cream mixes and melted ice creams

Rheological properties of fresh ice cream mixes and the corresponding aged ice cream mixes and melted ice creams were measured at 4°C by a controlled-stress rheometer with a cone and plate geometry.

7.4.6.1 Flow behaviour of ice cream emulsions

The flow curves assessed from fresh ice cream mix emulsions are shown in Figure 7.8 A. The results show that the apparent viscosity for the fresh ice cream mixes decreased with increasing shear rate over the whole tested shear rate between 0.5 s^{-1} and 500 s^{-1}, reflecting the non-Newtonian fluids with a shear-thinning behaviour (Rohn, 1995, Vlachopoulos and Polychronopoulos, 2012). For the ice cream mixes with different ratios of CO and SO, the ratio difference showed significant effects on the reduction in viscosity with increasing liquid oil content (i.e. SO). The ice cream mix with 100% SO had the lowest viscosity. The viscosity was also found to be affected by the protein concentration although its effect between two different concentrations of 0.3 and 1.1% seemed less than did the effect of different ratios of CO and SO. With an addition of CSMP, the protein content in fresh ice cream mixes was increased from 0.3% to 1.1%. The ice cream mixes (Formulations 100P, 75P and 50P) with added CSMP showed an increase in the viscosity, to some extent, compared to the corresponding samples (Formulations 100, 75 and 50) without added CSMP. For the other two samples (Formulations CM and DM with 1.1% protein) prepared from CM with 100% CO or DM with 100% dairy milk fat, the former had a high level of viscosity similar to the ice

cream mix (Formulation 100P) containing 100% CO with added CSMP, but the latter was quite low in its viscosity like the ice cream mixes (Formulations 50 and 0) with 50% CO or 100% SO.

After the fresh mixes were aged overnight, the aged mixes behaved in a similar manner as the fresh mixes as a non-Newtonian material with a shear-thinning behaviour (Figure 7.8B. The level of viscosity was still observed to remain high for the CM based aged mix followed by the CSM based ones containing 100% and 75% CO. The lowest viscosity was still observed from the CSM based containing 100% SO. It seemed like the apparent viscosity of fresh ice cream mixes increased further after ageing, except the DM based mix and CSM based one containing 100% SO that remained about the same (Figure 7.8).

Like the fresh and aged ice cream mixes, the same non-Newtonian with shear thinning flow behaviour was observed from a flow curve of the molten ice creams after melting at 4°C (Figure 7.8C). One major noticeable difference was that the level of viscosity at a low shear rate was much higher than that of the fresh and aged ice cream mixes. This could be due to more significant oil droplet flocculation and/or the larger size of oil droplets possessed by the molten ice cream samples (Tables 7.4 and 7.5). The dependence of viscosity upon the solid fat content was also observed in the melted ice creams. The melted ice creams with 100% CO had the highest viscosity, followed by the samples containing 75%, 50% and 0%, respectively. As described earlier, the addition of CSMP also showed a substantial effect on an increment in the viscosity of the melted ice cream samples compared to the samples without added CSMP.

It should also be noted that some samples of the ice cream mixes and molten ice creams based on CM or CSM containing 100% CO showed a distinctive combination of flow behaviours consisting of a shear thinning at a very low shear rate (less than 1 s^{-1}), a plateau region where the viscosity was independent of shear rates at a low shear rate range (between 1 and 10 s^{-1}) and the a shear thinning behaviour again from a moderate region towards high shear rate region (from 10 s^{-1} onwards). The combination of these

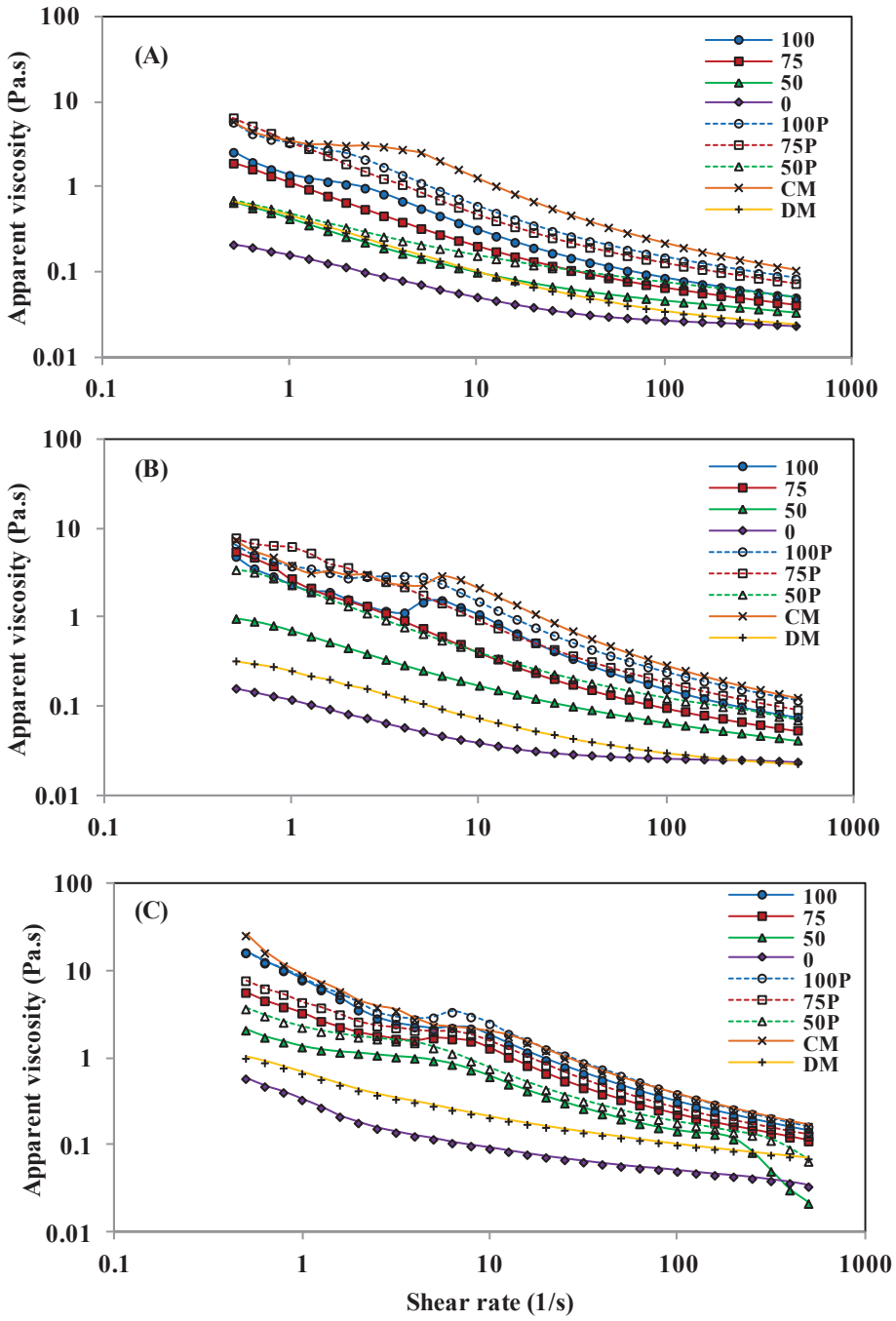

Figure 7.8 Apparent viscosity (η) of fresh ice cream mixes (A), aged ice cream mixes (B) and molten ice cream (C) prepared from CSM as an ingredient base. Abbreviations 100, 75, 50 and 0 represent the ratio of CO and SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP added into the ice cream mix formulations; CM and DM mean ice cream mixes from coconut milk and dairy milk, respectively.

flow behaviours may be explained by the fact that as the very low shear rates were applied, the particle-particle interactions in those samples could withstand these low shear forces and not breakdown, but as greater shear rates were applied, these forces overcame those interactions, resulting in the structural breakdown in those emulsions.

The increase in the viscosity of the aged ice cream mixes with increasing ratio of solid fat content (i.e., more CO over SO) observed in this study was in agreement with a study done by Goh et al. (2006). In their study, it was shown that the apparent viscosity of melted ice creams with varying ratios of milk fat to flaxseed oil decreased as the proportion of liquid oil (i.e., flaxseed oil) increased. This may have been because samples containing more liquid oil could be easily deformed by the applied shear forces, hence resulting in the lower viscosity (Goh et al., 2006, Takatori et al., 2004). The discrepancy was however shown in a study of Liew et al. (2001b) in that the viscosity of aged ice cream mixes containing fat blends consisting of anhydrous milk fat and palm kernel oil (solid fat) increased with decreasing solid fat content. They suggested that the aggregated fat droplets were responsible for the increased viscosity of the ice cream mixes after ageing.

The additional proteins may correspond to the more excess proteins available in the unfrozen mixes to form gel, which in turn corresponded to an increase in the viscosity of the mixes (Goff, 1997b). This is in accordance with that of Aime et al. (2001), Ruger et al. (2002) and Patel et al. (2006) who reported that the viscosity of ice cream mixes increased with increasing protein content. The cause of the increment of viscosity of the melted ice cream emulsions may be due to the presence of more protein gel of coconut milk proteins as the more amount of proteins added (Nakamura et al., 1984, Renkema et al., 2001) or the presence of more solid fat content in the melted ice creams as described earlier (Goh et al., 2006, Takatori et al., 2004). The additional explanation for an increase in the viscosity of the ice cream mixes may be due to the presence of stronger particle-particles interactions or flocculation in ice creams based on CM and CSM based one containing 100% CO than that of the CSM based ice creams containing 100% SO (Rao, 2007, Rohn, 1995, Vlachopoulos and Polychronopoulos, 2012). This observation is in agreement with those of oil-in-water emulsions stabilised with 11S

globulin proteins of soy proteins (Puppo et al., 2008) and is similar to the behaviours of emulsions prepared with soy-globular proteins in which the existence of particle-particle interactions or flocculated particles within those emulsions was proposed (Floury et al., 2000, Floury et al., 2002).

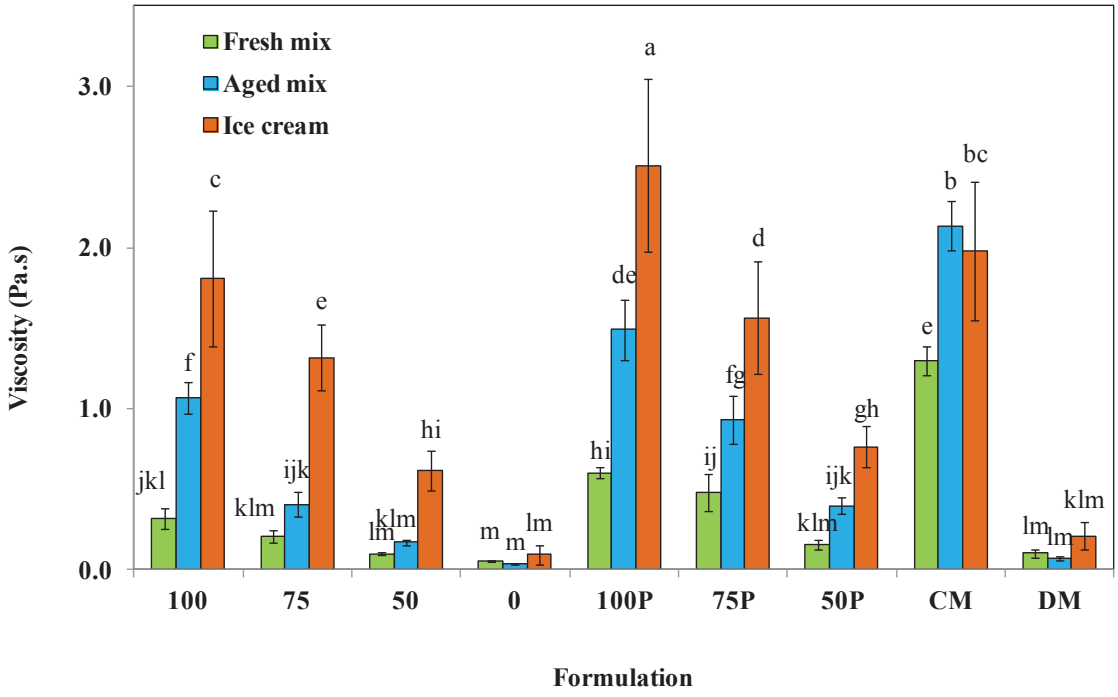

Figure 7.9 Apparent viscosity (η), at a shear rate of 10 s^{-1}, of fresh ice cream mixes ( ), aged ice cream mixes ( ) and molten ice creams ( ). Values with different letters indicate significant difference (n=6, P < 0.05). Abbreviations 100, 75, 50 and 0 represent the ratio of CO and SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the ice cream mix formulations; CM and DM mean ice cream mixes made from coconut milk and dairy milk, respectively.

In summary, the difference in the viscosities between samples was compared at the same shear rate of 10 s^{-1} applied. The results shown in Figure 7.9 indicate that the ratio of CO and SO and the protein concentration had a significant effect on the viscosity of samples. The viscosity increased with increasing solid fat content solid and protein concentration. As a result, the samples containing 100% SO was significantly lower in their viscosity compared to the other samples. The viscosity of ice cream mixes significantly increased after ageing. The melted ice creams were higher in viscosity

than the aged ice cream. The dairy milk ice cream had a significantly lower viscosity than the coconut milk based ice cream.

9.4.6.2 Stress sweep test

Prior to a small deformation oscillatory measurements, the stress sweep test was carried out to determine a critical stress (Pa) from the linear viscoelastic region (LVR) for a further use in the small deformation oscillatory test and to obtain an additional information on the viscoelastic behaviours of ice cream emulsions (Liew et al., 2001b). The critical stress is the stress where the viscoelastic parameters, i.e., a storage modulus (G′) which represents an elastic or solid-like property of materials or a loss modulus (G″) which represents viscous flow or liquid-like property of materials, become stress dependent (Liew et al., 2001b, Rohn, 1995). As the oscillatory test is carried out within the range of LVR, this ensures that the viscoelastic properties of materials are measured without the destruction of materials (Liew et al., 2001b, Rahalkar, 1992, Shoemaker, 1992).

The stress sweep test was performed between 0.06 Pa and 5.0 Pa at a frequency of 1 Hz using the same geometry and gap as used in the flow curve measurement mentioned earlier. The results of storage (G′) and loss (G″) moduli obtained by the stress sweep test are shown in Figure 7.10. The samples containing 75 or 100% CO, including the one made from CM, showed that changes in the storage and loss moduli occurred at a high shear stress of around 2.0-3.0 Pa, depending on the type of samples (fresh and age mixes or molten ice creams), with the longest LVR (between 0.06 and 2.0 Pa). This indicates the apparent strong intermolecular forces acting between fat droplets within the emulsion structure of these samples (Baird, 1981, Liew et al., 2001b), thus a high force was required to break down the structure. The magnitude of critical stress became lowered as the ratio of SO increased. The fresh ice cream mix samples containing 50% or 100% SO or made from dairy milk (DM) showed that both storage and loss moduli started to drop from a very low shear stress of approximately 0.15 Pa which was lower than the other ice cream mixes. The shortest range of stress assessed, particularly from the ice cream mix emulsion containing 100% SO, may indicate very weak forces acting between oil droplets, such as van der Waals attraction (Baird, 1981, Liew et al., 2001b).

Since the G′ and G″ values were shear stress independent at the stress from 0.1 Pa downwards, therefore, the shear stress of 0.1 Pa was selected and used as a constant stress for the further small deformation oscillatory measurements which will be described further in detail.

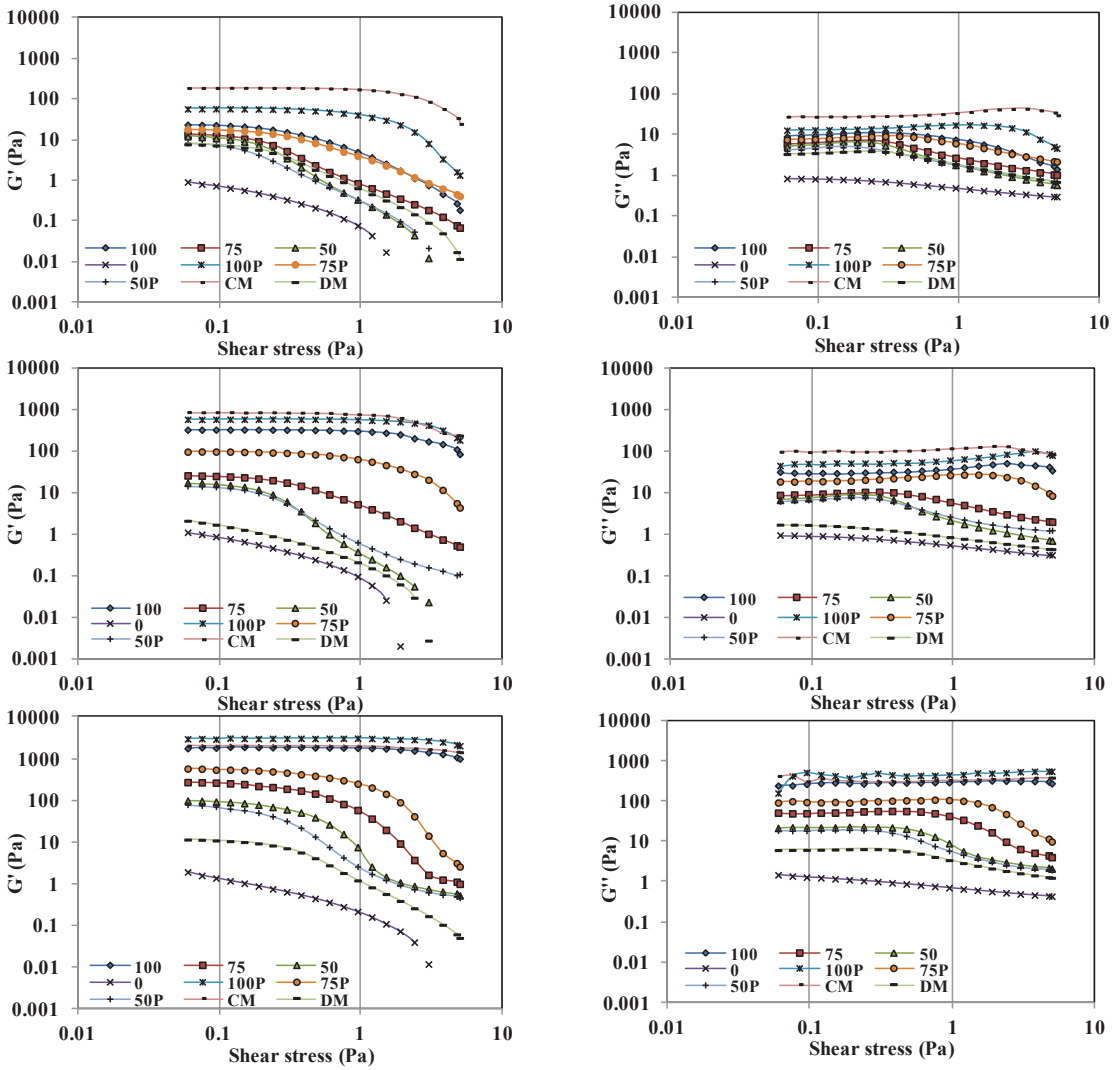

Figure 7.10 Storage modulus (G′) and loss modulus (G″) obtained from the stress sweep test of samples (A and B: fresh ice cream mixes; C and D: aged ice cream mixes; and E and F: molten ice creams). Abbreviations 100, 75, 50 and 0 represent the ratio of CO and SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the ice cream mix formulations; CM and DM mean ice cream mixes from coconut milk and dairy milk, respectively.

From this stress sweep test, the different ratios of CO and SO used in the ice cream mixes could be seen to have a substantial effect on the viscoelastic properties of ice cream mixes and ice creams. For all samples (fresh and aged ice cream mixes and melted ice creams), the storage modulus (G′) rose as more CO (solid fat) was incorporated in the formulation. Also, all these samples revealed rheological behaviours with higher storage modulus (G′) than loss modulus (G″), except for the samples containing 100% SO. This indicates that they were predominantly elastic solid-like properties, which could be resulting from the formation of a continuous network structure (Fang and Choi, 2012, Steffe, 1996). It should be mentioned that the storage modulus (G′) for the fresh and aged mixes and melted ice creams containing 100% SO was substantially lower compared to all other samples containing 100% CO or oil blends of CO and SO and was similar to or slightly lower than the loss modulus (G″). These results suggest the aged ice cream mixes and melted ice creams with 100% SO were predominantly liquid-like with a weakly entangled network structure (da Silva and Rao, 1992). The same finding has been shown in the literature for the ice cream mixes containing oil blends of palm kernel oil and sunflower oil, in which the storage modulus decreased with increasing liquid oil (Méndez-Velasco and Goff, 2011, 2012b). The two different concentrations of CSM proteins (0.3 and 1.1%) used in ice cream mixes were also observed to influence the measured storage and loss moduli. At a high protein concentration (1.1%), the rise of storage and loss moduli was observed from samples containing 100% and 75% CO but its effect was not significant for the samples containing 50% CO.

According to Granger et al. (2005a), stable emulsions could be characterised by the measured G′ that was always greater than G′ values. Before ageing, a group of stable ice cream mix emulsions was the mix containing CO (at all levels) and the one based on CM as G′ > G″, whereas the unstable one was evident in the ice cream mix containing 100% SO as G″ > G′. These results were in agreement with the results of the phase separation and viscosity measurements shown in Figures 7.7 and 7.9, respectively. As the more liquid oil was incorporated into the mix, the ice cream mix emulsion became less stable. The addition of CSMP enhanced the stability of emulsions of the ice cream

mixes containing 100% and 75% CO, but did not improve the stability of the ice cream mix emulsion with 50% CO.

After ageing, the storage and loss moduli were substantially increased compared to the ice cream mixes before ageing. The G′ for all emulsions containing CO (at all levels) rose, suggesting those emulsions became more stable with the solid-like property (G′ > G″) after ageing. The aged mixes which showed less stability possessed more liquid-like property (G″ > G′), including the aged ice cream mix emulsions containing 100% SO or made from DM. These two aged emulsions exhibited marked phase separation as shown in Figure 7.7. It should also be noted that the G′ of aged emulsions containing 100% CO, including the sample prepared from CM, was about tenfold higher than G″ (G′ >> G″). These samples could be characterised as a rubbery solid (Doublier et al., 1992, Ross-Murphy, 1994). The corresponding melted ice creams also showed less change in the G′ and G″ values throughout the whole shear stress range when compared to the fresh and aged ice cream mixes, indicating a more strong intermolecular attractive force existing within the network structure of molten ice cream emulsions.

By conducting a stress sweep test, the changes of shear strain (%) as a function of shear stress could be obtained (data not shown). A shear strain is defined by the change in the shape of a sample compared to its shape prior to the forces or shear stresses are applied (Rohn, 1995). The comparison of the percentages of shear strain between the samples at the same magnitude of shear force applied at 0.1 Pa is shown in Figure 7.11. A pronounced high percentage of shear strain was observed from the samples containing 100% SO or dairy milk fat made from DM. The relatively small change in shape was observed from all samples containing 100% CO.

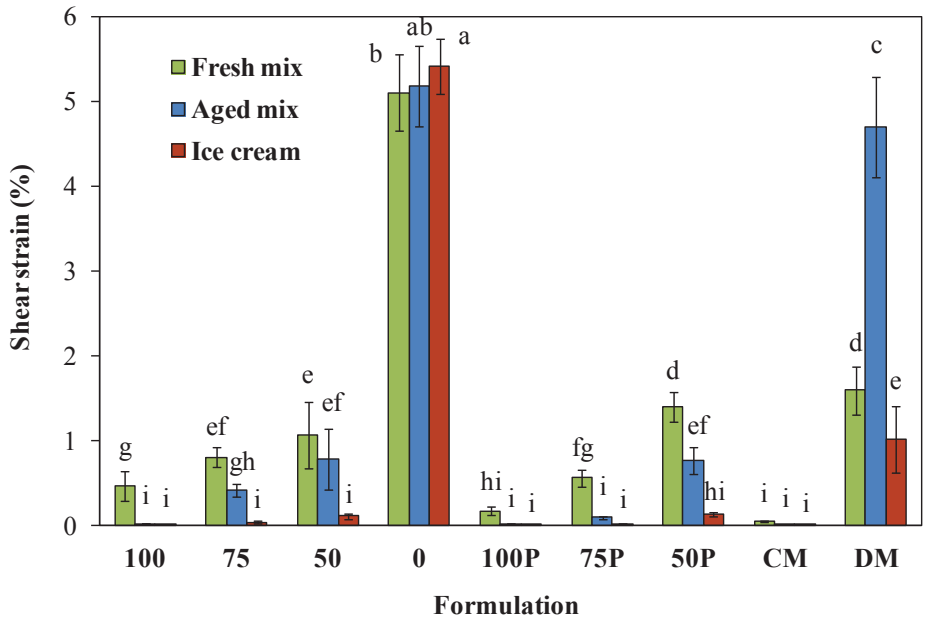

Figure 7.11 Comparison of shear strain percentage of ice cream fresh and aged mixes and molten ice creams at a shear stress of 0.1 Pa. Abbreviations: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP added into emulsions; CM and DM mean samples made from coconut milk and dairy milk, respectively. Error bars are the standard deviations calculated from shear strain data (n = 6). Mean values with the same letter are not significantly different (P < 0.05).

7.4.6.3 Frequency sweep test

The frequency dependency of viscoelastic moduli of all ice cream emulsions, e.g., fresh and aged mixes and melted ice creams, was analysed by a frequency sweep test at frequencies ranging from 0.01 to 10 Hz with a constant shear stress at 0.1 Pa. The logarithmic plots of storage modulus (G′) and loss modulus (G″) against frequency used are shown in Figure 7.12. From Table 7.6, the inclusion of liquid oil led to the significant decrease in the G′ value in the ice cream emulsions after preparation, ageing and turning into ice cream ($P < 0.05$). The inclusion of CSMP led to an increase in the G′ values of ice cream emulsions.

Table 7.6 Storage modulus (Pa) obtained from a frequency sweep test of ice cream emulsions measured at a frequency of 1 Hz.

Sample	Fresh ice cream mix	Aged ice cream Mix	Ice cream
100	112.86 ± 49.50^{c}	562.17 ± 122.26^{b}	1395.47 ± 761.76^{b}
75	23.95 ± 3.13^{d}	51.10 ± 3.40^{c}	404.40 ± 145.79^{c}
50	9.43 ± 2.10^{d}	17.54 ± 7.37^{c}	137.43 ± 54.06^{c}
0	1.22 ± 0.23^{d}	0.58 ± 0.35^{c}	0.97 ± 0.50^{c}
100P	198.63 ± 42.09^{b}	711.62 ± 156.26^{b}	2502.15 ± 1197.22^{a}
75P	45.55 ± 15.98^{d}	166.27 ± 41.95^{c}	678.05 ± 300.67^{c}
50P	10.34 ± 2.45^{d}	28.16 ± 6.64^{c}	181.17 ± 105.06^{c}
CM	352.13 ± 114.95^{a}	1045.27 ± 340.13^{a}	1941.52 ± 677.22^{ab}
DM	7.60 ± 2.12^{d}	3.72 ± 2.05^{c}	14.73 ± 3.40^{c}

Mean values in the same column with different alphabets indicate significant difference ($P < 0.05$). Abbreviation: 100, 75, 50 and 0 means the ratio of coconut oil to sunflower oil present in emulsions at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the mixes; CM means emulsions made from coconut milk; and DM means emulsions made from dairy milk.

As anticipated, G′ values were higher when the ratio of CO incorporated was higher. The very high G′ values were observed from samples containing 100% CO, regardless of the type of liquid aqueous base (100, 100P and CM), while the lowest G′ was seen from samples containing 100% SO. This was in agreement with studies reported by Méndez-Velasco and Goff (2011, 2012b) in those the solid fat content in ice cream mixes contributed to the solid-like property of ice cream mixes. The fresh mixes with 100% or 75% CO showed that the G′ was frequency independent as it was relatively constant and was also always higher than the G″ across the whole range of frequency from 0.01 to 10 Hz. As shown by the results of the frequency sweep test, these results suggest that the ice cream mixes with 100% or 75% CO have been characterised to have a gel-like structure or entanglement networks (Doublier et al., 1992, Ross-Murphy, 1994). The similar pattern of a less dependence on frequency has been shown in a study of Granger et al. (2005a). In that study, the storage modulus of ice cream emulsions

based on hydrogenated coconut oil added with unsaturated mono- and diglycerides was less dependent of the frequency in the range of 0.05-5 Hz and was always higher than the loss modulus. The frequency independence pattern of both moduli was also observed from the aged emulsions containing 100% and 75% CO which was quite similar before ageing.

The contrast phenomena were observed from fresh and aged ice cream mixes containing 50% CO with or without CSMP, 100% SO and DM as follows: a) a substantial drop of both G′ and G″ was observed from these samples compared to the samples containing 75% and 100% CO, b) the frequency crossover of G′ over G″ was observed, and c) the G″ representing the liquid-like property was dominant at the low range frequency but the elastic G′ dominated at the higher frequency range. These distinct characteristics have been reported to be normally found in semi-diluted polysaccharide solutions with a small entanglement network of weak structures compared to gels (Doublier et al., 1992, Ross-Murphy, 1994). In the aged mixes with 50% CO, the more pronounced solid-like property was observed from emulsions added with CSMP. In these aged mixes, the G′ values were higher than G″ values at the high frequency range, suggesting the development of the stronger intermolecular interactions created after ageing.

After the aged ice cream mixes were churned, structure of molten ice creams exhibited some changes in their mechanical spectra, compared to the corresponding fresh or aged ice cream mixes, as follows: a) the elastic solid-like G′ was always higher than the viscous liquid-like G″ over the whole frequency range, except for a few samples (100% SO or DM) that had no pronounced difference between both moduli; b) both mechanical spectra (G′ and G″) were higher than those of the ice cream mixes; c) the G″ increased particularly in emulsions containing fat blends; d) the elastic property was slightly-frequency dependent over the whole range of frequency; and e) there was no frequency cross-over between two moduli which mean the ice cream structure consists of a network with less likely to be broken down (Doublier et al., 1992). These results indicate that a more rigid network with a strong solid-like structure existed in ice cream structure compared to the aged ice cream mixes, which was harder to rupture due to the interaction or cross-links between particles of emulsions in the ice creams (Doublier et

al., 1992, Ross-Murphy, 1994). The possible cause of an increase in the elastic property of ice cream may be due to particle-particle interactions by covalent disulfide bonds which are present in the structure of coconut 11S globulins, which was confirmed by the results of SDS-PAGE already discussed earlier. A similar phenomenon was previously reported with regard to the disulfide bond linkage between emulsion droplets stabilised by globular soy proteins (Floury et al., 2002, Kim et al., 2002a, b). Another possible cause of the particle-particle interaction may be a result of the non-covalent bonds like hydrogen bonds, hydrophobic or electrostatic interactions (Ahmed et al., 2007, Floury et al., 2002, Renkema and van Vliet, 2002).

The physical states of emulsions can also be analysed and interpreted by the values of loss tangent or damping factor (Tan δ) (Ferry, 1980, Granger et al., 2005a, Rohn, 1995). Tan δ which is the ratio of loss modulus to storage modulus has been used to explain the viscoelastic properties of many emulsions (Granger et al., 2005a, Granger et al., 2005b, Nagano and Tokita, 2011). Emulsions are characterised as solid-like when Tan δ is less than 1 while they are liquid-like when Tan δ is greater than 1 (Granger et al., 2005a). The observed values of loss tangent from different ice cream emulsions plotted logarithmically against frequency are shown in Figure 7.13. The comparison of Tan δ values between samples at a frequency of 1 Hz is shown in Figure 7.14. Tan δ values for CSM based fresh and aged ice cream mixes containing 50% CO and 100% SO or prepared from DM showed predominantly liquid-like behaviour (Figures 7.13A and 7.13B). One noticeable finding is that at a very low frequency range, Tan δ for the molten ice cream samples containing 100% SO or made from DM was greater than 1 (Figures 7.13C and 7.14), suggesting the liquid flow property (G″) was predominant. This reflects a lack of cross-linked structures in those samples (Ferry, 1980).

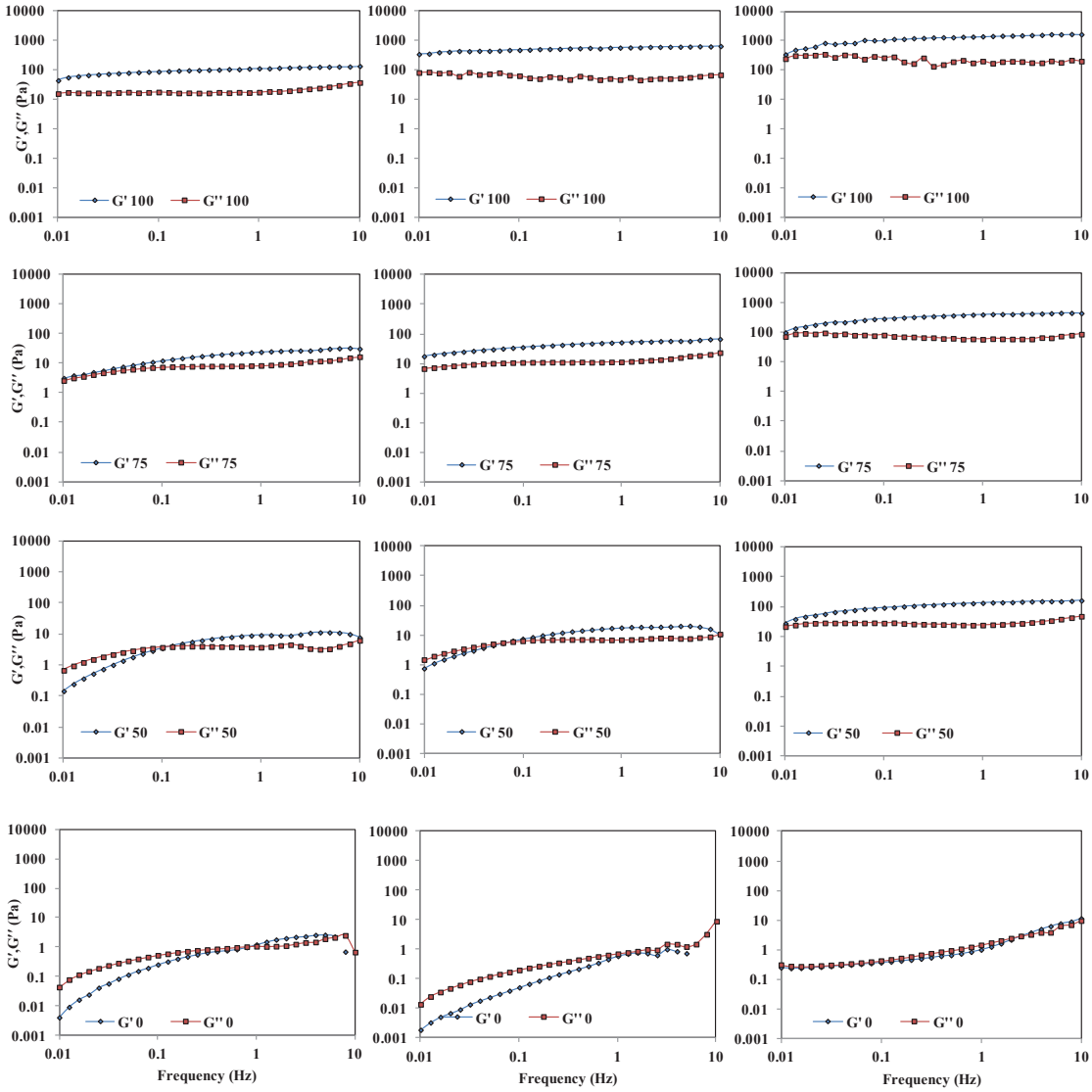

Figure 7.12 Mechanical spectra of storage modulus (G′) and loss modulus (G′′), plotted logarithmically as a function of log frequency between 0.01 and 10 Hz of fresh ice cream mixes (left), aged ice cream mixes (middle) and molten ice creams (right). Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into formulation; CM means ice cream emulsions made from coconut milk; and DM means ice cream emulsions made from dairy milk.

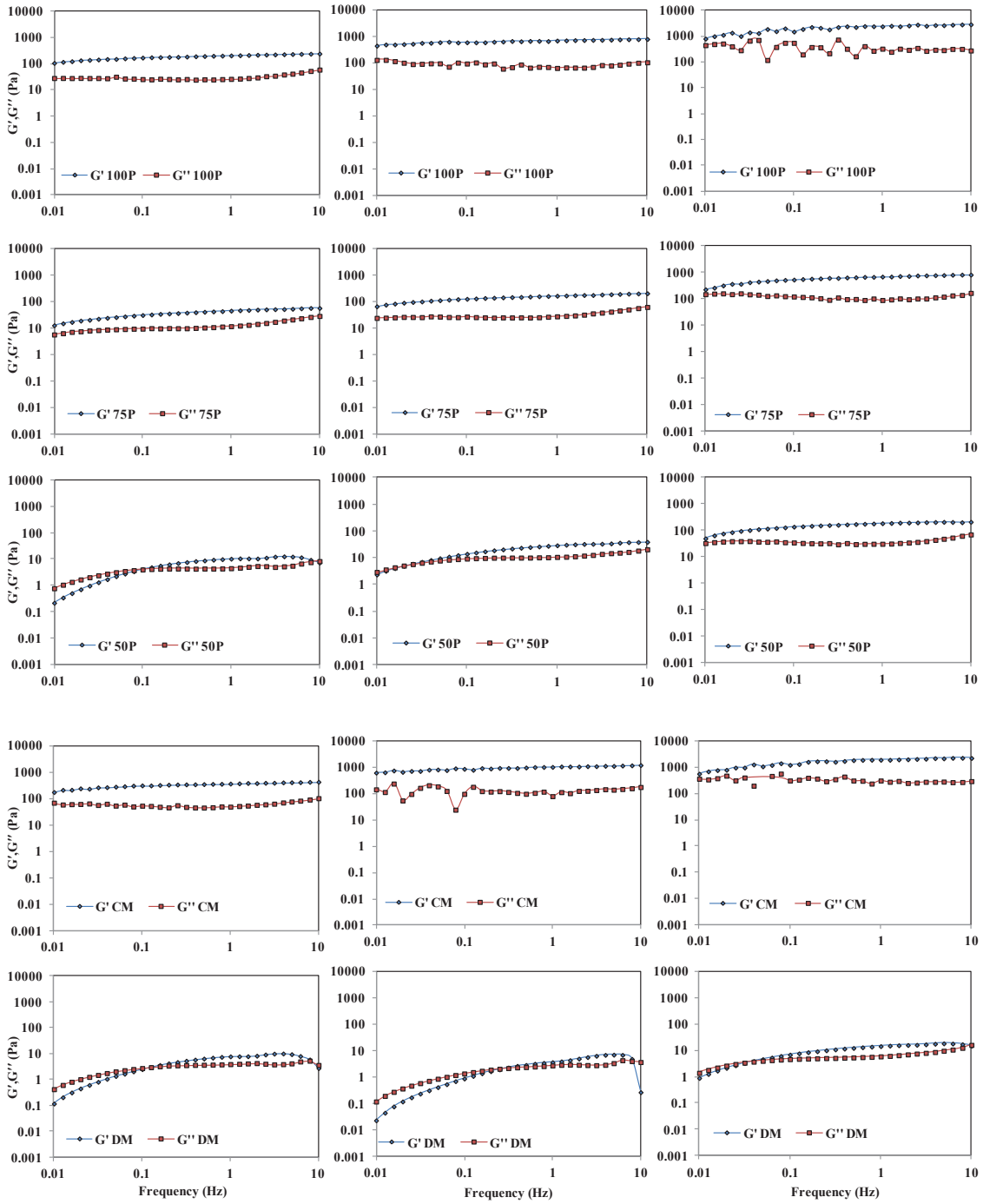

Figure 7.12 (Cont.) Mechanical spectra of storage modulus (G′) and loss modulus (G′′), plotted logarithmically as a function of log frequency between 0.01 and 10 Hz of fresh ice cream mixes (left), aged ice cream mixes (middle) and molten ice creams (right). Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into formulation; CM means ice cream emulsions made from coconut milk; and DM means ice cream emulsions made from dairy milk.

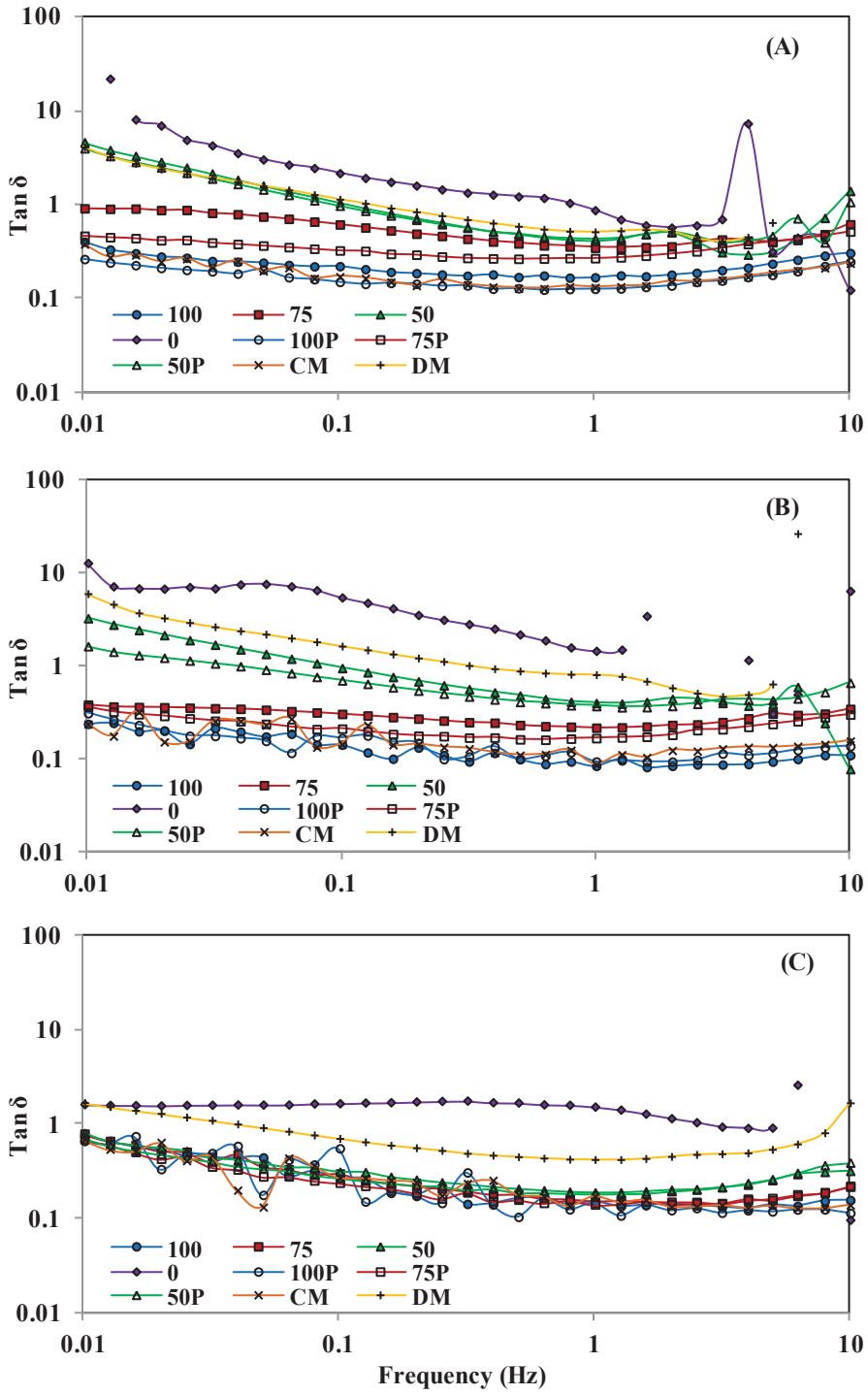

Figure 7.13 Loss tangent plotted logarithmically against log frequency between 0.01 and 10 Hz. Fresh ice cream mixes (A), aged ice cream mixes (B) and molten ice creams (C). Abbreviation: 100, 75, 50 and 0 represent the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the mixes; CM and DM mean ice cream emulsions prepared from coconut milk and dairy milk.

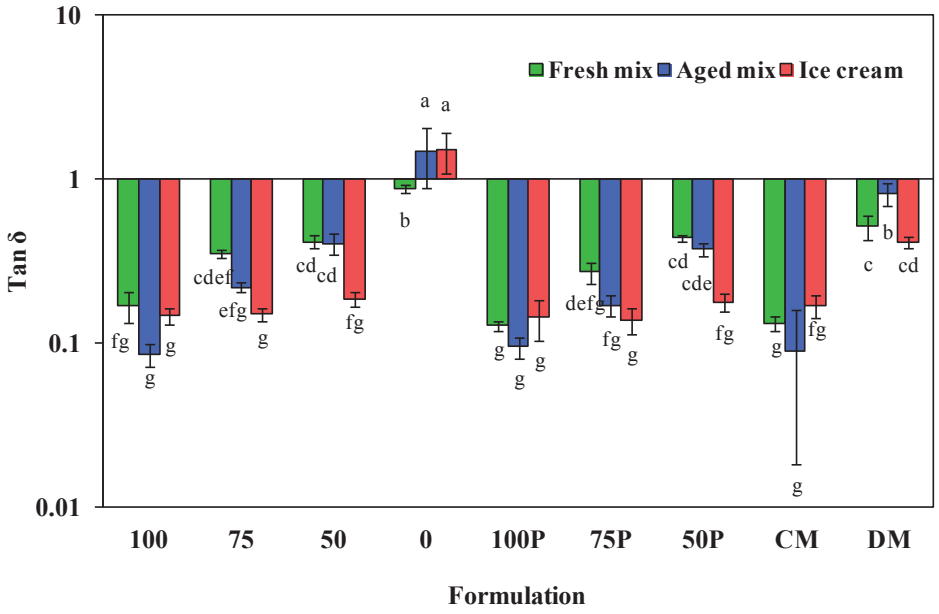

Figure 7.14 Comparison of loss tangent (Tan δ) of emulsions of fresh ice cream mix, aged ice cream mix and molten ice cream emulsions obtained from a frequency sweep test at 1 Hz. Values with the same letter are not significantly different (P < 0.05). Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the formulation; CM means ice cream emulsions made from coconut milk; and DM means ice cream emulsions made from dairy milk.

From Figures 7.13 and 7.14, it can be seen that ice cream mix emulsions after preparation exhibited from a slightly solid-like material for the samples containing 100% SO to a strong gel-like material for the samples containing 100% CO. After ageing, almost all aged ice cream mixes, except the one containing 100% SO, showed an increase in their solid-like properties but had no significant difference compared with their fresh ones ($P > 0.05$). After the freezing and churning process, the ice creams containing 75% and 50% CO or made from DM possessed tended to become stronger solid-like structures than their aged mixes, reflecting the formation of more coherent structures probably due to the fat droplet clumping in those ice creams, but were not as strong as the other samples containing 100% CO as Tan δ was still lower. The Tan δ of ice creams containing 100% CO however was not changed after freezing and churning of aged ice cream mixes.

7.4.6.4 Temperature Sweep test

Molten ice creams were observed for their viscoelastic parameters, G′ and G″, against temperatures increasing from 4°C to 30°C with a heating rate of 1°C/min. With increasing temperature, the G′ and G″ moduli of the melted ice cream samples showed different thermo-viscoelastic responses (Figure 7.15). Both moduli for the samples containing 100% CO (100, 100P and CM) increased gradually at a range of temperatures between 4°C and 18°C and then there was a sharp drop of both moduli when the temperature was increased further from 18°C to 25°C with a tendency of cross-point between both moduli at approximately 25°C for almost all samples (Figure 7.15). A slight increase in both moduli in emulsions containing 100% CO suggests the connectivity between the solidified coconut oil droplets that may be glued together by the liquid oil which was melted down as temperature increased.

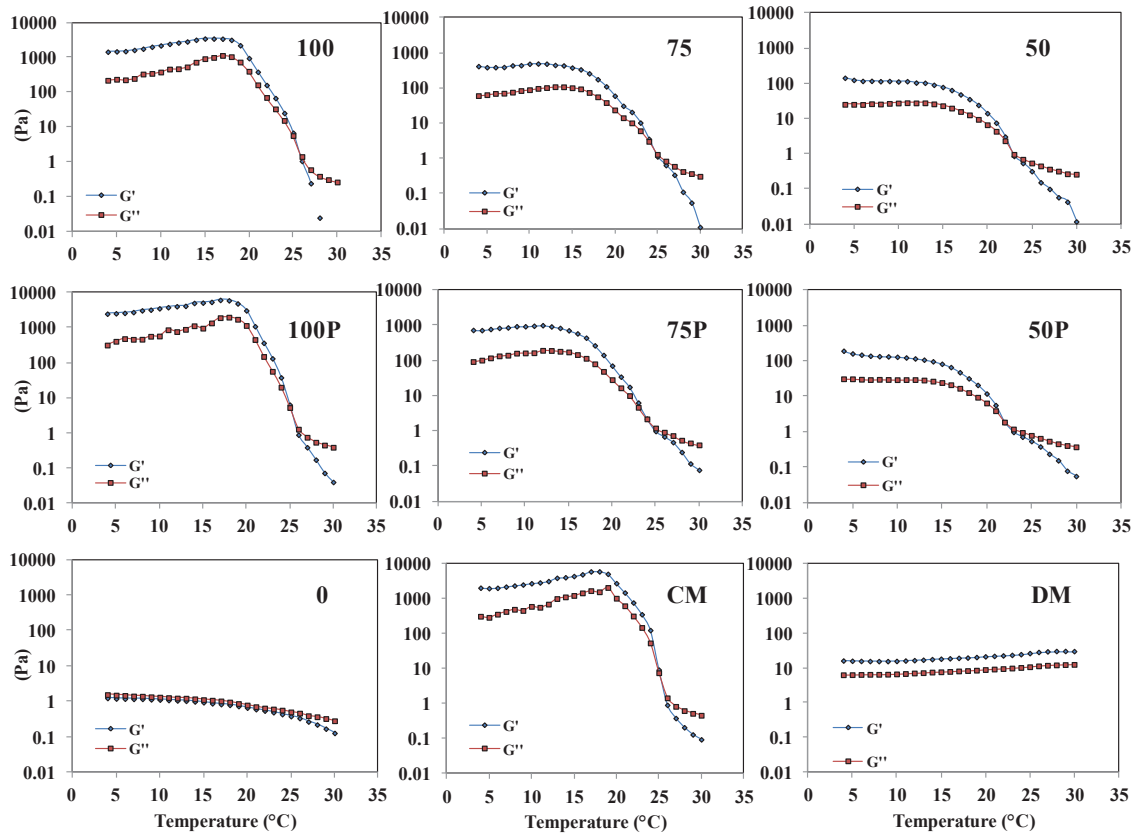

Figure 7.15 Mechanical spectra, storage (G′) and loss (G″) moduli, plotted logarithmically, against a range of temperatures between 4°C and 30°C, of molten ice creams. Abbreviation: 100, 75, 50 and 0 mean the ratio of CO:SO at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the formulation; CM means ice cream emulsions made from coconut milk; and DM means ice cream emulsions made from dairy milk.

Values of storage modulus (G′) at two different temperatures (4°C and 20°C) obtained from molten ice cream samples are shown in Table 7.7. From Table 7.7, a decrease in solid fat content led to a significant reduction in the G′ value in the molten ice cream emulsions ($P < 0.05$). An incorporation of SO into the ice cream formulation led to a significant drop in the elastic property of emulsion. The more liquid oil was added into the ice cream mixes, the more decrease in the G′ value was observed in the emulsions. Although the incorporation of proteins from CSMP led to an increase in both parameters in the molten ice cream compared to the one without it, the decrease in the G′ values could still be observed.

Table 7.7 Storage modulus (Pa) at two different temperatures of melted ice cream emulsions obtained from the temperature sweep test observed at the constant stress and frequency at 0.1 Pa and 1 Hz, respectively.

Sample	4°C	20°C
100	1438.35 ± 792.73^{b}	941.20 ± 607.52^{b}
75	414.98 ± 138.75^{c}	59.83 ± 14.71^{b}
50	142.70 ± 50.39^{c}	14.08 ± 11.08^{b}
0	1.25 ± 0.62^{c}	0.67 ± 0.29^{b}
100P	2541.05 ± 1190.43^{a}	3145.03 ± 1742.81^{a}
75P	700.72 ± 289.39^{c}	68.11 ± 47.40^{b}
50P	189.60 ± 108.42^{c}	11.79 ± 11.27^{b}
CM	1971.30 ± 642.07^{ab}	2655.65 ± 2185.56^{a}
DM	15.54 ± 3.83^{c}	20.45 ± 23.55^{b}

Mean values in the same column with different alphabets indicate significant difference ($P < 0.05$). Abbreviation: 100, 75, 50 and 0 means the ratio of coconut oil to sunflower oil present in emulsions at 100:0, 75:25, 50:50 and 0:100, respectively; P means CSMP was added into the mixes; CM means ice cream emulsions made from coconut milk; and DM means ice cream emulsions made from dairy milk.

A similar trend but with a relatively slight increase in both elastic and viscous moduli with increasing temperatures and then a sharp drop but at a lower temperature of 12^{o}C was seen from the molten ice cream samples containing 75% CO (Formulations 75 and 75P). The molten ice creams containing 50% CO (Formulations 50 and 50P) however showed only a rapid decrease in both moduli. In contrast, a relatively small change in both mechanical moduli was observed from the DM based ice cream and the CSM based ice cream containing 100% SO throughout the whole temperature range tested. It was interesting to see that the cross-point of both moduli where G′ = G″ of the molten ice creams based on CSM, with or without added CSMP, containing 100%, 75% and 50% CO was about 25°C which seemed to become slightly lower with increasing liquid oil (SO). No cross-point was observed in the ice creams containing 100% SO or made from DM.

The Tan δ values obtained from a temperature sweep test showed a similarity in the characteristics among the ice creams prepared from CSM and CM containing CO, irrespective of the ratio of CO and SO. The Tan δ values were less temperature dependent and quite constant over the range of temperatures between 4°C and 20°C, then became strong temperature dependent with a drastic increase from the temperature of 20°C onwards. This change was a good indicative of a collapse of a cohesive elastic gel-like structure (Owen et al., 1992) in the molten ice creams which was triggered by the increasing temperature. As expected, a contrast phenomenon was observed from the molten ice creams based on DM and CSM containing 100% SO as their values of Tan δ were temperature independent although there was a slight change by the increasing temperature throughout the temperature range. This suggests no structural breakdown in both melted ice creams with temperature changes.

The results suggest that the structural breakdown of intermolecular network of molten ice creams occurred dramatically at the temperature of around 18°C or 12^{o}C, except for the two samples containing 100% SO or dairy milk fat, and the networks were weakened further by increasing temperatures. The structural change started to occur at a lower temperature when the molten ice creams contained more SO and less CO.

As described and shown in Sections 7.4.6.2 and 7.4.6.3, the inclusion of a higher amount of CO was shown to provide the ice creams with the structural network behaving as solid-like materials at low temperature as their G′ values were higher with increasing CO. It is clearly seen that after ice cream samples were melted at 4°C, the ice cream emulsion with 100% SO exhibited a liquid-like material (Tan $\delta > 1$), whereas the other ice creams showed a solid-like material (Tan $\delta < 1$) (Figure 7.16). As the temperature increased, there was only a slightly change in the Tan δ values for the samples containing 100% SO or made from DM, suggesting the temperature independency of both ice cream emulsions. In other words, there was no structural breakdown due to the change in their temperatures. It is interesting to note that the melted ice cream made from DM remained a solid-like property at the entire temperature range of 4-30^{o}C as its Tan δ value remained smaller than 1 (Tan $\delta < 1$). In contrast, a dramatic change in the physical state from a solid-like to a liquid-like at temperatures beyond around 20-25°C was observed from the other molten ice creams. From the log loss tangent values, the temperature that Tan δ was higher than 1 from the ice cream emulsions containing 50%, 75% and 100% CO was at 22°C, 24°C and 25°C, respectively.

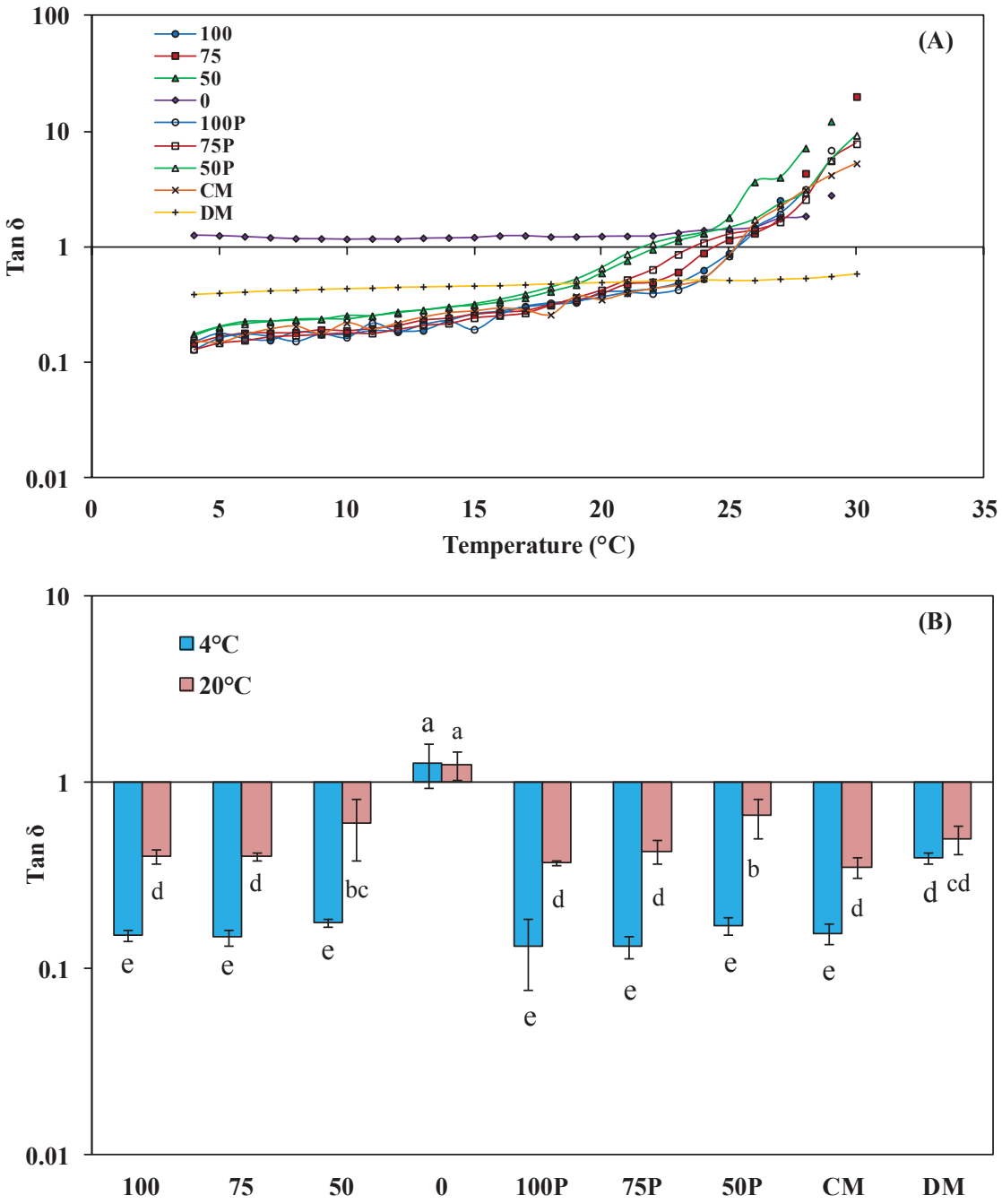

Figure 7.16 (A) loss tangent plotted logarithmically against log temperatures between 4°C and 30°C from molten ice cream emulsions and (B) comparison of logarithmic Tan δ at 4°C and 20°C between molten ice cream emulsions. Mean values with the same letter are not significantly different (P < 0.05). Error bars represent standard deviation of 6 replicates.

7.4.7 Ice cream overrun

Overrun which refers to an ability of ice cream structure to hold air bubbles was investigated by measuring a percentage increase in the volume of ice cream due to the whipping of ice cream mix during the freezing process (Muse and Hartel, 2004, Segall and Goff, 2002, Sung and Goff, 2010, Tong et al., 1984). Figure 7.17 shows that all ice cream samples, except for the one containing 100% SO, had 60-70% overrun. The overrun was observed to be highest when 25% SO was introduced into the ice cream mixes but it decreased with the addition of more or less than 25% SO, which may indicate the ratio of CO and SO at 75:25 rendered the optimum solid fat content in the dispersed fat droplets for the high overrun. This may also mean that there was an optimal amount of liquid oil present to bond the collision droplets together to form a continuous structural network to stabilise air cells (Fredrick et al., 2010). The presence of too much liquid oil leads to the collapse of air cells in the ice cream (Marshall et al., 2003). In other words, the high ability of holding air cells observed in the ice creams made from the ice cream mixes containing 100% or 75% CO could be due to the presence of fat aggregated network enhancing the foam stability (Eisner et al., 2005). The foamability of ice cream seemed to decrease with increasing unsaturated liquid oil (SO) at a level of > 50% SO, resulting in the low overrun. As a result, the least overrun was observed in the ice cream containing 100% SO which only had 20% overrun. Similar results have been reported by Méndez-Velasco and Goff (2012b) that the overrun of ice creams which contained a mixture of saturated palm kernel fat and unsaturated sunflower oil at different proportions increased with increasing solid fat content. Sung and Goff (2010) also reported that the percentage of overrun in ice cream containing fat blends of palm kernel oil and sunflower oil increased as the solid fat content increased.

The addition of CSMP which increased the total protein content in the ice cream mixes (75P and 50P) from 0.3% to 1.1% showed no pronounced enhancement of the air incorporation of ice creams compared with the corresponding samples without added CSMP (75 and 50). However, the ice cream containing 100% CO with the addition of CSMP (100P) showed a significant increase in the overrun.

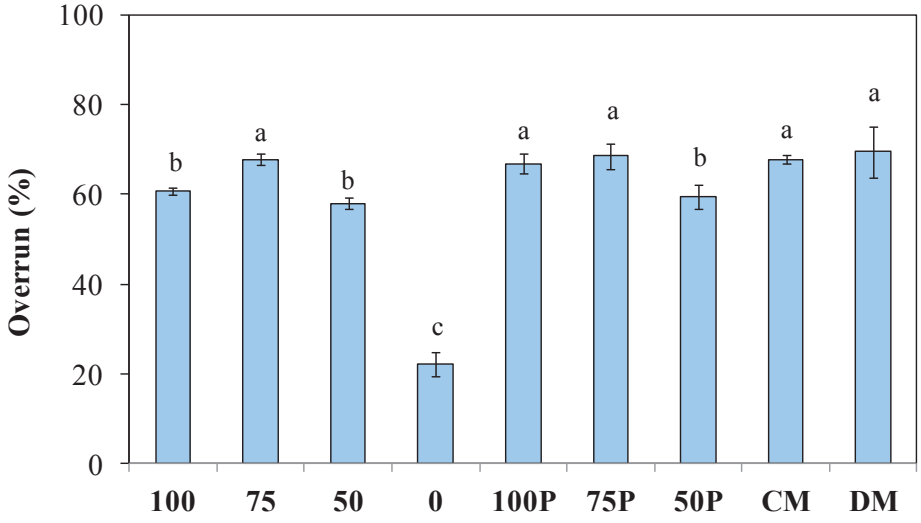

Figure 7.17 Overrun of ice creams. 100, 75, 50 and 0 represent the percentage of CO; P represents CSMP was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively. Mean values with the same letter are not significantly different ($P < 0.05$). Error bars represent standard deviation of 6 replicates.

In general, proteins do not directly involve in the stabilisation of air cells because proteins are normally displaced by added small molecule surfactants from the air-water interface during the freezing and whipping process (Méndez-Velasco and Goff, 2011). The increase in the concentration of protein would be expected to increase the viscosity of ice cream mix, thus preventing the coalescence of air cells (Eisner et al., 2005), as a result, enhancing the overrun. However, this was not clearly seen in this study as the overrun increase by the CSMP addition was only observed in the ice cream containing 100% CO, regardless of the viscosity increase (Figure 7.8B).

The ice creams based on CM and DM had an overrun of 68-69% which was similar to the ice creams with added CSMP (100P and 75P). These two ice creams had the same protein concentration and the similar or same solid fat content but the DM ice cream mix had a much lower viscosity than the CM ice cream mix. Overall, the results obtained indicate that the ratio of solid fat to liquid oil can lead to a significant difference in the overrun of ice cream. The overrun for the ice cream mix containing

100% CO may be further enhanced by an increase in the concentration of coconut milk proteins.

7.4.8 Melting properties of ice cream

The melting properties of ice cream samples were analysed by placing ice creams on a stainless steel mesh screen in a temperature-controlled incubator at 20 °C for 90 min. The weight of a portion of melted ice cream dripping through the mesh screen was measured every 10 min and expressed as a cumulative percentage of the initial weight of ice cream. The pictures of samples that remained on the mesh screen or dripped down after 90 min are shown in Figure 7.18. The plot of ice creams melting versus time is shown in Figure 7.19.

The solid fat content was found to have a strong correlation to the shape retention and melting resistance of ice creams. Ice creams containing 100% CO exhibited a quite strong melting resistance with more than half of the ice creams remained on the mesh screen without melting after 90 min (Figure 7.18). The incorporation of SO into ice cream mix provided the resulting ice cream with a higher or lower melting resistance and stand up properties during melting. The ice creams containing 25% SO showed a lower melting property and a higher shape retention ability than any other samples even the ice creams containing 100% CO. This could be possibly due to the presence of a network of large aggregated fat droplets contributing to the formation of steric hindrance to retard serum drainage through the fat network (Eisner et al., 2005, Koxholt et al., 2001). The formation of large aggregates was evidenced by the high FI value of this ice cream as shown previously in Table 7.5.

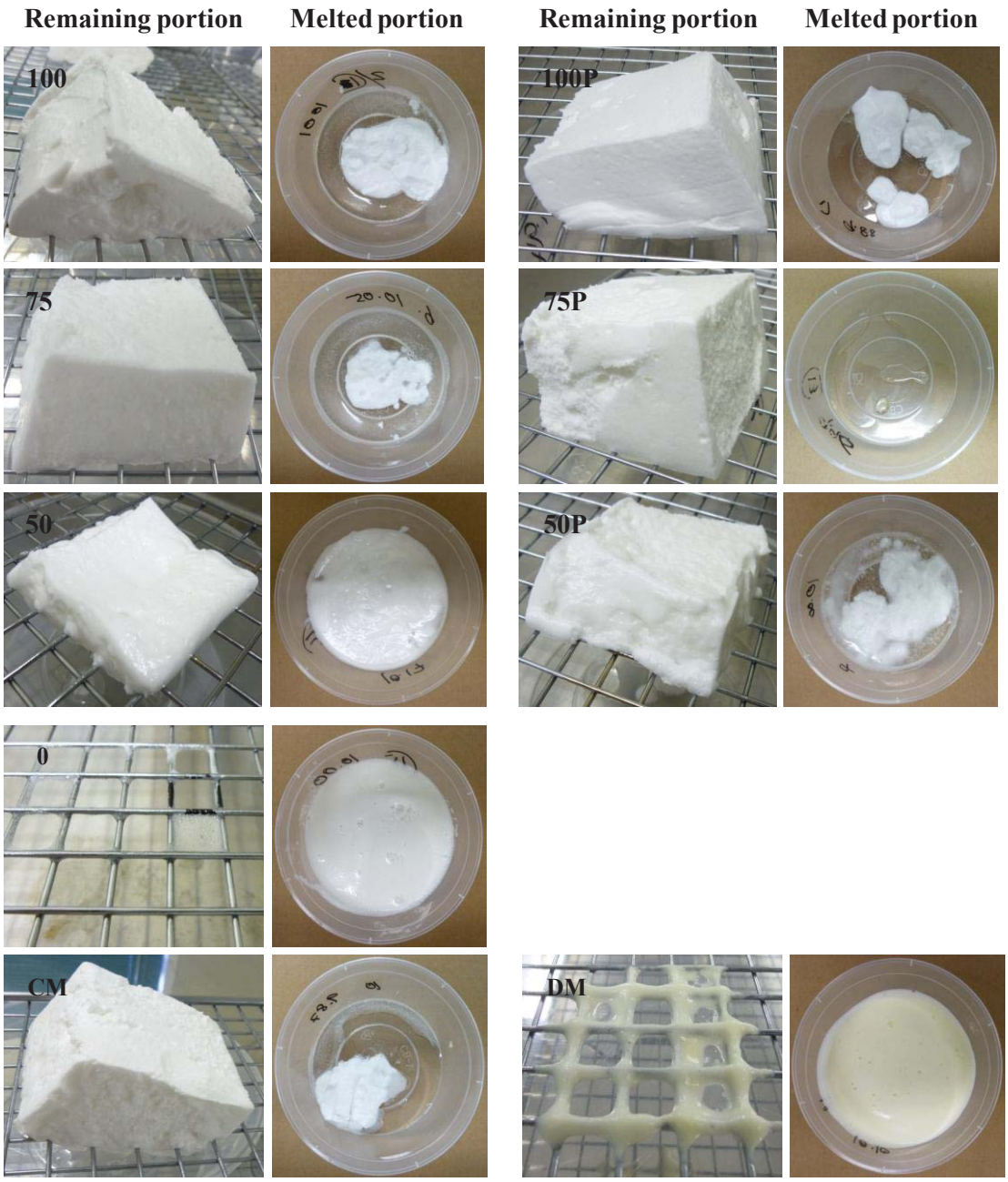

Figure 7.18 Digital images of ice cream samples after 90 min melting at 20°C. A portion of ice cream remaining above the mesh screen (left) and a melted portion of ice cream dripped through the mesh screen (right). 100, 75, 50 and 0 represent the percentage of coconut oil; P represents coconut skim milk powder (CSMP) was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively.

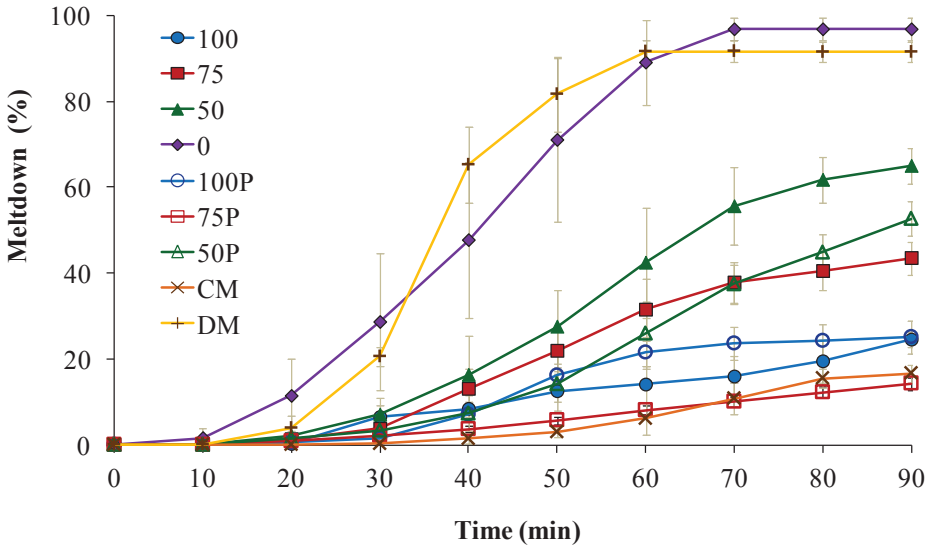

Figure 7.19 Melting rates of ice creams measured at 20°C for 90 min. 100, 75, 50 and 0 represent the percentage of coconut oil; P represents coconut skim milk powder (CSMP) was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively. Error bars represent standard deviation of 6 replicates.

The stand up property dramatically decreased as the ice creams contained more liquid oil. Ice cream containing 100% SO completely lost its shape and was melted before 90 min, indicating almost no structural network created in this ice cream (Pelan et al., 1997b). In other words, the extent of fat agglomeration occurred was very low, thereby resulting in weaker structures in the ice cream (Marshall et al., 2003). The melting resistance of the DM based ice cream was found to be poor as it was also completely melted before 90 min and its melting rate was similar to the ice cream containing 100% SO. However, the viscous melted ice cream sticking to the mesh screen was observed, suggesting the presence of fat aggregation network in the dairy based ice cream (Pelan et al., 1997b) which was also indicated by the high FI value shown in Table 7.5. In this ice cream melting portion, the small air cells were also observed visually, suggesting the ability of ice cream structure to stabilise air cells before melting (Pelan et al., 1997a).

It should be mentioned that the meltdown portion obtained from the ice creams based on DM and CSM with 50% or 100% SO without CSMP was in a homogeneous liquid state, whereas the melted portion dripped through the screen from the other ice creams

appeared to have a mixture of coarse lumps with the separation of some serum. The ice cream containing 75% CO with added CSMP was the only sample that gave a clear serum of dripped portion with no melted ice cream. This suggests the well-built structures of fat agglomeration in ice cream can resist melting and can hold molten ice crystals inside its structure (Regand and Goff, 2002).

The melting behaviours of ice creams with different solid fat contents obtained in this study contradict a study reported by Méndez-Velasco and Golf (2012b) in that more than 90% mass of the ice creams containing oil blends consisting of saturated fat (palm kernel oil) and unsaturated oil (sunflower oil) at ratios of 80:20 or 100:0 were lost after 90 min at 23°C. The possible reason for the slow melting rate of the ice creams containing > 75% CO observed in this study can be because CO has a melting point in the range of 23-26°C (Gunstone, 2004) which is higher than that of the melting point of milk fat or SO (Méndez-Velasco and Goff, 2012b). Therefore, at the temperature of 20^{o}C used in this study which was lower than the melting point of CO, the structural fat network in ice creams containing a high CO content might not be completely changed in its state and therefore could still retain its strength, resulting in the rigidity of the fat structures enough to maintain the ice cream shape without collapse. This finding was in agreement with the study of El-Rahman et al. (1997) that the dairy ice creams containing a very high melting milk fat fraction were highly resistant to meltdown and had a high stand up property than the normal standard dairy ice creams. In this study, as described above, the ice cream prepared from dairy milk was melted down completely after 90 min and the melting rate was similar to the ice cream sample containing 100% SO.

The increase in the protein concentration of ice cream formulations by the addition of CSMP also showed an improvement of melting resistance and shape retention in the ice creams containing 50% or 75% CO compared with the same ice creams without added CSMP (Figures 7.18 and 7.19). The most significant effect was observed from the ice creams containing 75% CO. Those ice creams were melted slower with a small amount of clear liquid dripping from samples after melting and they could maintain their shape similar to the shape of samples prior to the melting test. This was in agreement with the

enhanced ability of ice creams to retain their shape with increasing protein content reported elsewhere (Alvarez et al., 2005, Regand and Goff, 2002). However, it should be noted that the effect of protein was not observed for the samples containing 100% CO as there was no pronounced difference compared to the corresponding sample without added CSMP. It can be thought that the observed increase in the melting resistance for the other samples was due to an effect of the ratio of solid to liquid fat combined with the protein concentration. This suggests that the optimum meltdown properties of ice cream can be modulated by altering the solid fat content in conjunction with the protein concentration in the ice cream mixes.

The melting rate of ice cream samples with different solid fat contents shown in Figure 7.19 indicates clearly that the ice cream samples containing 100% SO or prepared from DM containing dairy milk fat melted faster than the other samples. The former and the latter ice creams completely melted after 70 and 60 min, respectively. This may be because at the temperature of 20°C used in the meltdown test, the fat structures in those ice creams were gradually melted from outside to inside, resulting in the collapse of the whole ice cream. This finding could be also supported by the rheological properties of these ice creams which were composed predominantly of liquid-like materials (Figure 7.15), thus could not resist melting. In contrast, most CSM based ice creams containing CO as well as CM based ones showed a slow melting rate. The slowest melting was observed from the ice cream containing 75% CO with added CSMP as this sample only had less than 20% weight loss after 90 min. The more SO was incorporated into the ice cream mixes, the faster melting rate of the ice cream samples. As it was mentioned above, the ability to retain the fat structures at 20°C of the ice creams containing 75 or 100% CO is believed to be associated with the slow melting properties observed in these samples. The presence of the stronger structures in these ice creams could also be supported by the low Tan δ values as mentioned above (Figure 7.16), which reflected a solid-like structure of these ice creams.

The dependency of ice cream melting on the solid fat content is also illustrated in Figure 7.20. This compares the meltdown rate of samples at a melting time of 60 min. The more SO was incorporated, the less melting resistance was the resulting ice creams. A

significant reduction in the dripped portion of ice cream samples by the addition of CSMP was also be seen, particularly from the ice creams containing 75% or 50% CO. Ice creams containing CSMP showed better melting resistance with low percentage of melting portion.

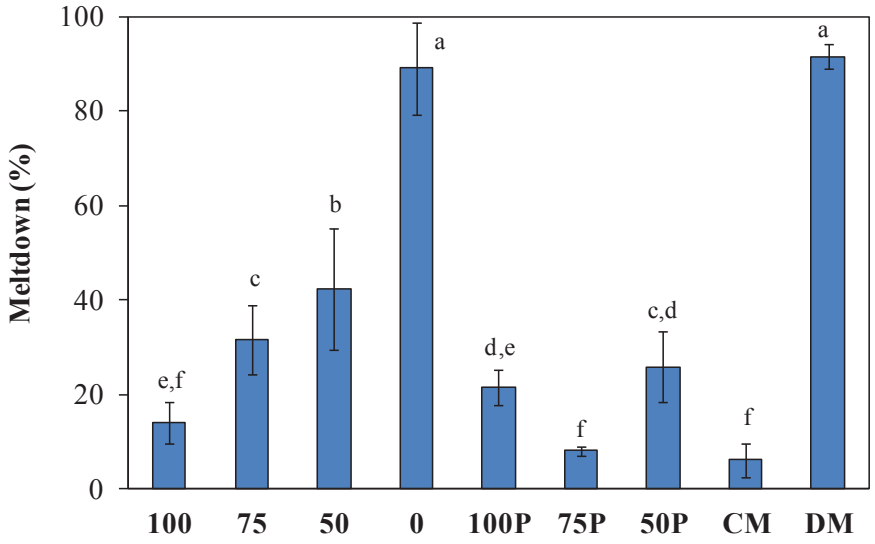

Figure 7.20 Percentage of ice creams melted down at 20°C after 60 min. 100, 75, 50 and 0 represent the percentage of coconut oil; P represents coconut skim milk powder (CSMP) was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively. Mean values with the same letter are not significantly different (P < 0.05). Error bars represent standard deviation of 6 replicates.

7.4.9 Solvent extractable fat content of ice cream

The amount of free fat in the ice creams prepared in this study was determined by the solvent extraction. The results shown in Figure 7.21 indicate that the percentage of solvent extractable free fat was significantly different between the samples, depending on the ratio of CO and SO (i.e. solid fat content) and the addition of CSMP in the ice cream mixes. The high percentage of free fat greater than >85% was observed from the ice creams containing 50%, 75% or 100% CO without added CSMP without noticeable differences between them. However, when the ice cream was produced with 100% SO, the extractable free fat significantly dropped to about 50%. This might be because the droplets in this melted ice cream containing 100% SO was quite stable resulting from the combined effects of its physical properties, such as low overrun, less flocculation, a

liquid like property and high stability against temperature, which are shown in Figure 7.17, Table 7.5 and Figure 7.16, respectively.

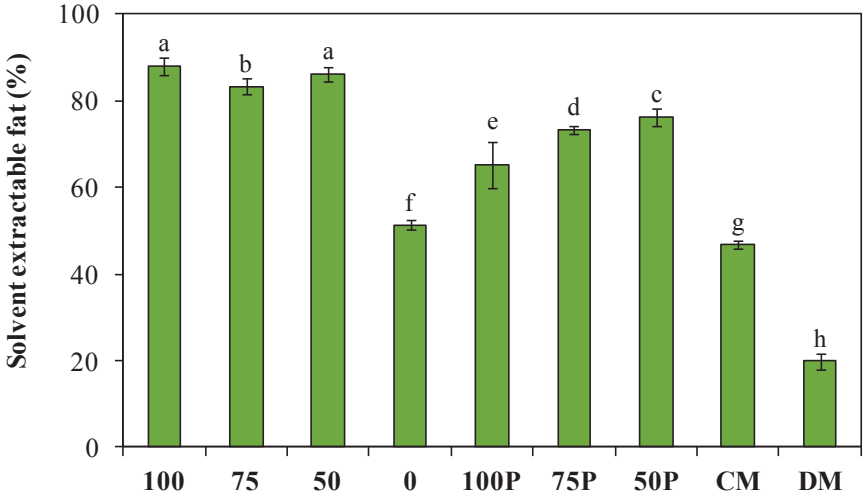

Figure 7.21 Percentage of solvent extractable fat in ice creams. 100, 75, 50 and 0 represent the percentage of coconut oil; P represents coconut skim milk powder (CSMP) was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively. Mean values with the same letter are not significantly different (P < 0.05). Error bars represent standard deviation of 6 replicates.

For ice creams with added CSMP (e.g. 100P, 75P, and 50P), it was observed that the extractable free fat was significantly reduced to 65-70% compared to >85% of free fat observed in the samples without added CSMP. In these samples, the extractable fat content increased gradually with increasing ratio of SO (liquid oil) in the ice creams. The addition of CSMP into the ice cream mixes was to study the effect of protein concentration on the ice cream mix and subsequent ice creams. Pelan et al (1997b) also showed that an increase in the level of milk protein in the ice cream mix reduced the extractable free fat content of ice creams formulated based on dairy milk by enhancing the emulsifying efficiency of fat droplets. For the ice cream samples made from CM or DM, the percentage of extractable fat was found to be much lower with 47% and 20%, respectively, compared to the other samples. The relatively low percentage of extractable fat observed from the DM ice cream could be because dairy milk proteins have much better functional properties to emulsify and stabilise oil droplets than coconut milk proteins.

It has been shown that the percentage of extractable fat from experimental dairy based ice creams was in the range of 16-93%, depending on the type and amount of small molecule surfactants added (Pelan et al., 1997a). Bolliger et al. (2000b) has reported the percentage of the extractable fat ranging from 5% to 60% from their experimental dairy ice cream samples. Ice cream with a higher extractable fat content also showed the higher melting resistance. In other words, ice cream samples with the fast melting rate (DM based and CSM based with 100% SO) were lower in the amount of extractable free fat although this was not applied to the ice cream made from CM as this sample had the relatively low extractable fat content but exhibited the slow melting rate.

The extractable free fat in ice cream can be defined as the de-emulsified fat which can be attributed to a reduction in the amount of adsorbed proteins at the interfacial layer by a displacement of small molecule surfactants, resulting in a less stable emulsion (Pelan et al., 1997a). As a result, content of fat from the destabilised droplets can be more readily dissolved and extracted by a non-polar organic solvent when a thawed ice cream is mixed with a solvent (Bolliger et al., 2000a, Marshall et al., 2003, Pelan et al., 1997a). The higher level of fat being extracted, the lesser stable emulsion and the high fat destabilisation in ice cream. Therefore, the amount of extractable fat content can be an indicative of fat destabilisation that occurred in ice cream (Pelan et al., 1997b).

7.4.10 Extent of fat destabilisation

The magnitude of fat destabilisation in ice creams was determined using the spectrophotometric method and expressed as fat destabilisation index (FDI). The FDI can reflect some properties of ice cream structures, such as fat-air interaction, size of fat clumps and the formation of fat network (Goff et al., 1999b, Marshall et al., 2003, Méndez-Velasco and Goff, 2012b, Rouimi et al., 2005). The FDI determination is based on the proportional difference in absorbance values between the ice cream mix and the subsequent ice cream. A higher value of FDI means the fat particles in ice cream mix underwent destabilisation more significantly during the freezing process, resulting in the formation of larger agglomerates in the ice cream.

The extent of fat destabilisation was high for all ice creams with a FDI value greater than 65%, except for the DM-based ice cream with a FDI value of about 10% (Figure 7.22). This suggests that the more agglomerated fat particles, relative to aged ice cream mix emulsions, were probably introduced into the CSM-based ice creams containing CO at all levels. These FDI values were significantly greater than the values obtained from the CM or DM based ice creams and the ice cream containing 100% SO. In particular, the DM based ice cream showed a far lower FDI value than any other samples. The term fat destabilisation may associate with the formation of a large fat network or the magnitude of fat droplets to form in different sizes of fat clumps (Méndez-Velasco and Goff, 2012b). This suggests that fat networks or fat clumps (agglomeration of oil droplets) were more pronounced in the CSM and CM based ice creams than the DM based ice cream.

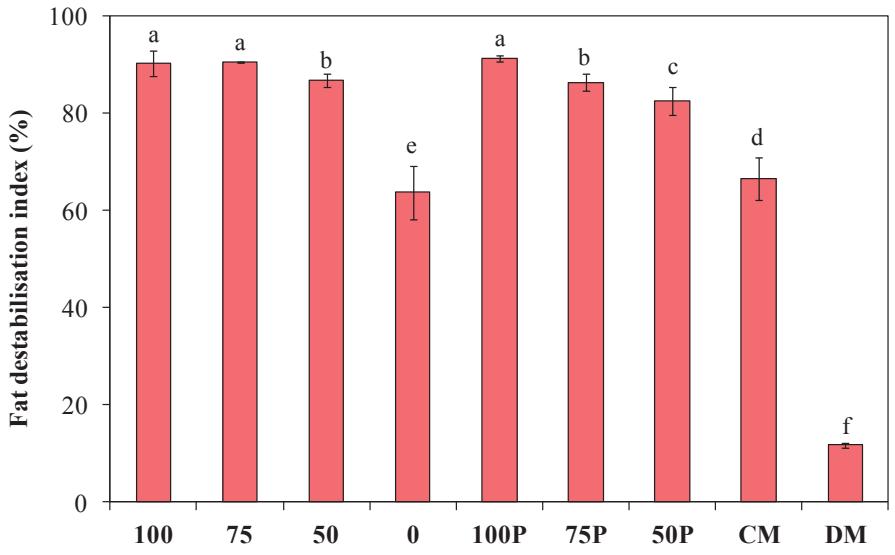

Figure 7.22 Amount of destabilised fat in ice creams. 100, 75, 50 and 0 represent the percentage of coconut oil; P represents coconut skim milk powder (CSMP) was added into ice cream mixes; CM and DM represent ice cream mixes made from coconut milk and dairy milk, respectively. Mean values with the same letter are not significantly different (P < 0.05).

As described earlier, a network of aggregated fat promotes the stabilisation of air cells in the ice cream. The fat clumps in the structures of ice creams based on CSM and CM as indicated by the FI values with all higher than 2 except for the ice cream containing 100% SO (Table 7.5) may explain that these ice creams had a good ability to entrap and

stabilise air cells into their ice cream structures, thus resulting in a a relatively high overrun compared to the ice cream containing 100% SO (Figure 7.17). The only one ice cream that did not follow this rule based on the FDI value (Figure 7.22) was an ice cream based on DM. Although a low magnitude of fat destabilisation was observed in the DM based ice cream but they had a high overrun. This may be due to the fact that milk proteins possess good foaming and emulsifying properties (Kuehler and Stine, 1974, Rouimi et al., 2005, Yu and Damodaran, 1991), therefore, a number of air cells could have been created and entrapped by flocculated fat clumps in the ice cream.

The FDI values were highly correlated, with the correlation coefficient (R^2) = 0.98, with the overrun of ice creams containing different ratios of CO and SO and without added CSMP, but a rather low correlation was established between ice creams with added CSMP. A high percentage of extractable fat has been described to indicate the presence of destabilised fat (Bolliger et al., 2000a, Koxholt et al., 2001, Pelan et al., 1997a, Segall and Goff, 2002). The FDI values obtained in this study were shown to be partly in agreement with the percentage of extractable fat. Between the ice cream samples without added CSMP, FDI was found to increase as the SEF content increased (R^2=0.96) while between samples with added CSMP, the FDI was inversely related to the SEF. The latter means that FDI increased as the SEF content decreased (R^2=0.97). This may be because the addition CSMP provided the oil droplets with more stability, thereby, less free fat extracted by a solvent. Although it is not clear, the cause of the increasing FDI values could be probably due to the hydrophobic interaction between oil droplets covered by the coconut skim milk proteins which are known as hydrophilic in their chemical properties (Kwon et al., 1996, Tangsuphoom and Coupland, 2009).

7.5 Conclusions

This study highlighted the effects of different solid fat contents on the properties of oil-in-water emulsions of ice cream mixes and ice creams prepared from CSM as a main base material. To study the effect of CSM protein concentrations, the freeze-dried CSMP was used to raise the protein concentration in the formulations from 0.32 to 1.12%. In ice cream making, during the ageing stage of the ice cream mixes, fat

droplets become crystallised and this leads to the presence of fat crystals in droplets containing solid fat. In ice cream mixes based on CSM, the ratios of solid to liquid fat affected the flowability of the aged mixes. The viscosity of the aged mixes increased when the mixes contained the solid fat content (SFC) greater than 68%, but had almost no effect on the increase in viscosity when the SFC of the mix formulation was less than 50%. No observed increase in viscosity was found in the ice cream mix containing 100% SO and dairy milk-based mixes. The increased viscosity also helped to reduce the phase separation of the ice cream mixes after ageing. Although the real droplet size was observed to be slightly different between the CSM-based ice creams containing fat blends, the difference in SFC had an effect on the apparent droplet size. The apparent droplets became bigger as the SFC increased. The high extent of flocculated droplets in the aged ice cream mixes was observed in the aged mix formulation containing 25% SO in the blend, suggesting the certain amount of liquid fat is needed to wet the protruding fat crystals which resulted in partial coalescence of the collided droplets.

After the aged mixes were churned into ice creams containing CO/SO blends at 75:25 and 50:50, the presence of liquid fat at 32% and 55%, respectively, in these samples, led to a high extent of aggregated fat clumps. Although the extent of fat clumps observed from the DM-based ice cream was higher than the CSM-based ice cream containing 50% SO, it was lower than the rate of fat clumps occurring in the ice cream containing 25% SO. This underlines the necessity of the liquid fat on the linkage of fat globules observed from the melted ice creams, which suggests it should be at least about 25%. The overrun of ice cream based on CSM increased as the liquid oil was involved into the formulation and became decreased with increasing liquid fat content in the formulation. The formulation of ice cream containing a 75:25 CO/SO blend had the highest overrun compared to the CSM-based ice cream containing other oil blend ratios and was incomparable with the DM and CM-based ice creams. Not only did the melting rate of these ice creams become slower than the ice creams made from DM and CM, these ice creams also exhibited better stand up property than the other two ice creams after melting for 90 min at 20°C.

The difference in the protein content of the ice cream mix formulations led to the differences in some properties of the aged ice cream mix and ice creams. The higher protein content led to the firmer structure of the ice cream mixes after ageing. In general, the emulsifying property of coconut skim milk proteins was lower than the dairy milk proteins. Ice cream mix with added CSMP showed less phase separation of the aged ice cream mixes, indicating it was attributed to the increased protein concentration by the addition of CSMP. In ice creams, the increased protein contents did not show a strong contribution to an increase in the overrun percentage but led to the slower melting rate of ice cream added with CSMP.

Chapter 8
Overall conclusions and recommendations

8.1 Conclusions

Several proteins are present in coconut milk, including 11S and 7S globulins and albumins. After homogenisation of coconut milk, the 11S globulins are reported to predominantly adsorb at the oil-water interface of emulsions in coconut milk, and albumins remain in the serum phase of the milk. In this study, both 11S and 7S globulins were observed at the interface although 11S globulins were the main proteins adsorbed on the surface of oil droplets. After homogenisation, more flocculated droplets were observed in the homogenised coconut milk and the particle size of oil droplets was also found not to be reduced by homogenisation pressures, suggesting the compositional and structural changes in the surface of original oil droplets causing the droplet aggregation. This may be possibly due to more exposure of hydrophobic sites of adsorbed proteins on the emulsion droplet surface which leads to an increase in hydrophobic interaction between the emulsion droplets.

The properties of oil-in-water emulsions based on coconut milk depend on the types of proteins present in coconut milk. Albumins which are the main proteins in coconut skim milk could form apparent smaller emulsion droplets with less flocculation than did globulins which are the proteins associated with the cream phase of coconut milk. In general, both albumin and globulin proteins could not provide a long-term stability to the emulsions stabilised by them, but the former proteins could render short-term stability to emulsion. It was also demonstrated that a high extent of flocculation in emulsions stabilised with proteins of coconut milk was driven by globulin proteins. Hydrophobic interaction is believed to be a driving force for aggregation between the oil droplets stabilised by globulin proteins.

The overall suggestion is that if small emulsion droplets with less flocculation are to be produced in emulsions, the proteins from coconut skim milk phase should be used and its concentration needs to be high enough to ensure that its emulsifying and stabilising capacities are not limited by the relatively low concentration of albumins in the skim milk phase, especially when the coconut skim milk liquid is used for making an emulsion containing a large volume of oil. This is also to ensure that the interfacial layer of albumins formed around the surface of oil droplets be rigid and thick enough to resist the break-down and disruption.

Properties of ice cream made from coconut milk depend largely on the amount of solid fat content. The too high or too low solid fat content in ice cream mixes renders aged mixes with too high or too low viscosity, respectively, and high or low solid-like properties, respectively, with less chance for inducing partial coalescence between fat droplets. This is important for the structural network formation of ice cream, e.g. a three-dimensional crystalline fat network. Although the inclusion of liquid oil in the range between 25 and 50% leads to a decrease in the viscosity and elastic properties of the resulting aged mixes, the chance for oil droplets to become partially coalesced is increased. The ice creams based on CSM with a high solid fat content showed a similar ability to incorporate air into their ice cream structures as did the ice cream made from dairy milk, resulting in a high overrun and a high extent of fat clumps. Those partially aggregated globules affected the melting property and shape retention of ice cream samples. Ice creams containing a blend of CO and SO at 75:25 (68% SFC) melted more slowly with less structural collapse than the dairy milk ice cream as well as the other CSM-based ice creams containing other solid fat contents.

8.2 Recommendations for future research

The research studies on coconut oil-in-water emulsions carried out in this project provided valuable insights into the properties and stability of coconut milk emulsion systems that are significantly affected by different proteins from various proteins present in coconut milk, mainly two types of coconut milk proteins, such as 11S globulins and albumins. In general, proteins provide the stabilising layer to emulsions droplets by steric and electrostatic stabilisation. A future research that needs to be considered is to investigate how the coconut proteins can be fabricated to provide steric and electrostatic stabilisation to the emulsion droplets of coconut oil in coconut milk. This will be worth investigating as this can have a significant impact on the utilisation of coconut milk proteins as functional proteins and make coconut milk as a more valuable raw material for its application in a range of food products. Also, a more extensive study on the composition, structure and thickness of the protective layer of oil droplets created by coconut proteins needs to be further investigated.

The oil blends of coconut oil and sunflower oil were used to prepare ice creams in this study. The results have shown that the properties of the resulting oil-in-water emulsions as well as the ice creams varied with oil blends with different ratios. However, the proportion of solid to liquid fat in different blends, in terms of solid fat content, was not demonstrated quantitatively and it needs to be further investigated. The current available methods to determine the amount of solid fat content at different temperatures are available, such as nuclear magnetic resonance (NMR) technique using the pulse NMR instrument and differential scanning calorimetry (DSC) technique.

The studies on the behaviours of emulsion droplets in the aged ice cream mixes and the melted ice creams were also conducted. The observation of droplets in the aged ice cream mixes was carried out by the light microscopy at ambient temperature. At this temperature, the properties of droplets may be altered from the original state in the aged mixes. Therefore, the observation of emulsion droplets at the same temperature as the droplets existed at 4^{o}C needs to be further conducted. In addition, the observation of ice cream structure has not been carried out due to a limitation of instrument availability.

The use of a microscopic technique at low temperature (e.g. below freezing point) would provide more valuable information about the structures of ice cream. For example, the observation of oil droplets in CSM or CM emulsions and the fresh and aged ice cream mixes as well as the structure of ice cream samples could be observed using a cryo-scanning electron microscopy (cryo-SEM) which would provide more information on the interaction between oil droplets. The structural network of ice cream could also been observed by this type of microscope.

Several standard analyses for ice cream samples, such as overrun and meltdown test, were carried out in this study. In addition to these measurements, the observation of the size of air cells and ice crystals by a means of microscopy needs to be further investigated. This may provide more information on the effects of the difference of ice cream formulations on the formation of air cells in ice cream samples.

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Appendix A
List of chemicals

A.1 Chemicals for sample preparation

- Sodium azide was purchased from Serva Electrophoresis GmbH, Heidelberg, Germany and used as antimicrobial agent.
- Sodium benzoate was purchased from Sigma Aldrich Chemical Co., St. Louis, MO, USA and used as antimicrobial agent.

A.2 Chemicals for Lowry method

- Whey protein isolate (ClearProtein^{TM} 895 WPI) (Fontera Co-operative group Ltd., Palmerston North, New Zealand). As stated from the manufacturer, WPI powder composed of 92% protein, 0.4% fat, 0.5% carbohydrate, 2.3% ash and 4.8% moisture.
- Folin-Ciocalteu's phenol reagent (2 N) (Sigma Aldrich Chemical Co., St. Louis, MO, USA)
- Potassium sodium tartrate tetrahydrate (Biolab (Aust) Ltd., Australia)
- Sodium hydroxide (Scharlab S.L., Sentmenat, Spain)
- Copper sulphate (Sigma Aldrich Chemical Co., St. Louis, MO, USA)
- Sodium carbonate (Scharlau Chemie S.A., Sentmenat, Spain)

A.3 Chemicals for protein determination by Kjeldahl method

- Kjeltabs (S/3.5; 3.5 g K_2SO_4 and 3.5 mg Se) (Foss Analytical AB, Hoeganaes, Sweden)
- Hydrochloric acid (Scharlab S.L., Sentmenat, Spain)
- L-Tryptophan (Sigma Aldrich Chemical Co., St. Louis, MO, USA)
- Sulphuric acid (Biolab (Aust) Ltd., Australia)
- Boric acid (Scharlab Chemie S.A., Sentmenat, Spain)

A.4 Chemicals for fat determination by mojonnier method

- Petroleum ether (Scharlab S.L., Sentmenat, Spain)
- Diethyl ether (Scharlab S.L., Sentmenat, Spain)

A.5 Chemicals for SDS-PAGE

- Molecular weight protein standard markers (Bio-Rad laboratories, Inc, Hercules, CA, USA)
- Acrylamide (99.9%, Bio-Rad laboratories, Inc, Hercules, CA, USA)
- 30% Acrylamide/Bis solution (37.5:1) (Bio_Rad catalogue no. 161-0158) (Bio-Rad laboratories, Inc, Hercules, CA, USA)
- Sodium dodecyl sulphate (SDS) (Fisher Scientific UK limited, Loughborough, UK)
- Tris base (Bio-Rad laboratories, Inc, Hercules, CA, USA)
- Glycerol (Biolab (Aust) Ltd., Australia)
- 2-mercaptoethanol (Scharlab S.L., Sentmenat, Spain)
- N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma Aldrich Chemical Co., St. Louis, MO, USA)
- Bromophenol blue (Bio-Rad laboratories, Inc, Hercules, CA, USA)
- Coomassie Brilliant Blue R-250 (Sigma Aldrich Chemical Co., St. Louis, MO, USA)
- Acetic acid (Biolab (Aust) Ltd., Australia)
- Methanol (Scharlab S.L., Sentmenat, Spain)

Appendix B
Preparation of reagents and standard solutions

B.1 Phosphate buffer (pH 6.2)

The phosphate buffer (pH 6.2) was prepared by dissolving 2.76 g of sodium dihydrogen orthophosphate monohydrate (Mw = 137.99 g/mol) and 2.84 g of sodium phosphate dibasic anhydrous (Mw = 141.96 g/mol) in distilled water in a volumetric flask and adjusting to pH 6.2 with 1N HCl and making it to the final volume of 1,000 ml with distilled water.

B.2 Reagents for Lowry method

- Complex-forming reagent: This reagent was prepared freshly every time before use by mixing the three stock solutions A, B and C at 100:1:1 (v:v:v), respectively.
  - Solution A: 2% (w/v) Na_2CO_3 in distilled water.
  - Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water.
  - Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

- 2N NaOH solution: NaOH (80g) was dissolved in 1000 ml distilled water.
- 1N Folin reagent: 2N Folin-Ciocalteu's phenol (10 ml) was diluted to 20 ml with distilled water.

- **Protein standard solutions**

The protein standard stock solution containing 4 mg/ml protein was prepared using whey protein isolate (WPI) by dissolving in distilled water. A series of standard WPI solutions with different concentrations were prepared by diluting the WPI stand stock solution with distilled water as shown in Table below.

Stock solution, µl	**0**	**1.25**	**2.50**	**6.25**	**12.5**	**25.0**	**32.5**	**125**	**250**
Water, µl	500	499	498	494	488	475	438	375	250
Protein, µl	0	10	20	50	100	200	500	1000	2000

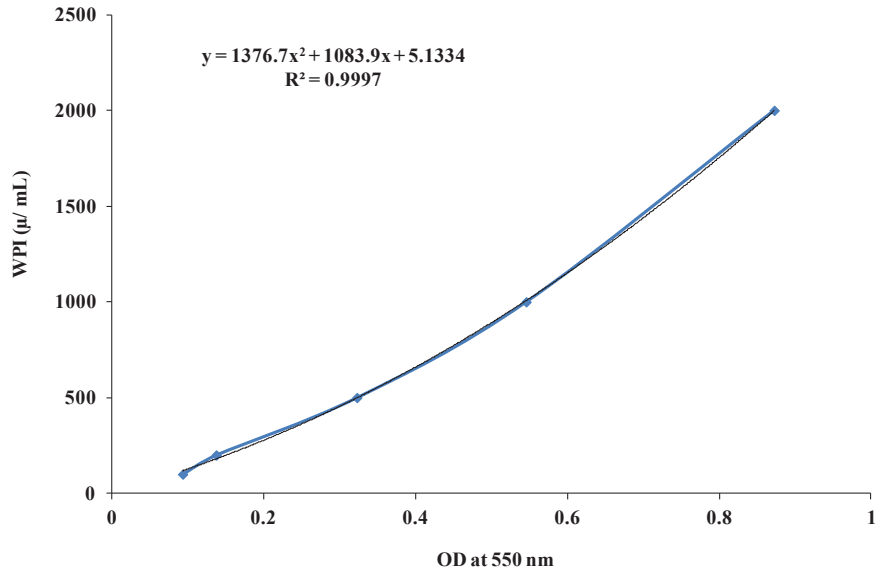

Figure A.1 Whey protein isolate standard curve

B.3 Reagents for Kjeldahl method

Sodium hydroxide solution
50% (w/w) NaOH solution was prepared by dissolving 50 g NaOH in 50 g distilled water.

Methyl red/ bromocresol green indicator solution
0.2 g methyl red was mixed with 100 ml in 95% ethanol. 1.0 g bromocresol green was dissolved in 500 ml 95% ethanol. One part of methyl red solution was then mixed with five parts of bromocresol green solution.

Boric acid solution
The 4% H_3BO_3 solution was prepared by dissolving 40 g H_3BO_3 in 500 ml distilled water and diluted to 1 litre of distilled water. Then, 3 ml of methyl red/bromocresol green solution was mixed with the H_3BO_3 solution.

Hydrochloric acid standard solution
The standard HCl solution at 0.1 M was prepared as follows. 43 ml of concentrated HCl was diluted to 5 L in distilled water. It was titrated against a standard alkali solution. The HCl standard solution was prepared to have the concentration in the range between 0.0995 M and 0.1005 M and used 0.1 M for the calculation of nitrogen percentage. The molarity of HCl was calculated using the following equation.

$$\text{HCl molarity} = \frac{\text{ml standard alkali x molarity of alkali}}{\text{ml HCl}}$$

B.4 Stock solutions and samples buffer for SDS-PAGE

Acrylamide/Bis solution: The solution of 30% acrylamide/Bis, 37.5:1 mixture (30% T, 2.67% concentration) was purchased from Bio-Rad (Bio-Rad, New Zealand) (Catalogue no. 161-0158).

10% (w/v) SDS solution: This solution was prepared by dissolving 10 g SDS (Mw = 28.38 g/mol) in 90 ml deionised water with gentle stirring and diluting to 100 ml in deionised water.

1.5 M Tris-HCl, pH 8.8: This solution was prepared by dissolving 18.15 g Tris base (w = 121.14 g/mol) in 80 ml deionised water with gentle stirring.

0.5 M Tris-HCl, pH 6.8: This solution was prepared by dissolving 6 g Tris base in 60 ml deionised water with gentle stirring. The solution was adjusted to pH 6.8 with 6 N HCl and made to 100 ml with distilled water.

10x Electrode (running) buffer, pH 8.3: The electrode buffer (10X) was prepared by dissolving 30.3 g Tris base, 144.0 g glycerol (Mw = 75.07 g/mol) and 10.0 g SDS in 500 ml deionised water and diluting to 1,000 ml with deionised water. Before each electrophoresis run, 50 ml of 10x electrode buffer was diluted to 500 ml with deionised water.

10% APS solution: This solution was prepared by dissolving 100 mg ammonium persulphate in 1 ml deionised water. This solution was prepared freshly before each use.

SDS sample buffer: The SDS sample buffer was prepared by mixing 3.55 ml deionised water, 1.25 ml 0.5M Tris-HCl, pH 6.8, 2.5 ml glycerol, 2.0 ml 10% (w/v) SDS and 0.2 ml 0.5% (w/v) bromophenol blue and then stored at room temperature. Before use, 50 µl of 2-mercaptoethanol was added into 950 µl sample buffer.

B.5 Preparation of SDS-PAGE gels

1. The first step was to prepare a 12% resolving gel. The stock solutions in Table below, except APS and TEMED, were mixed first. Then, APS and TEMED were added and mixed thoroughly just before pouring it into a gel cassette.

Volumes of stock solutions used to prepare the stacking and resolving gel

Stock solution	Resolving gel (12%)	Stacking gel (4%)
30% Acrylamide/bis	4.0 ml	1.32 ml
0.5 M Tris-HCl, pH 6.8	-	2.52 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	-
10% SDS	100 µl	100 µl
Deionised water	3.35 ml	6 ml
TEMED	5 µl	10 µl
10% APS	50 µl	50 µl
Total volume	10 ml	10 ml

2. The solution was poured into a gel cassette up to the height of approximately 1 cm below the well of the comb.
3. The resolving gel solution was immediately overlaid with distilled water. Then, it was allowed to polymerise for at least 45 min. After polymerisation, the water was drained off and the top surface of the resolving gel was washed with distilled water.
4. After washing and removing the water from the resolving gel, a gel comb was placed in the gel cassette in a tilt position with a slight angle.
5. The next step was to prepare a 4% stacking gel by combining all stock solutions shown in Table above and mixing well before gently pouring on top of the resolving gel until it reached the top of the gel cassette and covered all the comb teeth.
6. The comb was properly aligned down into the gel cassette.
7. The stacking gel was left to polymerise for 30-45 min.
8. The comb was taken off and the sample buffer was poured on top of the stacking gel.