Virgin Coconut Oil Prepared by Protease-Assisted Process: Characteristics and Application

Umesh Bapurao Patil

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Coconut milk and meat at three different maturity stages including immature coconut (IMC), mature coconut (MC) and overlay mature coconut (OMC) had varying proximate compositions. Compositions of coconut milk generally were in accordance with those found in coconut meat. Cocosin with molecular weight of 55 kDa was observed as the major protein in all coconut milks but its band intensity slightly decreased with increasing maturity stages. Oil droplet size increased as maturity stages increased. Nevertheless, virgin coconut oil (VCO) extracted from coconut with three different maturity stages had no impact on fatty acid composition and physicochemical properties. VCO separated using Alcalase showed the highest recovery (95.64%) when coconut milk from OMC was used as starting material. All VCO samples had water-like appearance and contained medium chain fatty acid (MCFA), especially lauric acid as a major fatty acid, (49.74-51.18 g/100g). Myristic acid in the range of 18.70-19.84 g/100g was present in all VCO. All VCO samples had low lipid hydrolysis and oxidation, indicating that maturity stages had no influence on oil stability.

Albumin and globulin were the predominant protein fractions in defatted coconut meat. Both fractions showed the differences in protein patterns and amino acid compositions. Varying emulsifying property was obtained between both fractions. Albumin, water-soluble protein fraction, exhibited lower emulsifying properties, compared to globulin (salt-soluble) counterpart. However, globulin fraction was more susceptible to hydrolysis by Alcalase, leading to the higher collapse of emulsion of coconut milk after being hydrolyzed. This contributed to the higher oil recovery from coconut milk.

Difference was observed in degree of hydrolysis (DH), oil recovery, microstructure and protein pattern of coconut milk hydrolyzed by partially purified protease from seabass pyloric caeca (PPSP) and commercial trypsin (CT) at different proteolytic

levels (5 and 10 units/g protein) at 60 °C for various hydrolysis times (0-150 min). The highest VCO yield (77.35%) was found when sample was hydrolyzed by PPSP (10 units/g protein) for 150 min. Based on DH and electrophoretic study, proteins in coconut milk were more prone to hydrolysis by PPSP, compared to CT. Therefore, PPSP could be used as an alternative processing aid and the efficiency was higher than CT.

PPSP was further used in combination with different treatments including micro-fluidization, chill-thawing and freeze-thawing for extraction of VCO. Coconut milk hydrolyzed by PPSP at 10 units/g protein, followed by freeze-thawing showed the highest yield among other samples ($p<0.05$). Conversely, the lowest VCO yield was attained for coconut milk homogenized at 4000 psi, followed by hydrolysis using PPSP (5 units/ g protein). Hydrolysis by PPSP, followed by freeze-thawing of 5 cycle rendered the highest yield of VCO (98.6%). However, no marked difference was observed in fatty acid profile, moisture content, free fatty acid content (FFA) and oxidative stability among all VCO extracted from aforementioned methods.

Because of high stability and various health benefits, VCO in combination with fish oil (FO) rich in n-3 fatty acids at different ratios (95:5, 90:10, 85:15, v/v) was used to prepare a functional mayonnaise. Chemical and physical changes were monitored during the storage of 30 days at room temperature (30-32 °C) in comparison with those of mayonnaise prepared using soybean oil (SO). Addition of FO up to 10% in VCO/FO blend could yield the mayonnaise with sensorial acceptability. Oxidative stability varied with mayonnaises containing different oils. Mayonnaise sample with VCO was less prone to lipid oxidation throughout storage of 30 days. Types of oil used for preparation of mayonnaise and storage time affected the color, textural and rheological properties of resulting mayonnaise. In general, mayonnaise containing VCO/FO (90:10) blend showed the property equivalent to that prepared using SO. Thus, VCO could be incorporated in combination with FO at appropriate level to prepare a functional mayonnaise with acceptability and oxidative stability.

Therefore, VCO could be successfully produced with the aid of fish trypsin in conjunction with repeated freeze-thawing cycles. The resulting VCO could be employed as food ingredient or other applications.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Coconuts (*Cocos nucifera* L.) are economically important and extensively used in many traditional foods of Asian and Pacific regions (DebMandal and Mandal, 2011). Coconut milk, which is a milky white oil-in-water emulsion, is generally obtained from grated coconut meat with or without added water. It has been used as a major ingredient for several cuisines such as curries and desserts (Tansakul and Chaisawang, 2006). Emulsion stability is crucial for the quality of coconut milk and it is governed by some proteins in aqueous phase (Peamprasart and Chiewchan, 2006). On the other hand, destabilization of coconut milk emulsion is required for the production of virgin coconut oil (VCO). VCO is generally extracted from wet coconut kernel (meat) by mechanical or natural means, with or without the use of heat. Chemical refining, bleaching or de-odorizing processes are not required. As a consequence, the nature of oil is not altered and is known VCO (Villarino *et al.*, 2007). Destabilization of coconut milk emulsion using different methods has been implemented (Raghavendra and Raghavarao, 2010). Thus, to maximize the yield of VCO, the emulsion of coconut milk must be collapsed to a high degree, in which oil can be released and separated effectively. To obtain VCO from wet extraction process, destabilization of coconut milk emulsion using different extraction methods has been implemented such as physical extraction, fermentation and enzymatic extraction. For physical extraction, VCO is obtained directly from coconut milk by pH adjustment, chilling-thawing, etc., the oil recovery is 62% and 92%, respectively. For fermentation process, microbial flora are allowed to ferment the coconut milk, in which the coconut oil will be separated into the top layer within 24-48 h. Some microorganisms may spoil the coconut milk, resulting in poor quality of VCO. The disadvantages of this process are low oil recovery (26%) and fermented odor, which mask the characteristics of coconut flavor of the oil (Raghavendra and Raghavarao, 2010). Amongst all processes, the enzyme-assisted separation process has been known to be effective and less time consuming. Additionally, high yield (98%) could be obtained from coconut milk with the aid of proteases such as Alcalase (Senphan

and Benjakul, 2016). The efficiency of enzyme in extraction of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu *et al.*, 2008). To provide the substrate available for hydrolysis, pretreatment such as high-pressure homogenization can be applied. As a result, the higher surface area of oil droplets where surrounding protein membranes can be cleaved more easily and effectively by proteases. Chill-thawing is important to enhance destabilization of the enzyme treated coconut milk emulsion. In which, high yield with prime quality of VCO could be achieved (Raghavendra and Raghavarao, 2010).

Protease, especially from microorganism, have been widely employed. Despite their availability, the cost is still one of the major concern for application, particularly for VCO production. Thus, the alternative and cheap protease are of attention, in which the operation cost can be reduced. Fish visceral proteases can be recovered with ease and introduced as the aid for VCO extraction. Overall, several proteases have been extracted from fish viscera mainly trypsin and pepsin (Klomklao *et al.*, 2007; Hoque *et al.*, 2010; Senphan and Benjakul, 2016). Thus, the proteases from cheap sources, especially fish processing byproducts, e.g. viscera can be alterative and promising for production of VCO and the cost of commercial protease can be reduced.

VCO is the purest form of coconut oil with natural distinctive coconut taste and smell. It is solidified at low temperature and becomes colorless like water when liquefied (Marina *et al*., 2009). VCO is different from other vegetable oils due to high content of medium chain fatty acids (MCFAs), mainly lauric acid (Dayrit, 2014). MCFAs are burned up immediately after consumption and therefore the body uses it instantly to make energy, instead of storing it as body fat (Enig, 1996). Lauric acid is converted into very valuable compound known as monolaurin, which has antiviral and antibacterial properties (DebMandal and Mandal, 2011). VCO is rapidly gaining an immense importance due to various health benefits and high stability (Carandang, 2008). The consumption of VCO may help to protect the body from infections. As VCO has many health benefits, it can be used as ingredient food system or fortified in some particular foods to bring about the health benefit. Additionally,

the indigenous antioxidants in VCO can contribute to prevention of lipid oxidation in foods, especially those containing PUFA (polyunsaturated fatty acid) susceptible to oxidation. To fulfil the extraction of VCO with prime quality and high yield, the combined methods should be developed, in which the shorter processing time and lower cost can be achieved. The information gained will be useful for VCO production, in which the effective extraction method of VCO can be gained. The application of VCO as ingredient and exploitation of its unique property can lead to the new products with desired characteristics.

Fish oil (FO) has been well known for health-promoting benefits. Therefore, health advisories recommend a higher intake of fish oil rich in polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hartvigsen *et al.*, 2000). Incorporation of PUFAs into foods can be of consumers' health benefit (Gorji *et al.*, 2016). Mayonnaise, as an oil in water emulsion, is a highly desirable salad dressing and widely consumed because of its desired flavor and texture (Rahmati *et al.*, 2015). Therefore, incorporation of VCO in combination with FO could be a means to prepare a functional mayonnaise for health benefits. The balance between saturated and unsaturated oil would achieve both targets: health promotion and oxidative stability of resulting mayonnaise.

1.2 Literature Reviews

1.2.1 Coconut Palm

Coconuts (*Cocos nucifera* L.) are monocotyledon palms from *Palmaceae* family (Ohler, 1999). It is commonly known as a coconut palm and is one of the most useful tree in the world. The coconut palm called as “tree of life” because of the many useful products at domestic, commercial and industrial levels. The well-known products include coconut water, coconut meat, coconut milk and coconut oil (Green and Mundial, 1991). It is estimated that about 70% of the coconuts are used for domestic consumption, in which over half of the produces is consumed fresh (Green and Mundial, 1991). The edible coconut products are mostly obtained from meat (solid endosperm) and water (liquid endosperm) (Grimwood, 1975). Coconut is also an important source of traditional medicine, crafting material and fuel (Morrison

et al. 1994). They are the most cultivated palms and economically important in all tropical regions around the world (Woodroof, 1979; Green and Mundial, 1991). Asia is the biggest coconut producer in the world and ninety percent of the world's total coconuts are cultivated in Indonesia, Philippines, India, Sri Lanka and Thailand (Maneepun *et al.*, 1988).

1.2.2 Classification / feature

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Subclass: Arecidae
Order: Arecales
Family: Arecaceae
Genus: *Cocos* L.
Species: *Cocos nucifera* L.

Coconut palms are medium-sized, solitary herbaceous plants. Although they are treelike in form, their trunks are not composed of wood, but of fibrous, stout, overlapping stems and may grow to 25 m tall (80 feet), topped by a crown of pinnately compound leaves up to 4 meters (15 feet) long (Ohler, 1999).

1.2.3 The fruit

Coconut fruit is considered most important part for human consumption. The entire development of fruit takes about one year (Ohler, 1999). First, the husk and shell grow mainly in size, not in thickness, and cavity of embryosac enlarges considerably. This cavity is filled with liquid. After about four months, the husk and shell become thicker. After six months, the solid endosperm begins to form against the inner wall of the cavity. This first layer is thin and gelatinous. About eight months later, the soft white endocarp starts hardening and turning dark brown. The fruit becomes mature within 12 months (Ohler, 1999).

The mature coconut fruit (about 12 months) contains 35% coir (middle fibrous coat of fruit), 12% shell (inner hard coat of fruit), 28% meat (solid endosperm) and 25% water (liquid endosperm) (Grimwood, 1975). Cross section of a coconut is shown in Figure 1.

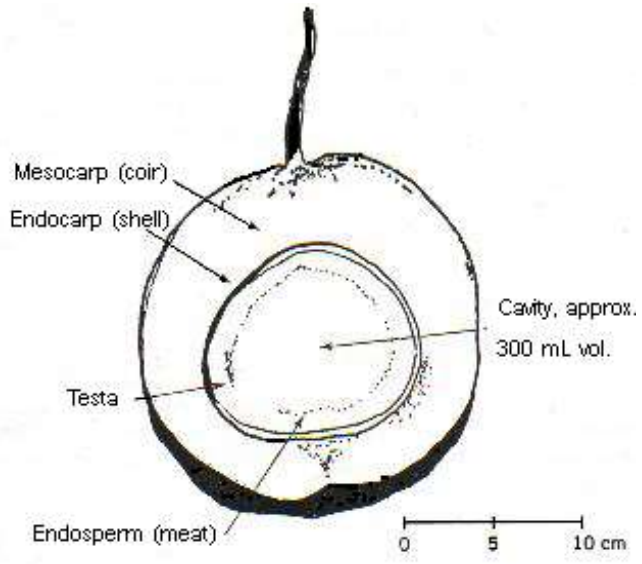

Figure 1. Cross section of a coconut
Source: Onsaard (2008)

1.2.4 Proximate composition of coconut meat

To obtain coconut meat, coconut is subjected to deshelling, paring and removal of water. Coconut kernel can be collected manually and grated using a rotary wedge cutter machine (Senphan and Benjakul, 2015). The composition of the mature kernel is dependent on variety, geographical location, cultural practices and maturity of the nut (Woodroof, 1979; Ohler, 1984). In general, it is rich in fat or oil. Proximate compositions of mature coconut meat are listed in Table 1. Coconut meat can be consumed fresh or grated, mixed with hot water and pressed to extract the coconut milk (Cancel, 1970).

1.2.5 Coconut milk

Coconut milk has been used as an important ingredient for Asian cuisine as well as in other parts of the world due to its unique flavor and other

desirable sensory characteristics. It has been estimated that 25% of the world's output of coconuts is consumed as coconut milk (Seow and Gwee, 1997).

Table 1. Proximate composition of mature coconut kernel

Composition						References
Moisture	**Protein**	**Oil**	**Crude fiber**	**Ash**	**Carbohydrates**	
44.0	3.6	38.1	3.1	1.3	9.9	Dendy and Timmins (1973)
42-48	4	36	2	-	7-20	Grimwood (1975)
35.37	5.5	44.01	3.05	0.77	14.35	Balachandran *et al.* (1985)
36	4.5	41.5	3.5	1.1	16.9	Chakraborty (1985)
40.9	3.8	35.2	-	-	-	Kwon *et al.* (1996)

1.2.5.1 Extraction of coconut milk

Coconut milk is generally produced from mature nuts of about twelve months in age of which the meat is hard and thick. The process begins with deshelling of fully mature coconuts followed by removing the thin brown layer that covers the kernel, i.e., testa. If present, it will impart a brown color and a slight bitter taste to the extracted milk (Seow and Gwee, 1997). Coconut milk is a milky white oil-in-water emulsion and can be obtained by extraction from grated coconut meat with or without added water (Seow and Gwee, 1997). Coconut milk can be prepared at home from grated meat by squeezing with hand, while industrial or commercial scale employs the hydraulic or screw press to press the coconut meat to obtain milk (Seow and Gwee, 1997). The efficiency of extraction as well as the composition of the coconut milk from coconut meat is varied with operation parameters such as the temperature of added water and the pressing condition (Grisingha, 1991). The difference in the water: coconut meat ratio, ranging from 1:1 to 20:1, had no effect on oil and protein extraction into coconut milk (Dendy and Timmins, 1973). Thungkao (1988) also found that protein contents were not affected by the variation of temperature used for coconut milk extraction (30, 55 and 80 ºC), when the grated coconut meat and water ratio of 1:1 was used. Nevertheless, the fat content of the coconut milk extracted at 55 ºC was highest, while those of coconut milk extracted at 30 ºC and 80 ºC were not

significantly different. Grisingha (1991) compared the oil and protein extractability in coconut milk prepared using three different methods and found that the fat and protein contents of the extracted coconut milk were not significantly different. The extraction of coconut milk is the first important step in the aqueous or wet processing of fresh coconut, which is an alternative method to traditional mechanical pressing of copra for the recovery of oil (Seow and Gwee, 1997). In this case, breakage of the emulsion is crucial to the efficient recovery of both oil and protein.

1.2.5.2 Composition and properties of coconut milk

The composition of coconut milk is much dependent on that of the coconut meat used for extraction. The solid endosperm of coconut is an abundant source of fat. Coconut milk is a complex oil-in-water (O/W) emulsion, which separates into two distinct phases, a lighter cream phase and a heavy aqueous phase (coconut skim milk) (Seow and Gwee, 1997). Coconut milk contains both water-soluble and water-insoluble components. The water-soluble component consists of sugar (generally sucrose), salt (mainly potassium salts) and soluble proteins, whereas the water-insoluble component consists principally of oil (Hagenmaier, 1977). The proximate compositions of coconut milk without any addition of water are given in Table 2.

Table 2. Proximate composition of coconut milk without addition of water.

Composition					References
Moisture	**Protein**	**Oil**	**Ash**	**Carbohydrates**	
50.0	2.8	39.8	1.2	6.2	Nathaneal (1954)
54.1	4.4	32.2	1.0	8.3	Seow and Gwee (1997)
50.0	3.0	40.0	1.5	5.5	Jeganathan (1970)
53.9	3.6*	34.7	1.2	6.6	Anon (1984)

*Conversion factor = 5.3

The size distribution of the oil droplets in coconut milk has been known to govern the characteristics of coconut milk. Dendy and Timmins (1973)

found that the diameter of the oil droplets was in the range of 0 - 35 μm and most oil droplets had diameter below 5 μm. Hagenmaier (1977) reported that the coconut milk obtained by hand squeezing through three layers of cheesecloth had pH of 6.5 and average diameter of oil globules was 10 μm. Some physicochemical properties of coconut milk are shown in Table 3 (Buccat *et al.*, 1973).

Table 3. Physicochemical properties of coconut milk

Physical properties	**Range**
Viscosity (mPa.s)	1.61-2.02
Specific gravity	1.0029-1.0080
Surface tension (dynes/cm^2)	97.76-125.43
Refractive index	1.3412-1.3446
pH	5.96-6.30

Coconut milk was extracted using water/coconut meat ratio of 1:1 (v/v)
Source: Buccat *et al.* (1973)

1.2.5.3 Stability of coconut milk emulsion

Coconut milk is naturally stabilized by proteins and phospholipids (Birosel *et al.*, 1963; Monera and Del Rosario, 1982). Hydrophilic and hydrophobic groups in these molecules are more likely to be adsorbed at the oil-water interface, thereby enhancing the stabilization of emulsion (Monera and Del Rosario, 1982). The oil droplets in coconut milk emulsion are surrounded by a film of interfacial active protein (Dendy and Timmins, 1973). Peamprasart and Chiewchan (2006) reported that some proteins present in the aqueous phase of the coconut milk emulsion could act as emulsifier to stabilize oil droplets. Proteins present in aqueous phase can lower the interfacial tension between two phases and promote the dispersion of the oil droplets in the aqueous phase. Hydrophobic domains of the proteins were able to interact with hydrocarbon chains on fatty acids, thus promoting physical entrapment of oil (Al-Kahtani and Abou-Arab, 1993). The freshly prepared coconut milk appears stable and homogenous. However, it is not physically stable and is prone to phase separation after a few hours. Cream separates from the aqueous phase within 5 to 10 h of

manufacture (Seow and Gwee, 1997). Droplet of the dispersed phase will be attached to each other, which will result in the separation of cream from aqueous phase. The separated milk, however, can be easily rehomogenized by shaking (Escueta, 1980). The major reason for the instability of coconut milk emulsion is the poor emulsifying properties and low surface activity of coconut proteins (Monera and Del Rosario, 1982). Onsaard *et al.* (2006) reported that proteins isolated from coconut skim milk are effective stabilizing emulsions that are fairly viscous, when droplet flocculation and creaming are not major problems. The lower efficacy of the proteins extracted from coconut cream was observed, compared to whey protein isolate, in either creating small droplets by the homogenizer or preventing droplet aggregation during or after homogenization to obtain a stable emulsion (Onsaard *et al.*, 2006). Moreover, the emulsifying properties of coconut proteins were affected by pH, ionic strength and especially temperature (Gonzales and Tanchuco, 1977; Kwon and Rhee, 1996; Onsaard *et al.*, 2005; Onsaard *et al.*, 2006). The coconut milk was also found to be poorly stable over the pH range of 3.5 to 5 and to exhibit two stability maxima at pH 1.5 to 2 and pH 6.5 (Monera and Del Rosario, 1982). In coconut milk, oil droplet size and pH are the most important factors that affect the emulsion stability. Coconut milk emulsion can be separated by adjusting pH of the coconut milk emulsion between pH 3 and 5.6 (Marina *et al.*, 2009). Raghavendra and Raghavarao (2010) studied the destabilization of coconut milk emulsion at different temperature and pH levels. It was found that coconut milk emulsions were highly unstable at pH 3-6 and at pH 7-8. Coconut milk proteins are easily coagulated and precipitated at pH 4 (Tangsuphoom and Coupland, 2008). Since proteins have polar groups, their intra and inter-molecular interaction are expected to be affected by changes in pH of emulsion. Furthermore, heating the coconut milk at higher temperature causes protein denaturation. Coconut proteins have been shown to denature and coagulate at 80 °C and higher temperature (Kwon *et al.*, 1996; Raghavendra and Raghavarao, 2010). Aggregation of oil droplets occurs due to thermal denaturation of proteins stabilizing coconut milk emulsion which in turn affect the surface charge of oil droplets. Droplets with lower surface charge interact each other and coalesce into larger ones. This results in unstable coconut milk emulsion. This will affect their emulsifying capabilities. The rate of emulsion destabilization is directly proportional to the oil droplet size. Processing

operations, which tend to produce smaller globules are expected to result in more stable emulsion (Onsaard *et al.*, 2005). Iswarin and Permadi (2012) studied the effects of ultrasound on coconut milk homogenization. Ultrasonic treatment with high power level (7 watts for 25 min) has an important effect on coconut milk homogenization and is an effective technique for the reduction of the fat globule size up to 3.64 μm. Effect of reduction in fat globule size represents the symptom of cavitation phenomenon (Iswarin and Permadi, 2012).

1.2.6 Coconut proteins

Apart from oil, coconuts is also served as a potential source of proteins with good nutritive value and relatively well balanced profile (Kwon *et al.*, 1996). Isolation and concentration protein from coconut skim milk were carried out by heat coagulation, isoelectric precipitation, salt precipitation, and ultrafiltration (Buccat *et al.*, 1973; Capulso *et al.*, 1981; Raghavendra and Raghavarao, 2010).

1.2.6.1 Extraction and isolation of coconut proteins

Different methods for isolation of protein from coconut skim milk including heat coagulation, isoelectric (pI) precipitation, combined isoelectric precipitation and heat coagulation, and co-precipitation with calcium salt have been used (Buccat *et al.*, 1973). Higher protein yield was obtained by heat coagulation than by pI precipitation and the combined technique. Similarly, Capulso *et al.* (1981) studied the effect of heat coagulation, isoelectric precipitation and simultaneous pH and heat coagulation on recovery of coconut proteins from skim milk. It was found that 84% of protein in the skim milk was precipitated with HCl at pH 4 and further precipitated by heat coagulation at 90 ºC for 30 min. Two major steps are involved in extraction of proteins by wet process; firstly, the extraction of an emulsion (coconut milk) from the coconut meat, and secondly, the breaking of this emulsion to separate oil and protein components. Oil and protein are separated from cream by a breaking down of the emulsion using different treatments (Raghavendra and Raghavarao, 2010).

1.2.6.2 Fractionation of coconut proteins

The Osborne classification is used to classify protein according to their solubility in various solvents. Coconut proteins are generally classified according to their solubility and amino acid composition (Rasyid *et al.*, 1992). Based on their solubility characteristics, the predominant proteins in coconut endosperm are classified as globulins (salt-soluble) and albumins (water-soluble), which account for 40% and 21% of the total protein, respectively (Samson *et al.*, 1971; Kwon *et al.*, 1996). Distribution of proteins in defatted coconut meal according to solubility is shown in Table 4. For protein content in coconut skim milk, 75% is accounted for globulin, while the remaining 25% is albumin (Garcia *et al.*, 2005). The large fraction of globulin in coconut proteins has high level of charged amino acids, i.e., glutamic acid, arginine, aspartic acid, and lysine (DeMason and Sekhar, 1990; Kwon *et al.*, 1996). The albumin fraction contains higher proportions of polar side chains. The relative proportion of each fraction affects the nutritional quality and the functional properties of the coconut protein (Johnson and Lay, 1974).

Table 4. Distribution of proteins in defatted coconut meal according to solubility

Fraction	Extraction solvents	Samson *et al.* (1971)	Kwon *et al.* (1996)	Sringam (1997)
Albumins	Water	30.6	21.0	22.7
Globulins	NaCl	61.9	40.1	46.1
Prolamines	Isopropyl alcohol	1.1	3.3	2.0
Glutelins-1	Glacial acetic acid	4.7	14.4	12.5
Glutelins-2	NaOH	-	4.8	1.2
Unextractable protein	Residue	-	16.4	13.4

1.2.6.3 Characterization of coconut proteins

Molecular weight of five protein fractions (albumin, globulin, prolamin, glutelin-1 and glutelin-2) from defatted coconut flour analyzed by SDS-PAGE with reducing agent (β-mercaptoethanol) was reported by Kwon *et al.* (1996) and Sringam (1997). The albumins fraction had molecular weight ranging from 18 to

52 kDa. Molecular weight of globulins fraction was below 60 kDa. Prolamin fraction had molecular weight ranging from 17 to 56 kDa, while the glutelins-1 fraction had molecular weights ranging from 14 to 100 kDa. Coconut globulins are consists of two major types, i.e., 11S and 7S globulins. The majority of the globulins in coconut endosperm are cocosin, i.e., one of a large class of seed storage proteins known as 11S globulin, which constitutes about 86% of the total globulins (Balasundaresan *et al.*, 2002). Cocosin is generally hexameric quaternary in structure, of which the molecular weight was about 300 to 360 kDa, with each subunit of 55 kDa. The subunits consist of the acidic (32 to 34 kDa) and basic (22 to 24 kDa) polypeptides linked by a disulfide bridge. Under reducing conditions, the acidic and basic chains are dissociated (Carr *et al.*, 1990; Garcia *et al.*, 2005). The 7S coconut globulin is a type of vicilins, which are characterized by a trimeric organization with an oligomeric molecular weight of 150 to 190 kDa, with each single chain subunit of about 55 kDa (Garcia *et al.*, 2005). The coconut 7S globulin is unglycosylated and lack of sulfur-containing amino acids (Carr *et al.*, 1990).

The molecular weight of coconut proteins has also been determined by size exclusion chromatography. The coconut protein can be classified as low molecular weight (LMW) and high molecular weight (HMW) fractions by a chromatographic analysis using Sephadex G-200 column and 0.95 M NaCl in 0.01M Na_2HPO_4 (pH 8.2) as elution buffer (Hagenmaier *et al.*, 1972). The HMW (150 kDa) and the LMW (24 kDa) were found approximately 84% and 14% of the total proteins, respectively. Kwon *et al.* (1996) separated the major protein fractions (albumin and globulin) from defatted coconut flour using Sephadex G-200 column and found that the albumin is separated into two major peaks of about 141 and 12 kDa and one minor peak of 27 kDa. The globulins showed five peaks with molecular weights of 186, 120, 46.7, 21.4, and 14.6 kDa, respectively.

1.2.6.4 Amino acid composition of coconut proteins

Coconut proteins generally provide good nutritional value with a relatively balanced amino acid profile (Gunetileke and Laurentius, 1974; Gonzales and Tanchuco, 1977; Chakraborty, 1985; Rasyid *et al.*, 1992; Kwon *et al.*, 1996).

Those proteins contain high amount of essential amino acids with a high biological value of 71% to 77% and a digestibility of 86% to 94% (Hagenmaier *et al.*, 1974). The most limiting amino acids of coconut skim milk are isoleucine, methionine, threonine, and tryptophan (Hagenmaier *et al.*, 1975). The amino acid compositions of three coconut protein fractions and coconut flour compared with Food and Agriculture Organization (FAO) amino acid scoring are shown in Table 5. In general, coconut proteins contain relatively high level of glutamic acid (17.0-27.2%), arginine (14.2-17.9%) and aspartic acid (5.6-8.9%) but are deficient in methionine (1.2-2.9%). Most amino acid levels are lower in the albumin fraction, except lysine, arginine and glutamic acid, which are higher than those found in globulins and glutelins-1 fractions. The coconut globulin contains higher content of essential amino acids including phenylalanine and valine but contains less glutamic acid, lysine, and arginine than the albumin (Kwon *et al.*, 1996). The leucine and phenylalanine of globulin fraction are comparable to those guided by FAO, while the globulin and glutelin-1 fractions have higher valine content. Methionine, cysteine and threonine appeared to be limiting amino acids for coconut proteins (Kwon *et al.*, 1996).

1.2.6.5 Functional properties of coconut proteins

Functional properties of coconut proteins depend strongly on their solubility (Chakraborty, 1985). The solubility of coconut proteins is generally low between pH 4 and 5, and is increased on both side of the pH scale (Gonzales and Tanchuco, 1977). The proteins of coconut endosperm from different regions were reported to have different solubility (Balachandran *et al.*, 1985), associated with different amino acid profiles. The minimum solubility of major protein components of coconut skim milk, coconut protein isolate and the extracts of coconut endosperm was observed between pH 4 and 5, which is the range of isoelectric point of those proteins (Hagenmaier *et al.*, 1972; Hagenmaier *et al.*, 1974; Gonzales and Tanchuco, 1977; Balasubramaniam and Sihotang, 1979; Kwon and Rhee, 1996), while the maximum solubility was reported at pH 10.3 (Balasubramaniam and Sihotang, 1979).

Table 5. Amino acid composition of three major protein fractions and coconut flour

Amino acid (g/100g of protein)	Albumins	Globulins	Glutelins-1	Coconut flour	FAO*
Isoleucine	2.8	4.1	3.7	4.2	-
Leucine	3.9	6.5	6.5	7.4	7.0
Lysine	5.1	3.5	3.5	4.7	5.5
Methionine	1.2	2.9	2.1	1.8	3.5
Phenylalanin	2.7	5.9	4.6	5.1	6.0
Tyrosine	3.0	3.7	3.1	1.8	-
Threonine	3.3	3.3	3.2	2.5	4.0
Trytophan	-	-	-	-	1.0
Valine	3.5	7.5	6.7	5.4	5.0
Histidine	1.8	1.9	1.9	1.8	
Aspartic acid	5.6	8.9	8.3	9.3	
Proline	2.7	3.4	3.2	3.6	
Serine	3.1	5.0	3.9	5.3	
Glutamic acid	24.5	17.5	17.0	22.4	
Glycine	4.0	4.9	4.5	5.1	
Alanine	2.9	4.1	3.9	4.8	
Arginine	17.9	15.0	14.2	12.3	

*Value guided by FAO

1.2.6.6 Thermal property of coconut proteins

Coconut proteins have been shown to be highly sensitive to temperature. They denature and coagulate upon heating to 80 ºC (Gonzalez *et al.*, 1990; Kwon *et al.*, 1996). Differential scanning calorimetric studies of raw undiluted coconut milk revealed several endothermic transitions over the temperature range of 80 ºC to 120 ºC, which reflects the complex protein composition and varying thermal denaturation behavior of the coconut proteins (Seow and Goh, 1994; Kwon *et al.*, 1996). The exposure to high temperatures for long time results in denaturation and precipitation of proteins in the coconut milk. The denaturation of coconut protein by heat is accelerated at the acidic and basic pH regions (Onsaard *et al.*, 2005). However, coconut protein is more resistant to heat denaturation in the presence of sugars, polyols, and salts (Seow and Goh, 1994).

1.2.7 Virgin coconut oil (VCO)

Coconut oil is generally extracted from wet coconut kernel (meat) by mechanical or natural means, with or without the use of heat. Chemical refining, bleaching or de-odorizing processes are not required. Therefore, the nature of oil is maintained and is known as virgin coconut oil (VCO). Coconut oil is the healthiest oil on earth (Aakansha *et al.*, 2010). VCO mainly consists of medium chain triglycerides and exhibits good digestibility (Man and Manaf, 2006). Purest form of coconut oil is visualized as water clear or colorless. It contains natural Vitamin E and has not undergone any hydrolytic and atmospheric oxidation as demonstrated by its very low FFA content and low peroxide value (Marina *et al.*, 2009). It has a fresh coconut aroma that can be mild to intense, depending on the oil extraction process used. Coconut oil is generally used as frying and cooking oil because of its excellent resistance to rancidity development.

1.2.7.1 Advantages of VCO

VCO is gaining popularity as a functional oil with increasing public awareness (Marina *et al.*, 2009). VCO serves as a significant source of energy in the diet (Boateng *et al.*, 2016). VCO provides lubricating action in dressing and enhances food flavor (Carandang, 2008). Additionally, medium chains are similar to the fat's presented in mother's milk which provides immunity for babies against disease. Similar advantageous effects are also found in adults (Maria and James, 2013). VCO possesses anti-inflammatory, antimicrobial and antioxidant properties and boosts the immune system (Carandang, 2008). VCO also showed high antimicrobial activity and inhibited various pathogenic bacteria for example *Listeria monocytogenes* (Wang and Johnson, 1992). It was also reported that coconut oil in combination with menhaden oil was able to reduce mammary tumor in animal study (Craig-Schmidt *et al.*, 1993). Effect of VCO on LDL oxidation in cholesterol, blood coagulation factors and lipid levels fed Sprague-Dawley rats were studied by Nevin and Rajamohan (2008). Antioxidant levels were higher and also reduced the triglyceride and cholesterol levels in VCO fed animals. VCO, without bile, can easily digest and goes directly to the liver for conversion into energy (DebMandal and Mandal, 2011). VCO has been using

to treat fat malabsorption patients, as it contains medium chain fatty acid (Carandang, 2008). The effect of consumption of VCO on HDL cholesterol and waist circumference (WC) in coronary artery disease patients (CAD) was studied by Cardoso *et al.* (2015). Diets rich in VCO decrease WC and increase HDL-cholesterol concentrations, thus supporting the secondary prevention for CAD patients. VCO increases the metabolism and therefore support weight management (Liau *et al.*, 2011). Protective effect of VCO against liver damage in albino rats challenged with the anti-folate combination, trimethoprim-sulfamethoxazole (TMP-SMX), was studied by Otuechere *et al.* (2014). The active components of VCO had protective effects against the toxic effects induced by TMP-SMX administration, mainly in the liver of rats. Arunima and Rajamohan (2014) studied the effects of VCO in comparison with olive oil and sunflower-seed oil on the synthesis and oxidation of fatty acids and the molecular regulation of fatty acid metabolism in normal rats. VCO had the beneficial effects on lipid parameters by decreasing lipogenesis and enhancing the rate of fatty acid catabolism, thus reducing coronary heart disease. VCO can be used for cooking and frying because of its high resistance against rancidity development. Gani *et al.* (2017) reported that VCO (5%) can be used as an alternative to other vegetable oils in surimi gel, as it content MCFAs, therefore, health advantages can be claimed.

1.2.8 Production of virgin coconut oil

Coconut is available as raw material for VCO production in two forms. Wet and dry materials are commonly known as wet coconut and dry coconut or copra. The oil can be extracted from both raw materials. Dry processing is the most widely used for extraction. In this process, clean ground copra is pressed by wedge press, screw press or hydraulic press to obtain coconut oil, which subsequently subjected to refining, bleaching and deodorizing (RBD) processes. Recently, wet process is very popular to produce coconut oil and does not need the RBD process. This process is more desirable as no chemical or high heat treatment is used. Consequently, the alteration of oil nature is negligible (Marina *et al.*, 2009).

1.2.8.1 Wet extraction

Wet processing or aqueous processing is the term used for the extraction of coconut oil directly from coconut milk. The wet process uses raw coconut rather than dried copra. Generally, the protein in the coconut creates an emulsion of oil and water (Grimwood, 1975). The more problematic step is breaking up the emulsion to recover the oil. The oil can be separated from the emulsion by means of boiling, enzymatic extraction, fermentation, refrigeration or mechanical centrifuge (Wen, 2010). Wet extraction eliminates the use of solvent, which may lower the investment cost and energy requirements. Furthermore, it does not require the RBD process (Marina *et al.*, 2009). The wet process appears more desirable due to the free usage of chemical solvents, thus more environmental friendly than the solvent extraction (Marina *et al.*, 2009). Even though the concept appears potentially attractive, the method yields comparatively low content of oil, which has discouraged its commercial application (Rosenthal *et al.*, 1996).

Separation of the oil from the water–oil emulsion can also be accomplished by breaking the emulsion using enzyme or aging for several hours, or by mechanical process using continuous centrifugation (Norulaini *et al.*, 2009). Destabilization of emulsion can be done through three mechanisms. The first stage is creaming by the action of gravitational force, resulting in two phases. Phase with the lower specific gravity moves to the top phase and the higher specific gravity phase moves downward. The second stage is flocculation or clustering, in which the oil phase moves as a group, which does not involve the rupture of the interfacial film that normally surrounds each oil droplet and therefore does not change the original oil droplet. The last stage, coalescence is the most critical phase in destabilization. During this stage, the interfacial area is ruptured; the globules join together and reduce the interfacial area (Wen, 2010).

1.2.8.2 Enzymatic extraction

Extraction of oil can be carried out by the use of enzymes in aqueous extraction process. Enzymatic pre-treatment has been known as a novel and effective means to improve the oil yield (Marina *et al.*, 2009). VCO can be separated from

coconut milk by means of enzymatic hydrolysis (Raghavendra and Raghavarao, 2010; Senphan and Benjakul, 2016). Amongst all processes, the enzyme-assisted extraction of coconut oil significantly increased the yield in aqueous system (Tano-Debrah and Ohta, 1997). Enzymatic extraction is effective to release the oil by breaking down and destabilization the coconut emulsion (Rahayu *et al.*, 2008). VCO prepared by enzymatic extraction method has more beneficial and safety effect than the oil produced from copra by traditional method, since they are often infected by insects or aflatoxin producing molds associated with the potential toxicity problem during manufacturing (Handayani *et al.*, 2009). The activity of enzyme is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Handayani *et al.*, 2009). Those factors determined oil extraction yield differently (Rahayu *et al.*, 2008). The effectiveness of protease in improving oil extraction yield was found in descending order as follows: alkaline protease > neutral protease > acid protease (de Moura *et al.*, 2008). Raghavendra and Raghavarao (2010) reported that oil could be recovered from coconut milk using papain with the yield of 60.09%. The maximal extraction yield of oil was obtained when using 0.6% (w/w) Viscozyme L, followed by Neutrase 1.5 MG (0.3%, w/w), in which total incubation time of 60 min, temperature of 60 °C, substrate/water ratio of 1:6 and pH of 7 were used (Sant'Anna *et al.*, 2003). Man *et al.* (1996) studied the use of an enzyme mixture (cellulase, α-amylase, polygalacturonase, and protease) at 1% (w/w) at pH 7.0 and an extraction temperature of 60 °C for coconut oil extraction. The recovery of 73.8% and good-quality oil were achieved. Senphan and Benjakul (2015) reported that VCO extracted using crude protease extract (from hepatopancreas from Pacific white shrimp) (10 unit/g protein) at room temperature for 6 h had the highest yield of 92.39%.

1.2.8.3 Physical extraction

Chilling, freezing and thawing techniques have been used to break the protein stabilized oil-in-water emulsion. Emulsion was centrifuged before chilling and thawing to allow better packing of the coconut oil globules (Seow and Gwee, 1997). The temperature used for chilling and freezing were 10 °C and −4 °C, respectively, while the thawing process was carried out at 40°C until the coconut cream reached

room temperature (25 °C). In addition, this action also helps in removing undissolved solids after extraction process. The removal of solids present in high percentages in the dispersion of oil droplet was important for efficient recovery of oil by centrifugation (Rosenthal *et al.*, 1996). The centrifugation step was followed to enable the packing of cream oil globule to crystallize upon lowering the temperature. Centrifugation process was carried out at 2000-5000xg for up to 6 min. During thawing, the oil coalesced and formed the large droplets of varying sizes. Raghavendra and Raghavarao (2010) reported that combination of treatments (Aspartic protease enzyme treatment at 37 °C followed by chilling and thawing) of coconut milk emulsion resulted in the highest yield of 94.5%.

1.2.8.4 Fermentation process

Natural fermentation is a method where the less proceeding conditions are involved for production of VCO. In the natural fermentation process, coconut is allowed for microbial fermentation (Marina *et al.*, 2009). Normal flora will ferment the coconut milk and separates the coconut oil into the top portion within 24 - 48 h. The fermentation associated with the microbial growth enhances the destabilization of emulsion, plausibly mediated by microbial proteases. The separated oil can be collected. However, there was a chance of contamination with microorganisms because the coconut milk is the rich source of proteins, carbohydrates and moisture, which can attract the microorganisms (Tansakul and Chaisawang, 2006). Some microorganisms may spoil the coconut milk, resulting in poor quality VCO (generally in yellow color) (Mansor *et al.*, 2012). Thus, the main disadvantages of this process are low oil recovery and fermented odor, which masks the characteristic coconut flavor of the oil (Raghavendra and Raghavarao, 2010). During fermentation of coconut milk, high FFA could be more produced by lipolytic enzymes in the presence of water. Coconut milk emulsion can also be separated by adjusting pH of the coconut milk emulsion between pH 3 and 5.6 and inoculated with bacteria cultures (Chen and Diosady, 2003). Che Man *et al.* (1997) investigated the use of pure culture, *Lactobacillus plantarum* 1041 IAM to extract coconut oil, which could be able to extract as much as 95% of the oil. VCO extraction by induced fermentation under semi-controlled conditions using probiotic organisms (*Lactobacillus plantarum*)

increased the yields of VCO (28.47%). The temperature 45 ºC, pH 5, inoculum concentration 2%, fermentation end time 48 h, anaerobic conditions were found as the most preferable conditions for the induced fermentative production of VCO (Satheesh and Prasad, 2014).

1.2.9 Quality of virgin coconut oil

Essential composition and quality parameters of VCO appointed by Asian Pacific Coconut Community (APCC) standards are enlisted in Table 6. Different types of raw materials namely incubated and desiccated coconut meat, incubated coconut milk as well as freeze-thawed coconut milk affected physicochemical properties of VCO, however the differences were not large enough to significantly affect the overall quality of the VCO. Physical and chemical qualities complied with the Codex standard for coconut oil and the Philippine standards for VCO (Dia *et al.*, 2005). Mansor *et al.* (2012) characterized VCO obtained from different methods including chilling, fermentation, fresh-drying and enzyme treatment. VCO was obtained from chilling method by centrifugation of coconut milk at 3600xg for 10 min and the upper layer of cream was removed for chilling. Chilling was done at 5 ºC for 24 h and then the chilled cream was thawed slowly in water bath at 50 ºC to extract the oil. VCO was obtained from fermentation method by fresh coconut milk added with distilled water at 1:1 ratio. In each 1 litre of the mixture, 2.0 g of Baker's yeast (*Saccharomyces cerevisiae*) was added as an inoculum for the fermentation process. The mixture was then left to stand for 36 h at room temperature. As the layers of oil and water became separated, the upper oil layer was simply decanted. VCO was also obtained via fresh-dry process by shredded the coconut meat and dried in an aerated oven at 35 ºC for approximately 48 h. After drying, it was screw-pressed for oil extraction. The collected oil was also filtered using Whatman filter paper no. 1 to remove some debris. For VCO obtained from enzymatic treatment, coconut milk was mixed with papain enzyme at 0.1% (w/w). It was left to stand for 3 h at 55 ºC. The mixture was later centrifuged at 4900xg for 25 min to obtain the oil. Various methods slightly affected the quality but the difference was not significant. Marina *et al.* (2009) described the chemical properties of commercial VCO available in Malaysia and Indonesia. Chemical properties of VCO did not vary

much. The iodine, peroxide, saponification and free fatty acid values obtained for commercial VCO samples were in accordance with the specification limit of Codex standard (2003) for refined coconut oil. Senphan and Benjakul (2015) also reported that the extraction of VCO using crude protease extract (from hepatopancrease of Pacific white shrimp) or Alcalase (10 unit/g protein) at 60°C for 90 min had no effect on the quality of resulting VCO.

Table 6. Essential composition and quality parameters of VCO appointed by Asian Pacific Coconut Community (APCC)

Sr. No.	APCC Parameters	APCC Standards
1.	Moisture (%)	Max 0.1
2.	Refractive index at 40 °C	1.4480 – 1.4492
3.	Relative density	0.915 – 0.920
4.	Specific gravity at 30 deg./30 deg. C	0.915– 0.920
5.	Iodine Value (g I_2/100g oil)	4.1 – 11
6.	Saponification Value (mg KOH/g oil)	250 – 260
7.	Free fatty acid (%)	Max 0.2
8.	Peroxide Value (meq O_2/kg)	Max 3

Fatty acid composition and other quality parameters for VCO Indian-Standard; IS: 6220-1971, Malaysian Standard and Philippines Standard, etc have been established. Additionally, Codex (Codex, 2003) and the Asian and Pacific Coconut Community (APCC, 2003) have proposed international standard. Fatty acid composition of virgin coconut oil (VCO) and refined, bleached and deodorized (RBD) coconut oil from various sources are enlisted in Table 7. Fatty acid composition of commercial VCO available in Malaysia and Indonesia was reported by Marina *et al.* (2009). Lauric acid with percentage ranging from 46 to 48% was dominant fatty acid and the content was within the limit of VCO standard according to Malaysian Standard (2007) and Asian and Pacific Coconut Community (APCC, 2003). Mansor *et al.* (2012) reported that there were some differences in the fatty acid profile of the VCO samples produced by different methods. Lauric acid (with the range of 46.36 - 48.42%) was found in all VCO samples.

Table 7. Fatty acid composition of virgin coconut oil (VCO) and refined, bleached and deodorized (RBD) coconut oil from various sources.

Fatty acid	Codex standard for RBD coconut oil	APCC standard for VCO	Malaysian standard for VCO	Marina *et al.* (2009)	Dia *et al.* (2005)
C6	nd-0.70	0.40-0.60	0.80-0.95	0.52-0.69	nd-0.60
C8	4.60-10.0	5.00-10.00	8.00-9.00	7.19-8.81	5.98-10.44
C10	5.0-8.0	4.50-8.00	5.00-7.00	5.65-6.59	5.37-6.60
C12	45.10-53.20	43.00-53.00	47.00-50.00	46.89-48.03	47.63-52.55
C14	16.80-21.00	16.00-21.00	17.00-18.50	16.23-18.90	16.79-20.08
C16	7.50-10.20	7.50-10.00	7.50-9.50	7.41-9.55	6.38-10.17
C18:0	2.00-4.00	2.00-4.00	2.50-3.50	2.81-3.57	7.45-10.73
C18:1	5.00-10.00	5.00-10.00	4.50-6.00	5.72-6.72	-
C18:2	1.00-2.50	1.00-2.50	0.70-1.50	0.90-1.60	nd-0.12
C18:3	nd-0.20	<0.5	nd	nd	nd

1.2.10 Antioxidant activity and phenolic compounds in VCO

VCO has been reported to contain indigenous antioxidant which help preventing lipid oxidation. α-Tocopherol was actually found in the coconut testa (the thin brown layer that clings to the white coconut meat) and only trace amount was detected in the VCO samples (Dia *et al.*, 2005). Since coconut testa was removed in the production of VCO, it was logical that the VCO samples contain only trace amount of α -Tocopherol. On the other hand, Manalac (1970) reported that coconut oil from copra contained natural tocopherol (721.06 and 777.1 mg/g). Mansor *et al.* (2012) analyzed tocopherol in VCO extracted using different extraction methods. Beta-tocopherol ranged from 0.04 to 0.05 mg/kg and gamma-tocopherol ranged from 0.01 to 0.05 mg/kg. However, delta-tocopherol was detected at a very low level for all VCO samples and alpha tocopherol was not detected.

Antioxidant activity in VCO was reported to be high in VCO, compared to the refined coconut oil (Dia *et al.*, 2005). The results also indicated that VCO with the highest total phenolic content also possessed the highest antioxidant

activity. The effect of different processing methods on antioxidant capacity of VCO was studied by Marina *et al.* (2009). VCO produced through fermentation had the strongest DPPH radical scavenging effect and the highest antioxidant activity, based on *β*-carotene-linoleate bleaching method. However, VCO obtained through chilling method had the highest reducing power. Very high correlations were found between total phenolic content and both scavenging activity and reducing power.

The phenolic acids identified in VCO were protocatechuic, vanillic, caffeic, syringic, ferulic and *ρ*-coumaric acids. Antioxidant activity in VCO could be due to those phenolic compounds. Seneviratne and Sudarshana (2008) also reported the presence of caffeic, *ρ*- coumaric and ferulic acids as well as catechin in the commercial and traditional coconut oil.

1.2.11 Sensory property of VCO

VCO has the different sensory characteristic from the refined coconut oil. Villarino *et al.* (2007) conducted a study to describe the sensory terms for VCO using trained panelists. A total of 14 attributes were generated. VCO was found to be almost colorless, while RBD coconut oil was described as having a distinct yellow color. The RBD sample had no perceptible aroma, while VCO samples have acid, cocojam (aroma associated with roasted coconut), latik (aroma of cooked coconut with sweet sensation), nutty and rancid aromas. VCO samples were found to have detectable sweet taste and nutty flavor. Since VCO is produced by natural methods without contamination by chemicals usage, its sensory profiles reflect the unique flavor.

1.2.12 Fish proteases

During fish processing, a large amount of waste including viscera is generated. Fish viscera are non-edible parts produced in large quantities and become a waste, creating the pollution (Kishimura *et al.*, 2008). These by-products are recognized as a potential source of digestive enzymes, especially proteases (Klomklao *et al.*, 2007), that may be of industrial applications (Simpson, 2000). According to the International Union of Applied Biochemists classification, proteases from fish and

aquatic invertebrates may be classified into four major groups i.e. aspartiyl proteases, serine proteases, cysteine proteases and metalloproteases (Simpson, 2000; Simpson, 2000).

1.2.12.1 Fish trypsins

Trypsin (EC 3.4.21.4) is one of the main digestive proteases in the group of serine proteases (Simpson, 2000). Trypsin has a catalytic triad of three essential amino acid residues including serine (Ser), histidine (His) and aspartate (Asp) within the S1 binding pocket. The high specificity of trypsin for lysine and arginine results from the negative charge of Asp at the S1 binding pocket of trypsin matching the positive charge of the P1 side chain of the substrate (Kishimura *et al.*, 2010). In the pancreas, trypsin not only functions as a digestive enzyme, but also is responsible for activating all the pancreatic enzymes by cleaving a short activation peptide from the amino-terminus of inactive zymogens (Kishimura *et al.*, 2010). Fish trypsin is unstable at acid pH, and contains a lower content of basic amino acid residues in the polypeptide chain in comparison with mammalian trypsins (Simpson, 2000). Trypsin consists of single peptide chain with molecular weight (MW) typically of 24 kDa (Rungruangsak Torrissen and Male, 2000). The MW of fish trypsins can vary with species. The optimal pH for the activation is between pH 7.5 and 10.5. This enzyme is more selective than any other enzyme, cleaving peptide bonds at the carboxyl terminus of lysine and arginine residues exclusively (Rungruangsak Torrissen and Male, 2000). Trypsins have been isolated and characterized from the pyloric caeca, pancreatic tissue, or intestines of several marine animals. The optimal conditions of fish trypsins are summarized in Table 8.

Table 8. Optimal conditions of various fish trypsins for hydrolysis of synthetic substrates

Identified species	Optimum pH	Optimum temperature (°C)	Substrates	References
Starfish (*Asterina Pectinifera*)	8	55	TAME	Kishimura and Hayashi (2002)
Japanese anchovy (*Engraulis japonica*)	8	60	TAME	Kishimura *et al.* (2005)
Monterey sardine (*Sardinops sagax caerulea*)	8	50	BAPNA	Castillo-Yánez *et al.* (2005)
Bigeye snapper (*Pricanthus macracanthus*)	8-11	55	BAPNA	Van Hau and Benjakul (2006)
Chinook salmon (*Oncorhynchus tshawytscha*)	8	60	BAPNA	Kurtovic *et al.* (2006)
New Zealand hoki (*Macruronus Novaezealandlae*)	9	60	BAPNA	Shi *et al.* (2007)
Bluefish (*Pomatomus saltatrix*)	9.5	55	BAPNA	Klomklao *et al.* (2007)
Skipjack tuna (*Katsuwonus pelamis*)	8.5	60	TAME	Klomklao *et al.* (2007)
Spotted goatfish (*Pseudupeneus maculatus*)	9	55	BAPNA	Souza *et al.* (2007)
Jacopever (*Sebastes schlegelii*)	8	60	TAME	Kishimura *et al.* (2007)
Elkhorn sculpin (*Alcichthys alcicornis*)	8	50	TAME	Kishimura *et al.* (2007)
Walleye pollock (*Theragra chalcogramma*)	8	50	TAME	Kishimura *et al.* (2008)
Threadfin hakeling (*Laemonema longipes*)	8	50	TAME	Kishimura *et al.* (2010)
Pacific cod (*Gadus macrocephalus*)	8	50	TAME	Fuchise *et al.* (2009)
Saffron cod (*Eleginus gracilis*)	8	50	TAME	Fuchise *et al.* (2009)
Hybrid tilapia (*Oreochromis niloticus* x *O. aureus*)	9	60	Casein	Wang *et al.* (2009)

Identified species	**Optimum pH**	**Optimum temperature (°C)**	**Substrates**	**References**
Threadfin hakeling (*Laemonema longipes*)	8	50	TAME	Kishimura *et al.* (2010)
Brownstripe red snapper (*Lutjanus vitta*)	8.5	60	BAPNA	Khantaphant and Benjakul (2010)
Barbel (*Barbuds callensis*)	10	55	BAPNA	Sila *et al.* (2012)
Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*)	8	60	TAME	Klomklao *et al.* (2011)
Sardinelle (*Sardinella aurita*)	9	50-55	BAPNA	Khaled *et al.* (2011)
Silver mojarra (*Diapterus rhombeus*)	8.5	55	BAPNA	Silva *et al.* (2011)
Zebra blenny (*Salaria basilisca*)	9.5	60	BAPNA	Ktari *et al.* (2012)

1.2.12.2 Applications of trypsin

Trypsin is extensively exploited in food and beverage industries. It can be used to improve the workability of dough, in tenderizing of meat, in production of protein hydrolysates, to improve the texture of fish products and to enhance cold stabilization of beer (Stoytcheva *et al.*, 2013). In biochemistry trypsin is used in the development of cell and tissue culture protocols (Banumathi *et al.*, 2009). In medicine, the test of trypsin activity serves as a specific and reliable diagnostic tool to monitor pancreatic function and its alteration: cystic fibrosis, chronic pancreatitis, etc. (Chen *et al.*, 2009). Trypsin has been used widely for hydrolysis purposes. It is able to cleave peptide bonds of various proteins from animal and plant origins. Simpson and Haard (1985) extracted carotenoprotein from shrimp processing waste with the aid of trypsin.

Proteins extracted from defatted oats were chemically modified by partial hydrolysis with trypsin (Ma and Wood, 1987). Water hydration capacity and foaming properties were improved by trypsin hydrolysis. When bovine serum albumin (BSA) was hydrolyzed with trypsin, emulsifying activity index was increased about 40% above its original level (Saito *et al.*, 1993). Peptide with MW of 24 kDa generated after hydrolysis showed good emulsifying properties. Fresh and frozen shrimp were hydrolyzed with trypsin, and their proximate composition, pH, amino acid content, and sensory properties were studied by Simpson *et al.* (1998). The hydrolysates had high levels of glycine, proline, arginine and valine, considered to be important in crustacean flavors.

Enzymatic hydrolysis of whey protein via trypsin is generally used to produce whey protein hydrolysates to improve heat stability, reduce allergenicity, produce bioactive peptides, tailor amounts and size of peptides for special diets and alter the functional properties of gelation, foaming and emulsification (Foegeding *et al.*, 2002). Casein in milk generates several bioactive peptides when hydrolyzed by trypsin (Kitts and Weiler, 2003; Korhonen and Pihlanto, 2006). Lorenzen and Meisel (2005) demonstrated that trypsin treatment of yoghurt milk prior to fermentation with cultures resulted in a release of phosphopeptide-rich fractions. However, trypsin

treatment of skim milk led to increased fermentation times and substantially lowered the production of L-lactic acid. The functional properties of the milk proteins were decreased and was less bitter than samples from non-treated milk.

Trypsin has been used to prepare protein hydrolysate with bioactivity. Abedin *et al.* (2014) isolated α_1 and β chains of the collagen from the sea cucumber (*Stichopus vastus*) and then hydrolyzed by trypsin. The molecular mass distribution of the hydrolysates ranged from 5 to 25 kDa and hydrolysates exhibited excellent radical-scavenging activity. Chi *et al.* (2016) produced acid soluble collagen hydrolysates from the cartilages of *Sphyrna lewini, Dasyatis akjei* and *Raja porosa* using different hydrolysis conditions by trypsin. The hydrolysates obtained from hydrolysis at pH 2.5 for 3 h at 37 ºC showed high foaming and emulsifying capacities. Conversely, hydrolysates obtained from hydrolysis at pH 7.8 for 3 min at 37 ºC exhibited strong scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, hydroxyl radical and moderate reducing power. Enzymatic hydrolysis via trypsin on wheat gluten protein improved its solubility and produced hydrolysates with high foaming properties which might find applications in food products (Wouters *et al.*, 2017). Recently, proteases (especially trypsin) from shrimp hepatopancreas were used for extraction of VCO (Senphan and Benjakul, 2015).

1.2.13 Mayonnaise

Mayonnaise is one of the most widely used sauces or condiments in the world today (Huang *et al.*, 2016). It was firstly produced commercially in early 1900s and has become popular in America (Depree and Savage, 2001). Mayonnaise is a low-pH oil-in-water emulsion, consisting of three different components: 70-80% oil (the dispersed phase), vinegar (the continuous phase) and egg yolk as an emulsifier at the interface (Li *et al.*, 2014). It may contain salt, spices, natural sweeteners and various natural flavoring ingredients (Harrison and Cunningham, 1985). This emulsion is formed by first mixing the eggs, sugar, salt and vinegar and then slowly blending in the oil (Huang *et al.*, 2016).

The pH of mayonnaise can have a dramatic effect on the structure of the emulsion. The pH of mayonnaise ranges from 3.6 to 4.0 (Krishnamurthy and

Witte, 1996). The highest viscoelasticity and stability of mayonnaise is achieved when the pH is close to the isoelectric point of the egg yolk because of the minimum charge on the proteins (Depree and Savage, 2001). Kiosseoglou and Sherman (1983) found that the viscoelasticity of their mayonnaise was highest at a pH of 3.9.

1.2.13.1 Stability of mayonnaise

Mayonnaise is a high oil containing product. Therefore it is susceptible to oxidation, which results in quality deterioration and the formation of undesirable components such as free radicals and reactive aldehydes (Gorji *et al.*, 2016). Its stability is depending on the type of oil used (Depree and Savage, 2001). In mayonnaise, very large area of oil is exposed to an aqueous phase, which may contain substantial dissolved oxygen. Wills and Cheong (1979) studied the rancidity in a commercial mayonnaise stored at 20 ºC and found that peroxide values increased and reached the maximum of about 3.5 meq/kg after 15 days. Subsequently, the values were declined.

The oxidation of mayonnaise in response to light of different wavelengths and the effect of packaging was studied by Lennersten and Lingnert (2000). Light with a wavelength of 365 nm promoted the oxidation of unsaturated fats due to photosynthesized oxidation. Visible light in the blue range also promoted oxidation and discoloration of the mayonnaise, but light of wavelengths above 470 nm had no effect. Hence, it is important to protect mayonnaise from wavelengths shorter than 470 nm. Packaging materials, which contained PEN (polyethylene naphtalate), blocked the ultraviolet light, but substantial oxidation of the mayonnaise still occurred due to blue light. Cool fluorescent lights similar to those used in supermarkets were a significant source of light at 365 nm and in the 410-450 nm range (Lagunes-Galvez *et al.*, 2002).

Some salts and minerals have been known to act as pro-oxidant in mayonnaise. Lahtinen and Ndabikunze (1990) investigated the effect of two concentrations (0.85 and 1.45%) of three types of salt on the oxidation of the mayonnaise prepared with or without the addition of the antioxidants including butylated hydroxy anisole and butylated hydroxytoluene. The salts were NaCl,

Morton Lite salt (50% NaCl, 50% KCl) and mineral salt (65% NaCl, 25% KCl, 10% $MgSO_4.6H_2O$). After storage for 60 days at room temperature, both NaCl and mineral salt promoted oxidation of the oil in the absence of antioxidant, whereas Morton Lite salt did not. This effect was largely lowered in the presence of antioxidant, although a significant increase in oxidation still occurred in mayonnaise containing 0.85% NaCl.

Types of oil present in mayonnaise also affect its oxidative stability. Hsieh and Regenstein (1992) prepared mayonnaise containing 70% fish oil, corn oil or soya oil. That with fish oil was oxidized very rapidly, followed by those containing corn oil and soya oil. Susceptibility of a lipid molecule to oxidation is determined by its chemical structure, in particular, the number and location of the double bonds (McClements and Decker, 2000). Generally, saturated lipids are more stable to lipid oxidation than unsaturated lipids. Using two different types of oil in mayonnaise can affect the oxidative stability of mayonnaise. Mayonnaise mixed with saturated medium triglyceride and unsaturated linseed oil was less prone to oxidation than that made with unsaturated linseed oil (Raudsepp *et al.*, 2014). Oxidative stability of mayonnaise was therefore totally dependent on the lipid type, while the rheological properties of the mayonnaise was influenced by the structure of the lipid (Jacobsen *et al.*, 2003).

1.2.13.2 Fish oil enriched mayonnaise

Fish oil enriched mayonnaises are more prone to development of unpleasant off-odors and off-flavors than the mayonnaise without fish oil. Four volatile oxidation compounds, namely 3-furaldehyde, 2,4-heptadienal, 2,4-decadienal and ethyl benzene, appeared to correlate to the fishy and rancid off-flavors developed in mayonnaises (Jacobsen *et al.*, 1999). To prevent oxidation, synthetic antioxidants such as BHT, BHA, TBHQ and EDTA have been used in mayonnaise (Sanhueza *et al.*, 2000). Nowadays, consumers are more concerned of health. Therefore, there is a growing trend in consumer preferences toward natural antioxidant (Gorji *et al.*, 2016). Natural antioxidants such as gallic acid, ascorbic acid, tocopherol, rosemary, etc. have been used in mayonnaise to prevent the oxidation. Jacobsen *et al.* (1999) examined the effect of the water-dispersible tocopherol preparation, Grindox 1032, and the oil-

soluble tocopherol preparation, Toco 70, on the oxidative stability of fish oil-enriched mayonnaise. Tocopherol did not appear to be an efficient antioxidant in fish oil-enriched mayonnaise, because it could not prevent the metal-catalyzed decomposition of peroxides. Jacobsen *et al.* (1999) examines the effect of ascorbic acid (0−800 ppm) on the sensory perception of mayonnaises containing 16% fish oil and on the levels of iron and copper in the aqueous phase. Ascorbic acid increased the formation of fishy off-flavors in fresh mayonnaise. The ascorbic acid-iron complex subsequently reacts with lipid hydroperoxides, resulting in the increased lipid oxidation and in the immediate formation of rancid and fishy off-flavors. Jacobsen *et al.* (2001) investigated the antioxidative effects of gallic acid and EDTA in mayonnaise enriched with 16% fish oil. EDTA reduced the formation of free radicals, lipid hydroperoxides, volatiles, and fishy and rancid off-flavors. The antioxidative effect of EDTA was attributed to its ability to chelate free metal ions and iron from egg yolk located at the oil-water interface. Gallic acid also reduced the levels of both free radicals and lipid hydroperoxides but promoted slightly the oxidative flavor deterioration in mayonnaise. Mayonnaise samples with 16% fish oil and 64% rapeseed oil (w/w) were supplemented with either lactoferrin (8−32 μM), phytic acid (16−124 μM), or EDTA (16−64 μM) and were stored at 20 °C for up to 4 weeks. Lactoferrin reduced the oxidation when added in concentrations of 8 μM in the mayonnaise, whereas it was a prooxidant at higher concentrations. EDTA was an effective metal chelator even at 16 μM, whereas phytic acid did not exert a distinct protective effect against oxidation (Nielsen *et al.*, 2004). Alemán *et al.* (2015) assessed the antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise. Caffeic acid esterified with fatty alcohols of different chain lengths (C1–C20) were better antioxidants than the original phenolic compound. Fish oil enriched mayonnaise added with caffeates of medium alkyl chain length (butyl, octyl and dodecyl) resulted in a better oxidative stability than those with shorter (methyl) or longer (octadecyl) alkyl chains. Effects of cashew leaf extract (CE) or BHT at levels of 100 and 200 ppm on the oxidative stability of fish oil enriched mayonnaise during storage of 30 days at 30 °C were investigated by Chotphruethipong and Benjakul (2017). It was found that CE and BHT at the concentration of 200 ppm effectively inhibited lipid oxidation of fish oil enriched mayonnaise during storage.

1.3 Objectives

1. To study physicochemical properties and emulsion stability of coconut milk from different maturity stages.
2. To examine the characteristics and quality of virgin coconut oil extracted from different maturity stages.
3. To fractionate proteins in coconut meat and elucidate their role in emulsion stability.
4. To comparative study on extraction of VCO with the aid of partially purified protease from seabass pyloric caeca and commercial trypsin.
5. To study the production of virgin coconut oil with aid of protease from seabass pyloric caeca in combination with different treatments.
6. To study the oxidative stability of mayonnaise prepared using virgin coconut oil/fish oil blend.
7. To study the physical and rheological properties of mayonnaise prepared using virgin coconut oil/fish oil blend.

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CHAPTER 2
A COMPARATIVE STUDY OF THE PHYSICOCHEMICAL PROPERTIES AND EMULSION STABILITY OF COCONUT MILK AT DIFFERENT MATURITY STAGES

2.1 Abstract

Based on chemical analysis, mature coconut (MC) milk had the highest moisture content ($p<0.05$), followed by immature coconut (IMC) and overlay mature coconut (OMC) milk, respectively. OMC milk had the highest lipid content while IMC milk showed the lowest lipid content ($p<0.05$). The lowest protein and carbohydrate contents were found in MC milk ($p<0.05$). Cocosin with MW of 55 kDa was observed as the major protein in all coconut milks; however, the band intensity slightly decreased with increasing maturity stages. Increase in oil droplet size was observed with increasing maturity stages. Therefore, maturity stages have an influence on the chemical compositions, properties and emulsion stability of coconut milk.

2.2 Introduction

Coconuts (*Cocos nucifera* L.) are extensively used in many traditional foods of the Asian and Pacific regions (Onsaard *et al*., 2005). Coconut milk is commonly used in several cuisines such as curries and desserts (Tansakul and Chaisawang, 2006). It contains high amounts of medium chain saturated fatty acids (MCFAs), especially lauric acid (Raghavendra and Raghavarao, 2010). Lauric acid is converted into a very valuable compound known as monolaurin, which has antiviral and antibacterial properties. The consumption of coconut milk may help to protect the body from infections (DebMandal and Mandal, 2011).

Coconut milk is a milky white oil-in-water emulsion extracted from grated coconut meat with or without the addition of water. The emulsion in coconut milk is naturally stabilised by coconut proteins (globulins and albumins), as well as phospholipids (Raghavendra and Raghavarao, 2011). The major protein (~65%) in coconut endosperm is an 11S globulin known as cocosin with a molecular weight (MW) of 55 kDa (Garcia *et al*., 2005), and is believed to play a significant role in stabilizing the coconut milk emulsion (Tangsuphoom and Coupland, 2008). Generally, both

intrinsic factors (e.g. protein compositions, etc.) and environmental conditions (e.g. pH, temperatures, etc.) can affect the stability of the coconut milk emulsion (Raghavendra and Raghavarao, 2010).

On the other hand, the instability of the coconut milk emulsion is required for the production of virgin coconut oil (VCO). In recent years, coconut milk is immensely used for the extraction of VCO. Moreover, VCO has gained much popularity in the scientific community due to the presence of MCFAs, its high degree of saturation and good stability. It can be obtained by breaking the emulsion of coconut milk using different extraction methods (Raghavendra and Raghavarao, 2010). Thus, to maximize the yield of VCO, coconut milk emulsion must be destabilized to a high degree, so that oil can be released and separated effectively.

The quality and stability of coconut milk emulsion could be governed by intrinsic factors, especially at different maturity stages. However, no information exists regarding the influence of maturity stages on the characteristics and emulsion stability of coconut milk. A better understanding of the physicochemical properties and emulsion stability of coconut milk at different maturity stages could be beneficial in the manufacturing of VCO with prime quality and high yield. Therefore, this comparative study was carried out to evaluate the physicochemical properties and emulsion stability of milk obtained from coconut at three different maturity stages.

2.3 Objective

To study physicochemical properties and emulsion stability of coconut milk from different maturity stages.

2.4 Materials and methods

2.4.1 Chemicals

Sodium hydroxide, boric acid and Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulfate and isooctane were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, chloroform, petroleum ether, hydrochloric acid, sulfuric acid, n-hexane and cyclohexane were procured from Lab-Scan (Bangkok, Thailand). Chemicals for electrophoresis were

obtained from Biorad (Richmond, VA, USA) and protein molecular weight marker was procured from GE healthcare (Buckinghamshire, UK).

2.4.2 Preparation of coconut meat and coconut milk

Coconuts at three different maturity stages including immature coconut (IMC) (9-10 months old from pollination), mature coconut (MC) (11-12 months old from pollination) and overlay mature coconut (OMC) (14-15 months old from pollination) with the same size in each maturity stages were purchased from a plantation site in Yaring District, Pattani Province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was collected manually and grated using a rotary wedge cutter machine. To prepare coconut milk, the grated coconut meat was pressed using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand) with a maximum pressure of 10.35 MPa for 2 min. Thereafter, coconut milk was collected and analyzed.

2.4.3 Proximate analysis of coconut meat and coconut milk

Coconut meat and coconut milk at three different maturity stages were analyzed for moisture, ash, lipid and protein contents according to the method of AOAC (AOAC, 2000). The protein content was calculated using 6.25 (as the factor) and the carbohydrate content was calculated as the difference from the sum total of the aforementioned proximate analysis components. The values were expressed as g/100 g (wet weight basis).

2.4.4 Color determination

Coconut milk color was measured using a colorimeter (HunterLab, Model colourFlex, VA, USA). The color was reported as L^*, a^*, b^* values, indicating lightness, redness/greenness and yellowness/blueness, respectively.

2.4.5 pH measurement

A digital pH meter (Eutech, pH700 Thermo Scientific, USA) was used to measure the pH values of coconut milk.

2.4.6 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of coconut milk were determined by SDS-PAGE, according to the method of Laemmli (1970), using 4% staking gel and 12% separating gel. The coconut milk samples (10 mL) were homogenized with 10 mL of 50 g/L SDS at a speed of 12,000 rpm for 1 min. The homogenate was heated at 95°C for 1 h, followed by centrifugation at 7000 ×g for 10 min at 25°C using a centrifuge (Beckman coulter, Allegra™ centrifuge, CA, USA). The protein concentration of the supernatant was determined by the Biuret method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as a standard. The prepared samples were mixed with sample buffer containing 2% SDS, 10% glycerol and 0.05% bromophenol blue in 0.5 M Tris-HCl, and the resulting solution had a pH of 6.8. Under reducing condition, β-mercaptoethanol was added to the sample buffer in order to obtain a final concentration of 5% and the mixtures were heated at 95°C for 3 min prior to loading. The prepared mixtures (20 μg protein) were loaded onto the gel. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, VA, USA) at a constant voltage of 20 mA/gel. After electrophoresis, the gels were stained with 0.5 g/L Coomassie Brilliant Blue R-250 in 500 mL/L methanol and 75 mL/L acetic acid for 30 min. Finally, they were destained with a mixture of 500 mL/L methanol and 75 mL/L acetic acid for 30 min and destained again with a mixture of 50 mL/L methanol and 75 mL/L acetic acid for 1 h. The relative mobility (Rf) of proteins was calculated and their molecular weight was estimated from the plot between Rf and log (MW) of standards.

2.4.7 Microstructure determination of oil droplets

2.4.7.1 Confocal laser scanning microscopy (CLSM)

The microstructures of coconut milk samples were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan.). The samples were dissolved in Nile blue A solution (1:10) and manually stirred until uniformity was obtained. Fifty microlitres of sample solutions were smeared on the microscope slide. The CLMS was operated in the fluorescence mode at excitation and emission wavelengths of 533 and 630 nm, respectively; using a Helium Neon Red laser (HeNe-R) for lipid analysis. A magnification of 400x was used.

2.4.7.2 Phase contrast microscopy

Oil droplets in coconut milk were observed under a phase contrast microscope (Model IX50; Olympus, Tokyo, Japan) equipped with camera. Samples were placed on a glass slide, covered with cover slip and observed at 400x magnification.

2.4.8 Determination of particle size

The particle size distribution of coconut milk emulsion was determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani *et al*. (2006). Prior to analysis, the sample (5 mL) was diluted with 1 mL sodium dodecyl sulfate (SDS) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

2.4.9 Determination of coalescence and flocculation

Coconut milk samples were diluted with distilled water in the presence and absence of SDS. The coalescence index (*Ci*) and flocculation factor (*Ff*) were calculated using the following equations (Intarasirisawat *et al*., 2014):

$$F_f = \frac{d_{43} - \text{SDS}}{d_{43} + \text{SDS}}$$

$$C_i = \frac{(d_{43} + \text{SDS, t} - d_{43} + \text{SDS, in})}{d_{43} + \text{SDS, in}} \times 100$$

where d_{43}+SDS and d_{43}-SDS are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 1% SDS, respectively; d_{43}+SDS,in and d_{43}+SDS,t are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 1% SDS at 0 h and the designated storage time (24 h). The determination was conducted at room temperature.

2.4.10 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For paired comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.5 Results and discussion

2.5.1 Proximate compositions of coconut meat and milk

The proximate compositions of coconut meat and milk at three different maturity stages are shown in Table 9. MC meat had the highest moisture content (61.07 g/100 g), followed by IMC (53.94 g/100 g) and OMC (39.50g/100 g), respectively. A similar trend was observed in coconut milk, in which MC had the highest moisture content (61.55 g/100 g), followed by IMC (55.36 g/100 g) and OMC (36.59 g/100 g), respectively. The high moisture content of MC meat and milk was more likely due to the absorption of coconut water inside the endosperm, since the beginning of germination. Water uptake is an essential step towards germination (Bewley and Black, 1994). On the other hand, low moisture content was observed in OMC meat and milk. The result suggested that the absorbed water in the endosperm was utilized during embryo development (Bewley and Black, 1994). It was found that there was a general increase of lipid content in coconut meat and milk with increasing maturity. OMC meat

and milk were found to have the highest lipid content ($p<0.05$). The lipid content of coconut increased with maturity stage due to the accumulation of lipids in the endosperm (López-Villalobos *et al*., 2001). Lower protein content was observed in both MC meat and milk as compared with those of IMC and OMC ($p<0.05$). Cocosin is a reserve protein found in coconut endosperm and serves as nitrogen source during germination (Balasundaresan *et al*., 2002). The result suggested that proteins could be degraded and utilized at the beginning of germination. Since water was utilized during the germination of OMC, the proportion of proteins was slightly increased. The ash content of both meat and milk decreased with maturity. The ash contents are indices of the mineral content (Obasi *et al*., 2012). It has been reported that coconut water contains sugars, vitamins, minerals, amino acids and phytohormones (Yong *et al*., 2009). The decrease in ash content in OMC meat and milk suggested that minerals are more likely used up during the germination process. A significantly lower carbohydrate content was observed in MC meat and milk ($p<0.05$). The obtained results are in accordance with that of Jeganathan (1970) who found that coconut milk at the mature stage, had a carbohydrate content of 5.5%. White *et al.* (1989) found that coconut milk at the mature stage is composed predominantly of galactose and arabinose with a small amount of mannose and glucose.

Table 9. Proximate composition of coconut meat and milk with three different maturity stages

Content	Coconut Meat			Coconut Milk		
(g/100 g)	IMC	MC	OMC	IMC	MC	OMC
Moisture	53.94±0.60b	61.07±1.02a	39.50±0.82c	55.36±0.15b	61.55±0.13a	36.59±1.05c
Lipid	18.59±0.89b	20.86±0.95b	32.45±0.35a	17.28±1.46c	30.34±0.96b	44.20±0.85a
Protein	4.79±0.16a	3.95±0.09b	4.45±0.56a	3.35±0.29a	2.90±0.06b	3.34±0.49a
Ash	1.15±0.02a	1.14±0.04a	1.04±0.03b	1.03±0.05a	1.00±0.04a	0.80±0.03b
Carbohydrate	21.53±0.98a	13.05±0.95b	22.34±0.85a	22.98±1.21a	4.21±0.93c	15.07±1.63b

IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut.
Values are given as mean ± SD (n=3). Different lowercase letters in the same row within the same commodity indicate significant difference ($p<0.05$).

Balasubramaniam (1976) reported that galactomannans and cellulose are present in the kernel of maturing and matured coconuts, whereas mannans are almost absent from very immature kernel and increased with maturation. Endosperm rich nutrients appear to function as a food reservoir for embryo development (Balasundaresan *et al*., 2002). Thus, reserved materials, particularly carbohydrates, were degraded and utilized during maturity. The decrease in carbohydrate plausibly led to the increased proportion of lipid in MC, as compared with the IMC sample. The results revealed that different maturity stages had marked impact on the chemical composition of coconut meat and milk.

2.5.2 Color of coconut milk

*L**, *a** and *b** values of coconut milks at three different stages of maturity are shown in Table 10. The coconut milks were milky white in color as evidenced by high *L** value (lightness). In general, coconut milk is an oil-in-water emulsion, where oil droplets are dispersed in the water phase. Light scattering of oil droplets is mostly associated with the white color of coconut milk. All samples had low *a** and *b** values, suggesting that deterioration caused by some microorganisms did not occur in all samples (Chiewchan *et al.*, 2006). The highest *L** value was found in the MC sample ($p<0.05$). The turbidity, cloudiness, or opaque appearance of emulsion is dependent on light scattering which is mediated by the dispersed oil droplets (McClements, 2002). Lightness is not only determined by lipids or oils, but also by the size of oil droplets, which is another prime factor governing the color, particularly the lightness of coconut milk.

Table 10. Color and pH of coconut milk with three different maturity stages

Samples	*L**	*a**	*b**	pH
IMC	92.90±0.24c	0.10±0.05a	5.09±0.06a	7.00±0.01a
MC	94.86±1.83a	-0.28±0.04b	4.48±0.12a	6.39±0.02b
OMC	93.09±0.18b	-0.35±0.03c	4.22±0.08b	5.58±0.13c

IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut. Values are given as mean ±SD (n=3). Different lowercase letters in the same row within the same commodity indicate significant difference ($p<0.05$).

2.5.3 pH of coconut milk

The pHs of freshly prepared coconut milks at three different stages of maturity are shown in Table 10. IMC milk was found to have a pH of 7.0 while MC milk was slightly acidic in pH (pH 6.39). The lowest pH (5.58) was obtained in OMC milk ($p<0.05$). Reserved food materials such as proteins, carbohydrates and lipids provide nourishment to growing embryo (Samson *et al*., 1971). The breakdown of these stored food materials by some enzymes, possibly occurred for embryo development. It was presumed that acidic metabolites or degradation products such as acidic amino acids may contribute to the lowered pH. The pH of 5.58 found in OMC milk is close to the isoelectric point of coconut proteins (pI = 4-5) (Samson *et al*., 1971; Monera and Del Rosario, 1982; Kwon *et al*., 1996). Therefore, pH may affect the emulsifying properties of proteins in coconut milk, especially those stabilizing oil droplets in the aqueous phase.

2.5.4 Electrophoretic patterns of coconut milk proteins

The protein patterns of coconut milks at different stages of maturity under reducing and non-reducing conditions are shown in Figure 2. Under non-reducing condition, there were six protein bands with MW of 55, 46, 33, 25, 18 and 16 kDa. The major protein in coconut endosperm is 11S globulin, which is referred to as cocosin, with MW of 55 kDa (Garcia *et al*., 2005). A hexamer (55 kDa) consists of acidic (32-34 kDa) and basic (22-24 kDa) subunits, which are linked by a disulphide bridge (Garcia *et al*., 2005; Tangsuphoom and Coupland, 2008). Some proteins present in the aqueous phase of coconut milk could act as emulsifier to stabilize fat globules (Peamprasart and Chiewchan, 2006). Cocosin plays a prominent role in regulating the stability of coconut milk (Tangsuphoom and Coupland, 2008). In the present study, smaller bands of proteins with MW higher than 55 kDa were found. Bands with higher intensity were found in IMC as compared with others. In the OMC sample, protein bands with MW of 70 and 46 kDa almost disappeared, indicating the degradation of proteins during germination. Under the reducing condition, several major protein bands with MW of 55, 33, 31, 25, 21, 20, 18 and 16 kDa were observed, which are in agreement with previous reports of Garcia *et al.* (2005) and Demason and Sekhar

(1990). Under the reducing condition, cocosin dissociated into acidic and basic polypeptides with the coincidental appearance of proteins with MW of 32 and 22 kDa. Other proteins with MW greater than 55 kDa were not found. The occurrence of protein with MW of 20 kDa was also observed under reducing conditions. Protein with MW of 36 kDa was found only in the MC sample. In general, the OMC sample showed the lower band intensity of most proteins as compared with others. The result confirmed that the protein composition of coconut milk changed with maturity stages.

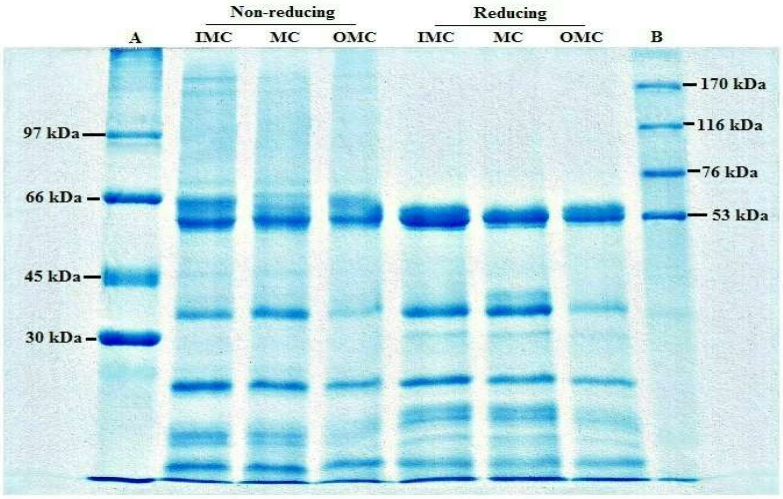

Figure 2. SDS-PAGE patterns of coconut milk proteins with three different maturity stages. A: low molecular weight standards; B: high molecular weight standards; IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut.

2.5.5 Microstructure of oil droplets in coconut milk

The microscopic structures of coconut milk emulsions at three different stages of maturity were visualized by confocal laser scanning microscopy (CLSM) and phase contrast microscopy (Figure 3). In the same coconut milk sample, similar results were observed when both CLSM and phase contrast microscopies were used. CLSM generally provides higher clarity and better resolution images of the emulsion microstructure than convention optical microscopy. The observation of the microstructure of the emulsion was facilitated using a fluorescence dye such as Nile blue A, in order to label the lipid.

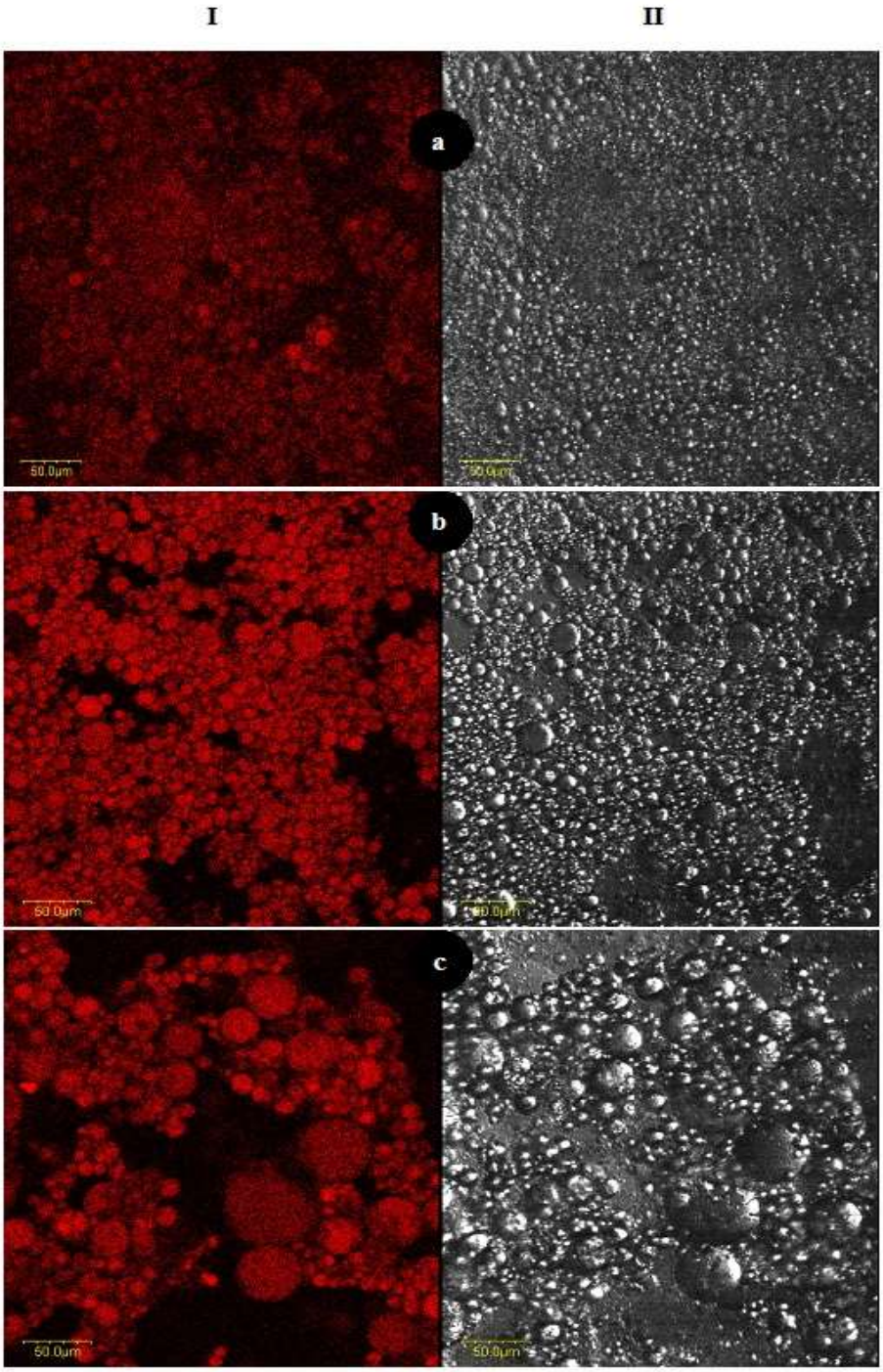

Figure 3. Confocal laser scanning micrographs (I) and phase contrast microscopy (II) of coconut milk with three different maturity stages: (a) IMC: Immature coconut; (b) MC: Mature coconut; (c) OMC: Overlay mature coconut. Magnification: 400×. Scale bar = 50 µm.

However, phase contrast microscopy provides excellent contrast, and a halo is formed even around a small oil droplet. For IMC (Figure 3a), smaller oil droplets with uniform shape and size were distributed uniformly in the aqueous phase. An emulsion with the same size of oil droplets is referred to as a monodisperse emulsion, whereas that containing a range of droplet sizes is referred to as a polydisperse emulsion (McClements, 2004). Coconut milk is an oil-in-water emulsion naturally stabilized by coconut proteins (Birosel *et al*., 1963). In the present study, IMC had higher protein/lipid ratio as compared with the MC sample (Table 9). High protein content can lead to efficient localization of protein films at the oil-water interphase. Thus, this could increase the emulsion stability of coconut milk. Moreover, proteins could stabilize the coconut milk emulsion by lowering the interfacial tension between two phases, in which oil droplets are dispersed uniformly throughout the water phase. However, polydisperse emulsion was observed in MC and OMC (Figure 3b and c) with a wide range of oil droplet sizes. Large sizes of oil droplets were abundantly observed in the OMC. In OMC, coconut milk contained a high amount of lipid. Thus, the present proteins may not be sufficient to stabilize the emulsion. The low pH of OMC milk could be another factor enhancing the destabilization of emulsion, by lowering the repulsion of protein film surrounding the oil droplets. In general, the emulsion was less stable as evidenced by the larger droplets with non-uniform distribution. The results clearly indicated that maturity stages affected oil droplet size.

2.5.6 Particle size distribution

Particle size distributions expressed as d_{32} and d_{43} of coconut milk emulsions at three different stages of maturity are shown in Table 11. The d_{32} increased from 3.38 μm (IMC) to 5.48 μm (OMC), while $d4_3$ increased from 5.29 μm (IMC) to 13.38 μm (OMC) with increasing maturity stages. Coconut milk from OMC contained the largest oil droplets (d_{43} and d_{32}), followed by those from MC and IMC. The d_{32} is related to the average surface area of droplet exposed to the continuous phase per unit volume of emulsion. The smaller d_{32} indicates higher specific surface area (Intarasirisawat *et al*., 2014). The d_{43} is the sum of the volume ratio of droplets in each size class multiplied by the mid-point diameter of the size class. The d_{43} can be used as the index of coalescence and flocculation (Hebishy *et al*., 2013). The proteins in

coconut milk are known to function as emulsifier, which stabilizes the oil droplets in coconut milk (Dionisio, 1963; Monera and Del Rosario, 1982). The largest size of oil droplets in OMC can be attributed to the pH of the OMC milk, close to pI. As a result, there was a decrease in repulsion between protein films surrounding the oil droplets, thereby facilitating the coalescence. When the repulsive forces dominate, the droplets tend to remain as individual entities (McClements, 2004) and form a stable emulsion. After 24 h of storage at room temperature, both d_{32} and d_{43} increased (Table 11), indicating the coalescence of oil droplets. Among all samples, a slight increase in d_{32} and d_{43} were observed in the OMC sample. The result suggested that the collapse of emulsion in the OMC milk was pronounced at the initial time and less coalescence occurred after 24 h. Conversely, the emulsion collapsed continuously in the MC and IMC samples.

Table 11. Droplet size and stability of coconut milk with three different maturity stages

Samples	Storage Time (h)	d_{32} (µm)	d_{43} (µm)	F_f	C_i
IMC	0	3.38±0.10C	5.29±0.20C	1.18±0.03A	-
	24	4.16±0.40b	8.10±0.10c	0.77±0.04b	53.22±0.59a
MC	0	5.07±0.25B	12.31±0.15B	0.90±0.90A	-
	24	5.55±0.60a	14.22±0.21a	0.78±0.03b	15.52±0.89b
OMC	0	5.48±0.13A	13.38±0.03A	1.26±0.04A	-
	24	5.66±0.26a	13.44±0.01b	1.26±0.01a	0.45±0.50c

IMC: Immature Coconut, MC: Mature Coconut and OMC: Overlay Mature Coconut.
Values are given as mean ±SD (n=3). F_f: Flocculation factor, C_i: Coalescence index
Different uppercase letters in the same column at the initial storage time (0 h) indicate significant difference ($p<0.05$).
Different lowercase letters in the same column after the designated storage time (24 h) indicate significant difference ($p<0.05$).

2.5.7 Coalescence and flocculation

The coalescence index (*Ci*) and flocculation factor (*Ff*) of the coconut milk emulsions were investigated to determine the instability of the emulsion as shown

in Table 11. To mimic the traditional method, the coconut milk was allowed to stand at room temperature for 24-72 h (Senphan and Benjakul, 2017). Emulsions are thermodynamically unstable due to the unfavorable contact between oil and water (Fredrick *et al*., 2010) and their physical structures are likely to change over time by various mechanisms including coalescence and flocculation. In IMC, higher *Ci* was observed after 24 h, as compared with the MC and OMC samples ($p<0.05$). On the other hand, *Ff* decreased, suggesting that the individual oil droplets assembled to form larger oil droplets as evidenced by the increase in droplet size. The increase in d_{43} also reconfirmed the assembly of individual droplets into larger flocs (Intarasirisawat *et al*., 2014). The formation of larger oil droplets indicates poor emulsion stability (Fredrick *et al*., 2010). The interactions between oil droplets depend on the quality and quantity of proteins (Damodaran, 2005). The proteins in IMC were plausibly not effective in stabilizing the coconut milk emulsion, especially after the extended storage. However, the lowest rate of *Ci* was observed for OMC. This coincided with the lowest rate of changes in d_{32} and d_{43} of OMC. For OMC, initial pH close to pI might not favor the solubility of proteins and as such, it was presumed to have poor emulsifying property. Additionally, the partial crystallization of lipid within the oil droplets could be another factor that favors the destabilization of emulsion by coalescence (Rousseau, 2000). Nevertheless, the emulsion initially found was slightly altered upon storage time. The results suggested that the oil droplet size and emulsion stability of coconut milk depended on the maturity stages.

2.6 Conclusion

Coconut milk and meat at three different maturity stages had varying proximate compositions. Cocosin with MW of 55kDa was predominantly observed in coconut milks, regardless of the maturity stage. Polydisperse emulsion was observed in coconut milk at the mature and overlay mature stages, whilst the monodisperse counterpart was found in coconut milk of the immature stage. The stability of coconut milk emulsion depends on intrinsic factors, mainly pH and protein content. Thus, the maturity stages had influence on the physicochemical properties and emulsion stability of coconut milk. The present study has provided a better understanding of the impact of maturity stage on the characteristics and emulsion stability of coconut milk, used as

the starting material for VCO production. OMC was more appropriate for the production of VCO with higher lipid as compared with other stages.

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CHAPTER 3
CHARACTERISTICS AND QUALITY OF VIRGIN COCONUT OIL AS INFLUENCED BY MATURITY STAGES

3.1 Abstract

Characteristics and quality of Virgin Coconut Oil (VCO) extracted from coconut with three different maturity stages including immature coconut (IMC), mature coconut (MC) and overlay mature coconut (OMC) were comparatively studied. The highest recovery (95.64%) was found in VCO from OMC ($p<0.05$), followed by those from MC (84.40%) and IMC (61.06%), respectively. All VCO samples had water-like appearance and contained medium chain fatty acid (MCFA), especially lauric acid (C12:0) as a major fatty acid, (49.74-51.18 g/100g). Myristic acid (C14:0) in the range of 18.70-19.84 g/100g was present in all VCO. Quality parameters of all VCO samples complied with Asian Pacific Coconut Community (APCC) standards. All VCO samples had low lipid hydrolysis and oxidation, indicating that maturity stages had no influence on oil stability. Thus, maturity stages played an essential role in recovery, but showed no impact on fatty acid composition and physicochemical properties of resulting VCO.

3.2 Introduction

Coconut oil is one of important oils used extensively in many traditional foods of Asian and Pacific regions (Onsaard *et al*., 2005). Coconut oil is different from other vegetables oils due to high content of medium chain fatty acids (MCFAs), particularly lauric acid. MCFAs are burned up immediately after consumption and therefore the body uses it instantly to make energy, instead of storing it as body fat (Enig, 1996). Lauric acid is converted into a very valuable compound known as monolaurin, which has antiviral and antibacterial properties (DebMandal and Mandal, 2011). It is therefore assumed that consumption of coconut oil may help to protect the body from infections. Coconut oil is generally extracted from wet coconut kernel (meat) by mechanical or natural means, with or without the use of heat. Chemical refining, bleaching or de-odorizing processes are not required. As a consequence, the nature of oil is not altered and is known as virgin coconut oil

(VCO) (Villarino *et al.*, 2007). VCO is rapidly gaining immense importance due to various health benefits, high degree of saturation and good stability. VCO is the purest form of coconut oil with natural distinctive coconut taste and smell. It is solidified at low temperature and becomes colorless like water when liquefied (Marina *et al*., 2009).

Coconut milk, a milky white oil-in-water emulsion, has been used as a starting material for VCO production. The emulsion in coconut milk was naturally stabilized by coconut proteins: globulins and albumins, as well as phospholipids (Raghavendra and Raghavarao, 2011). To obtain VCO, destabilization of coconut milk emulsion using different extraction methods has been implemented (Raghavendra and Raghavarao, 2010). Amongst all processes, the enzyme-assisted separation process has been known to be effective and less time consuming. Additionally, high yield could be obtained from coconut milk with the aid of protease like Alcalase (Man *et al*., 1996). The efficiency of enzyme in extraction of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu *et al*., 2008).

Yield is generally one of prime parameters in VCO extraction. Properties of VCO are also essential and must meet the specification set by the organization with authority. Both yield and quality of VCO could be affected by several factors, both intrinsic and extrinsic ones. Maturity stage of coconut could be another factor influencing oil level in coconut kernel associated with oil yield as well as properties of VCO obtained. Although OMC showed the higher oil content, VCO is mostly produced from MC. The objective of this study was to comparatively investigate the recovery, fatty acid composition and properties of VCO from coconut milk with three different maturity stages.

3.3 Objective

To examine the characteristics and quality of virgin coconut oil extracted from different maturity stages.

3.4 Materials and methods

3.4.1 Chemicals

Potassium hydroxide and *ρ*-anisidine were purchased from Sigma (St. Louis. MO, USA). Sodium thiosulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, chloroform, petroleum ether, hydrochloric acid, sulfuric acid, n-hexane and cyclohexane were procured from Lab-Scan (Bangkok, Thailand). Alcalase (2.4L) with the activity of 2.4 AU/g and a density of 1.17 g/mL was purchased from Brenntag (Toronto, Canada).

3.4.2 Preparation of coconut milk with different maturity stages

Coconuts from three different maturity stages including immature coconut (IMC) (9-10 months old from pollination), mature coconut (MC) (11-12 months old from pollination) and overlay mature coconut (OMC) (14-15 months old from pollination) were purchased from a plantation site in Yaring district, Pattani province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was removed manually and grated using a rotary wedge cutter machine. Coconut milk was then prepared from grated kernel without water addition using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand). Coconut milk from IMC, MC and OMC had oil contents of 17.28%, 30.18% and 46.2%, respectively, as determined by the Bligh and Dyer method (Bligh and Dyer, 1959).

3.4.3 Separation of virgin coconut oil (VCO) using Alcalase

Coconut milk was adjusted to pH 8 using 1 M NaOH and added with Alcalase at a level of 0.5% (v/v). The hydrolysis was performed at 60 C for 90 min in a shaking water bath (W350, Memmert, Schwabach, Germany). After the designated time, the mixtures were centrifuged at 4900xg for 30 min at room temperature (26-28 °C) using a centrifuge (Beckman coulter, Allegra™ centrifuge, CA, USA). VCO was

collected from the upper oil phase. All VCO samples from coconuts with different maturity stages were calculated for the recovery and subjected to analyses.

3.4.4 Oil recovery

The Bligh and Dyer method (Bligh and Dyer, 1959) was employed to determine total oil content of coconut milk. The weight of oil separated after Alcalase aided process (VCO) was determined. Oil recovery was calculated using the following equation (Mansor *et al*., 2012):

$$\text{Oil recovery (\%)} = \frac{\text{Weight of VCO}}{\text{Weight of total oil in emulsion}} \times 100$$

3.4.5 Characteristics and quality of VCO

3.4.5.1 Determination of fatty acid profile

Fatty acid profile of VCO samples was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (AOAC, 2000). The prepared FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m ′ 0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 °C. The oven was programmed from 170 to 225 °C at a rate of 1 °C /min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

3.4.5.2 Fourier transform infrared (FTIR) spectra analysis

FTIR analysis of VCO samples was performed in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run.

For spectra analysis, VCO sample (200µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra, in the range of 4000– 400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

3.4.5.3 Color determination

Color of VCO samples were measured using a colorimeter (HunterLab, Model colourFlex, VA, USA). The color was reported in L^*, a^*, b^* values representing lightness, redness/ greenness and yellowness/ blueness, respectively. Total difference in color (ΔE^*) and the difference in chroma (ΔC^*) were also calculated using following equations:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where, ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard (L^*= 93.55, a^* = 0.84, b^* = 0.37).

$$\Delta C^* = C^*_{sample} - C^*_{Standard}$$

where, $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

3.4.5.4 Determination of moisture content and volatile matters

VCO samples were analyzed for moisture content according to the method of AOAC (AOAC, 2000) at 105 °C.

3.4.5.5 Measurement of viscosity

Viscosity of VCO samples was determined using a controlled-stress rheometer (Rheostress RS1, Thermo Hakke, Germany) with parallel plates (35 mm

diameter). The samples were sheared at a constant shear rate of $100s^{-1}$ over temperatures ranging from 25 to 80 °C and their viscosity was then measured.

3.4.5.6 Measurement of refractive index (RI), relative density and specific gravity

RI of VCO samples was determined using a refractometer (Model AR3D-AR64, Abbe, Hamburg, Germany). Measurements were performed at 40 °C. The relative density and specific gravity of all VCO samples were measured according to the AOAC method (AOAC, 2000). Measurements were performed at 30 °C.

3.4.5.7 Measurement of iodine value (IV), saponification value (SV), peroxide value (PV) and *ρ*-anisidine value (AnV)

IV, SV. PV and AnV of all VCO samples were analyzed according to the method of AOCS (AOCS, 2009).

3.4.5.8 Measurement of acid value and free fatty acid (FFA)

Acid value of all VCO samples was determined by the method of AOCS (AOCS, 2009) and FFA was determined by the following equation, using the conversion factor 2.81 for lauric acid. Acid value = % FFA × 2.81

3.4.5.9 Thermal analysis by DSC

Thermal properties of VCO samples were determined using a differential scanning calorimeter (PerkinElmer, Model DSC-7, Norwalk, CT, USA). The DSC instrument was calibrated with indium (m.p. 156.6 °C, ΔHf = 28.45 J/g). Samples (10-12 mg) were weighed into aluminum pans and were hermetically sealed. Prior to analysis of samples, the baseline was obtained from an empty hermetically sealed aluminum pan. Samples were subjected to the following temperature program: 80 °C for 5 min, cooled at 5 °C/min to -80 °C and held for 5 min. The same sample was then heated from -80 to 80 °C at the same rate. Melting and crystallization temperatures and enthalpy of samples were determined.

3.4.5.10 Cloud and pour points

Cloud and pour points of all VCO samples were determined according to ASTM D 2500 and ASTM D 97 standard method, respectively. Cloud and pour point analyzer (Walter Herzog GmbH, Lauda-Königshofen, Germany) was used. The samples (50 mL) were cooled to -40 °C with continuous agitation. The temperature of first crystallization, at which a thermometer in the sample was no longer visible, was considered as 'cloud point'. Pour point was defined as the temperature, at which oil sample was solidified and is not able to flow.

3.4.5.11 Flash point

Flash point of VCO samples were determined according to the ASTM D 93 standard method using a flash point tester (Pensky-Martens Model HFP 386, Walter Herzog GmbH, Lauda-Königshofen, Germany). The samples (100 mL) were heated using an electric heater. The temperature, at which the first flash appeared on the surface of sample, was recorded.

3.4.5.12 Sensory characteristics

Thirty non-trained panelists (aged between 25 and 40 years) were used for difference-from-control test. They were the students and staffs from the Department of Food Technology, who were acquainted with VCO. VCO samples from three different maturity stages were coded with 3-digit random numbers. VCO samples and one blind control MC sample, with a volume of 20 mL were served at room temperature under the fluorescent daylight-type illumination. The panelists were asked to evaluate the difference from the blind control for color, odor, viscosity, flavor and overall of VCO samples using 10-point scale (0, no difference; 9, extreme difference) (Meilgaard *et al*., 2006). Between samples, the panelists were asked to rinse their mouth with warm water.

3.4.6 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Mean comparison

was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1960). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussions

3.5.1 Oil recovery

Varying oil recovery was observed, depending on maturity stages. The highest oil recovery (95.64%) was found when coconut milk from OMC was used ($p<0.05$), followed by MC (84.45%) and IMC (61.06%). The result suggested that the emulsion of coconut milk from OMC was less stable than others. Therefore, Alcalase might be able to hydrolyze the proteins stabilizing oil droplets in OMC milk emulsion more effectively. Coconut milk is naturally stabilized by coconut proteins (McGlone *et al*., 1986). During germination, proteins in kernel were plausibly hydrolyzed, in which free amino acids could be used for embryo. Man *et al*. (1996) reported that Alcalase played a significant role in destabilization of coconut milk emulsion, thereby liberating free oil. On the other hand, low oil recovery (61.06%) was obtained when IMC milk was used. Proteins in coconut kernel at this stage might be more resistant, due to the high complexation. Therefore, the proteins could stabilize oil droplets in coconut milk effectively and thus oil released was lower. The result suggested that maturity stage of coconut fruit was an important factor governing the oil released from coconut milk emulsion when Alcalase was employed.

3.5.2 Characteristics and quality of VCO

3.5.2.1 Fatty acid compositions

Fatty acid profiles of VCO separated from coconut milk with three different maturity stages are shown in Table 12. Regardless of maturity stages, all VCO samples dominantly contained medium chain fatty acids (MCFA) especially lauric acid (49.74-51.18%). The second major fatty acid was myristic acid with 18.70-19.84%. The VCO from OMC had the highest lauric acid, whilst that from MC showed the highest myristic acid ($p<0.05$). The most abundant MCFA present in coconut oil is lauric acid, which is responsible for health benefits (1996). MCFAs

have 6 to 12 carbons, including caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids (Papamandjaris *et al*., 1998). Palmitic acid ranged from 8.16 to 8.87 g/100g. Caprylic acid and capric acid constituted 7.20-7.45 and 6.11-6.52 g/100g, respectively. Oleic acid was found in the range of 3.73-4.58 g/100g and the lowest content was found in VCO from MC milk ($p<0.05$). Stearic acid was found at low level, but VCO from MC showed the highest content, compared to others. In general, there was no marked difference in fatty acid profiles between VCO extracted from coconut milk having different maturity stages. Furthermore, the fatty acid composition was comparable to those guided by APCC standards. Due to the high level of saturated fatty acids, VCO could be solidified at low temperature with ease.

Table 12. Fatty acid profile of virgin coconut oil with three different stages.

Fatty acids (g/100 g oil)	**IMC**	**MC**	**OMC**	**APCC Standards**
Caproic acid (C6:0)	0.32±0.03b	0.41±0.05a	0.31±0.02b	0.10 – 0.95
Caprylic acid (C8:0)	7.30±0.37a	7.45±0.41a	7.20±0.34a	4 – 10
Capric acid (C10:0)	6.37±0.11a	6.11±0.07b	6.52±0.10a	4 – 8
Lauric acid (C12:0)	50.32±0.23b	49.74±0.27c	51.18±0.10a	45 – 56
Myristic acid (C14:0)	18.98±0.16b	19.84±0.15a	18.70±0.16b	16 – 21
Palmitic acid (C16:0)	8.47±0.06b	8.87±0.07a	8.16±0.09c	7.5 – 10.2
Stearic acid (C18:0)	2.62±0.01b	3.08±0.02a	2.56±0.03c	2 – 4
Cis-9-Octadecanoic acid (C18:1 n-9)	4.58±0.03a	3.73±0.03b	4.58±0.06a	4.5 - 10
Cis-9,12-Octadecadienoic acid (C18:2 n-6)	0.93±0.00a	0.65±0.00c	0.70±0.01b	0.7 – 2.5
Arachidic acid (C20:0)	0.05±0.00a	0.05±0.00a	0.05±0.00a	-
Unidentified peak	0.07±0.00a	0.06±0.01a	0.03±0.00b	-

Values are mean ± standard deviation (n=3). Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut

APCC: Asian and Pacific Coconut Community.

3.5.2.2 Fourier transform infrared (FTIR) spectra

FTIR spectra at wavenumber ranging from 4000 to 500 cm^{-1} of VCO prepared from coconut milk with three different maturity stages are shown in Figure 4. Infrared mid region of the spectrum has been used for characterization of vegetable oils and the absorption bands are associated with the vibration of particular functional groups (Guillén and Cabo, 1997). For all VCO samples, the major peak was found at wavenumber of 1742 cm^{-1}. Generally, the carbonyl absorption of the triglyceride ester linkage was observed at 1741-1743 cm^{-1} (Chaijan *et al*., 2006). Sánchez-Alonso *et al*. (2012) reported that the ester bond of hake lipids was found in the 1743-1740 cm^{-1} region. The result indicated that triglyceride containing ester bonds constituted in all oil samples. Major peaks were also observed at a wavenumber range of 2922 cm^{-1} and 2853 cm^{-1} indicating asymmetrical and symmetrical stretching of –CH2, respectively. Furthermore, peaks at the wavenumbers of 1462 (–CH2 bending), 1418 (cis =C–H bending), 1375 (–CH3 bending), 1228 (–C–O stretch), 1153 (–C–O stretch; –CH2 bending), 1109 (–C–O stretch), 963 (trans-CH=CH– bending out of plane), and 722 cm^{-1} (cis-CH=CH– bending) (Guillén and Cabo, 1997; Lerma-García *et al*., 2010) were also observed in all samples, irrespective of maturity stages.

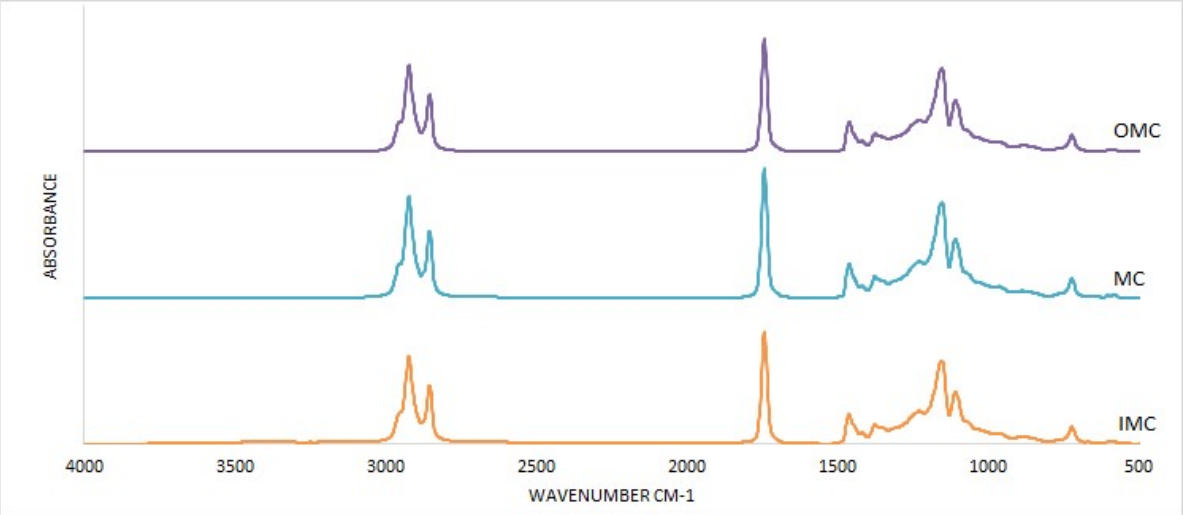

Figure 4. Fourier transform infrared (FTIR) spectra of virgin coconut oil with three different maturity stages. IMC: Immature coconut; MC: Mature coconut; OMC: Overlay mature coconut.

Overall, the similar spectra were observed in all VCO samples, which was in agreement with similar fatty acid profiles of the oils (Table 12). The peak near 3006 cm^{-1} was associated to the stretching vibration of C=C bonds. These bonds are mostly presented in unsaturated fatty acids (Henna Lu and Tan, 2009). However, the peak near 3006 cm^{-1} was absent in all VCO samples, indicating the low degree of unsaturation. This was in agreement with fatty acid profile (Table 12). From the spectra, there was no peaks at 3470 cm^{-1} and 1711 cm^{-1} detected in all samples, representing hydroperoxides and FFAs, respectively (Van de Voort *et al*., 1994; Guillén and Cabo, 1997). The result indicated that coconut milk from different maturity stages had no impact on oxidative stability of the resulting VCO.

3.5.2.3 Color

The colors of VCO extracted from coconut milk with different maturity stages expressed as L^*, a^* and b^* values, are shown in Table 13. All VCOs were clear like water and showed high L^* value with the distinct coconut aroma. VCO separated from OMC milk showed the lowest L^* value ($p<0.05$), whereas the highest a^* and b^* values were found for the oil of IMC ($p<0.05$). The VCO was extracted from a fresh coconut kernel, negligible pigments associated with deterioration were contaminated into the separated oil. The ΔE^* and ΔC^* were observed for the VCO of OMC ($p<0.05$). This was plausibly due to the differences in indigenous pigments found in kernel from coconut with different maturity stages. VCO is generally colorless and has natural pleasant coconut smell.

Table 13. Color of virgin coconut oil with three different maturity stages.

Sample	L^*	a^*	b^*	ΔE^*	ΔC^*
IMC	97.13±0.20a	-1.68±0.03b	1.78±0.08a	4.15±0.26a	2.25±0.11a
MC	97.53±0.07a	-1.51±0.02a	1.26±0.02b	4.19±0.08a	1.56±0.04b
OMC	96.86±0.04b	-1.54±0.02a	0.96±0.04c	3.20±0.07b	1.29±0.05c

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference between means ($p<0.05$).

IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut.

The refining, bleaching, and deodorizing (RBD) process may cause yellow color of coconut oil (Marina *et al*., 2009). In the present study, no further refining process was implemented. As a result, color and smell of VCO had natural characteristics.

3.5.2.4 Moisture content

Moisture content of all VCO samples was negligible (~0.1%) (Table 14). As per APCC standard, VCO must have moisture in the limited range (max 0.1%) (APCC, 2003). High moisture content indicated that VCO could be prone to hydrolysis process, leading to the enhanced rancidity (Raghavendra and Raghavarao, 2010). In the present study, all VCO samples had very low moisture content, thereby assuring their long shelf-life. Furthermore, oil fraction could be separated from aqueous phase effectively after Alcalase aided process. This was evidenced by low moisture content of separated VCO.

3.5.2.5 Viscosity

Viscosity of all VCO samples as a function of temperatures ranging from 25 to 80 °C is shown in Figure 5. All VCO samples showed a non-linear decrease in viscosity with increasing temperature. VCO with different maturity stages showed the viscosity over the temperatures tested. Heat applied to oil has been recognized to reduce intermolecular interactions by thermal molecular movement (Santos *et al*., 2005). Coconut oil mainly consists of medium chain fatty acid (MCFA) and hence its viscosity is lower as compared to oils with long chain fatty acid (Akhtar *et al*., 2009). Additionally, fatty acids with more double bonds do not have a rigid and fixed structure. The loosely packed structure is associated with more fluid-like nature (Abramovic and Klofutar, 1998). Although all VCO samples had slight difference in fatty acid composition, similar viscosity was noticeable. The results suggested that there was no marked difference in viscosity of VCO samples with three different maturity stages.

Table 14. Properties of virgin coconut oil with three different maturity stages.

Sr. No.	APCC Parameters	IMC	MC	OMC	APCC Standards
1.	Moisture (%)	0.1±0.03a	0.1±0.01a	0.1±0.01a	Max 0.1
2.	Refractive index at 40 °C	1.4486±0.00a	1.4486±0.00a	1.4486±0.00a	1.4480– 1.4492
3.	Relative density	0.921±0.00a	0.921±0.01a	0.921±0.00a	0.915 – 0.920
4.	Specific gravity at 30 deg./30 deg. C	0.921±0.00a	0.921±0.01a	0.921±0.00a	0.915 – 0.920
5.	Iodine Value (g I_2/100g oil)	5.54±0.25a	4.70±0.10b	5.39±0.04a	4.1 – 11
6.	Saponification Value (mg KOH/g oil)	258.42±0.77a	256.76±0.75c	257.29±0.63b	250 – 260
7.	Free fatty acid (%)	0.06±0.03a	0.06±0.02a	0.06±0.02a	Max 0.2
8.	Peroxide Value (meq O_2/kg)	1.66±0.57a	1.66±0.58a	1.66±0.57a	Max 3
9.	*ρ*-anisidine	0.57±0.02a	0.57±0.02a	0.57±0.03a	-

The mean ± standard deviation, n=3. Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut. APCC: Asian and Pacific Coconut Community.

3.5.2.6 Refractive index (RI), relative density and specific gravity

RI values of different VCO samples are shown in Table 14. All samples had the same RI (1.4486), which was within the standard range (1.4480 – 1.4492) of VCO guided by APCC (APCC, 2003). Refractive Index (RI) is related with the degree of unsaturation in lipid. RI increases proportionally with increasing number of double bonds. It is also used to indicate impurity of the oil since each substance has a different RI (Kamariah *et al*., 2008).

Both relative density and specific gravity were similar between all VCO samples obtained from coconut milk with various maturity stages. The values were 0.921, which were close to the standard range (0.915 - 0.920) following the guidance of APCC (APCC, 2003). Specific gravity is ratio of the density of a substance to the density of a reference substance at the same temperature. The density varies according to the temperature of oil reported by Kamariah *et al*. (2008). The result indicated that VCO from coconut milk having different maturity stages more likely had similar compositions, especially lipids or fatty acids. This was confirmed by similar fatty acid compositions between samples (Table 12).

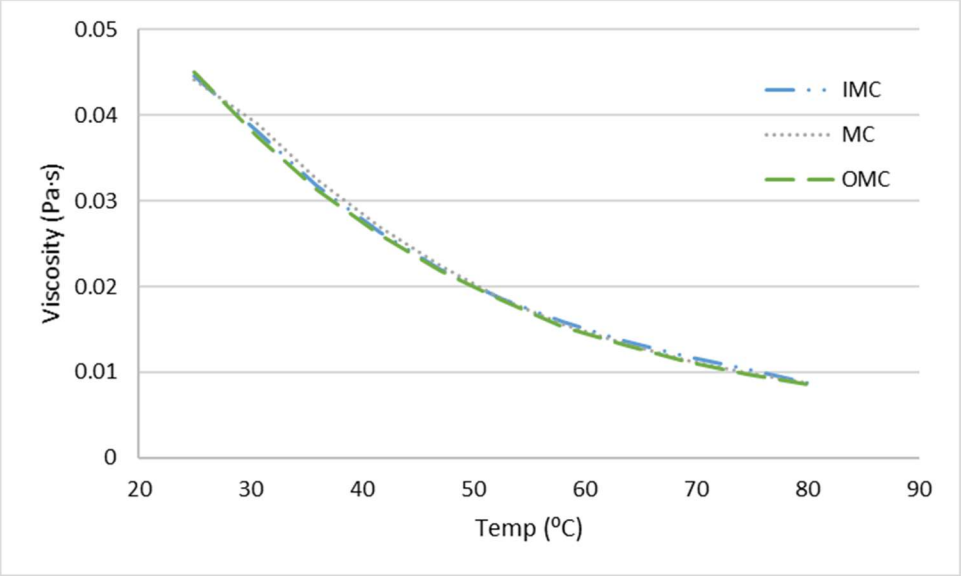

Figure 5. Viscosity of virgin coconut oil with three different maturity stages as a function of temperatures. IMC: Immature coconut; MC: mature coconut; OMC: Overlay mature coconut.

3.5.2.7 Iodine value (IV)

All VCO samples had IV with the range of 4.70-5.54 g I_2/100g oil (Table 14), which were in accordance with APCC standard (APCC, 2003). The Iodine value (IV) is used to measure the degree of unsaturation in fatty acids. The lowest IV was observed for the oil of MC ($p<0.05$), which was correlated with lower contents of oleic acid (C18:1) and linoleic acid (C18:2) (Table 12). The low IV found in all VCO samples indicated that the VCO samples were highly saturated, which ensured their

stability against oxidative rancidity. Additionally, they could be solidified at low temperature, e.g. refrigerated temperature.

3.5.2.8 Saponification value (SV)

The relative high SV were found for all VCO samples, ranging from 256.76 to 258.42 mg KOH/ g (Table 14). High SV of all VCO samples indicated that VCOs contained high amount of short chain or medium chain fatty acids (Marina *et al*., 2009). This coincided with the high contents of fatty acids with chain length of C8-C16 (Table 12). Amongst all VCO samples, slight differences in SV were observed. This was probably due to same differences in fatty acid composition. The values were within the standard range (250 – 260 mg KOH/ g) guided by APCC standard (APCC, 2003). Saponification value (SV) is an index of the average molecular weight of triglycerides present in sample. Saponification is the process of breakdown of a fat sample into glycerol and fatty acids by treatment with strong base (KOH). SV is inversely proportional to the molecular weight of triglycerides (Opoku-Boahen *et al*., 2012). The long chain fatty acid on glycerol backbone gives a low SV, however short chain counterpart renders a high SV (Marina *et al*., 2009).

3.5.2.9 Peroxide value (PV)

Low PV (1.66 meq O_2/kg) was found in all VCO samples (Table 14). PV of VCO samples were within the standard range (max 3 meq O_2/kg) guided by to APCC standard (APCC, 2003) PV is used to determine the primary oxidation products of oil (Choe and Min, 2006). Low PV in VCO indicated that low lipid oxidation took place in all samples. This was more likely due to the low content of unsaturated fatty acids confirmed by iodine value. Conversely, saturated fatty acids were prevalent (Table 12). This made VCO more stable to oxidation. When comparing PV between samples, the similar PV was observed, suggesting the similar oxidative stability of VCO from coconut milk with different maturity stages. This was in agreement with FTIR spectra, in which no peak representing hydroperoxide was detected (Figure 4).

3.5.2.10 Anisidine value (AnV)

Low AnV was observed in all VCO samples (0.57) (Table 14). There were no differences in AnV among the samples ($p>0.05$). According to Rossell (1983), oils with an AnV below 10 were considered as good quality. AnV is used to determine the secondary oxidation products of oil. The primary oxidation products i.e. peroxides are not stable and decomposed to the secondary products such as aldehydes, which are responsible for rancid odor and taste. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Low AnV of all samples was in agreement with low PV, regardless of maturity stages. The results reconfirmed high oxidative stability of all VCO samples.

3.5.2.11 Free fatty acid (FFA)

FFA contents of VCOs separated from coconut milk with various maturity stages are shown in Table 14. All VCO samples had the low FFA content (0.06%). The result indicating that ester bonds of triglycerides were cleaved to low degree. This was in agreement with FTIR spectra, in which no peak at 1711 cm^{-1} representing the free fatty acid was noticeable (Figure 4). FFAs are responsible for unacceptable taste and aroma in oils and fats. It was reported that VCO produced by enzymatic process contained low FFA with a good smell and long lifetime (Rahayu *et al*., 2008). Marina *et al*. (2009) found that FFAs were high in coconut oils having high moisture content. There was no difference in FFA content amongst VCOs obtained from coconut with different maturity stages. FFA contents of all VCO samples were below APCC standard (APCC, 2003), indicating their good initial quality.

3.5.2.12 Thermal behavior

Melting and crystallization temperatures of all VCO samples are shown in Table 15. Endothermic peaks, representing melting point, were observed for VCO from MC, OMC and IMC at 24.87, 23.94 and 23.30 °C, respectively. Exothermic peaks of VCO from MC, OMC and IMC were found at -1.13, -1.74 and -1.89 °C, respectively. The onset and endset temperatures indicate the start and the end of the melting process. Different onset and endset temperatures were observed

between all the samples. Onset temperatures were observed for VCO from MC, OMC and IMC at 12.63, 9.46 and 9.52 °C, respectively. Endset temperatures of VCO from MC, OMC and IMC were found at 28.86, 27.99 and 26.40 °C, respectively. Generally, the highly saturated oil melts at higher temperatures than the unsaturated oil. The former oil also undergoes crystallization at higher temperature. For crystallization process, onset temperatures were observed for VCO from MC, IMC and OMC at 4.72, 4.43 and 4.17 °C, respectively. Endset temperature of VCO from MC, OMC and IMC were found at -4.07, -4.25 and -4.62 °C, respectively. Differences in the onset and endset temperatures for melting and crystallization process were plausibly due to differences in chemical compositions of VCO from different maturity stages. VCO sample from MC had lower degree of unsaturation than others as indicated by lower IV (Table 14). Thus, it was expected to melt and crystallize at higher temperature when compared to OMC and IMC samples. Melting and crystallization, two commonly used physical events to characterize thermal behavior of VCO samples, require the intake or release of thermal enthalpy, respectively. The melting and crystallization enthalpies of all VCO samples are shown in Table 15. VCO samples from MC showed the highest melting enthalpy at 117.96 J/g ($p<0.05$), followed by OMC (114.17 J/g) and IMC (112.16 J/g), respectively. Crystallization enthalpy of MC was -110.73 J/g, and OMC (-105.78 J/g) and IMC (-104.29 J/g). Generally, the oil samples with high degrees of saturation showed slightly higher enthalpy than those with a high degree of unsaturation (Tan and Che Man, 2000). VCO sample from MC had lower degree of unsaturation. As a result, higher enthalpy was required for melting process and less energy was released during crystallization process. The obtained results were in accordance with those reported by Tan and Che Man (2000) for coconut oil, in which melting and crystallization temperatures were observed at 22.45 and -0.70 °C. Melting and crystallizing enthalpies were 120.6 J/g and -106.5 J/g, respectively. DSC results indicated that the differences in the thermal behavior of VCO samples with three different maturity stages were more likely associated with different chemical compositions.

Table 15. Melting and crystallization temperatures and enthalpy of virgin coconut oil with three different maturity stages.

Samples	Melting (Endotherm)				Crystallization (Exotherm)			
	Onset (°C)	**Peak (°C)**	**Endset (°C)**	**Enthalpy (J/g)**	**Onset (°C)**	**Peak (°C)**	**Endset (°C)**	**Enthalpy (J/g)**
IMC	9.52±0.46b	23.30±0.37b	26.40±0.33c	112.16±1.05b	4.43±0.39b	-1.89±0.07b	-4.62±0.41b	-104.29±1.68a
MC	12.63±0.93a	24.87±0.40a	28.86±0.62a	117.96±2.49a	4.72±0.57a	-1.13±0.26a	-4.07±0.75a	-110.73±0.75b
OMC	9.46±0.35b	23.94±0.49ab	27.99±0.77b	114.17±0.91b	4.17±0.69c	-1.74±0.18b	-4.25±0.36b	-105.78±0.37a

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference ($p<0.05$). IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut.

3.5.2.1 Cloud and pour points

Cloud and pour points of VCO samples with three different maturity stages are shown in Table 16. In general, there was no difference in both cloud and pour points amongst all samples ($p>0.05$). Cloud and pour points of VCO samples in the present study were higher than those reported by Akhtar, *et al*. (2009), in which coconut oil had the higher cloud and pour points (13.1 and 12.7 °C) than other oils. This was plausibly owing to the differences in process, variety, and fatty acid compositions. The cloud point is the temperature at which a cloud is formed in the oil due to the beginning of crystallization and pour point is the temperature at which the oil is solidified enough to resist the flow (Akhtar *et al*., 2009; Bello *et al*., 2015). The chain length of fatty acids affects the cloud and pour points. With the shorter chain length, oil has higher cloud and pour points (Akhtar *et al*., 2009). VCO contained high amounts of short chain or medium chain fatty acids (Marina *et al*., 2009). The results suggested that high cloud and pour point of VCO samples were more likely due to the presence of short or medium chain fatty acids.

Table 16. Cloud, pour and flash points of VCO with three different maturity stages.

Samples	Cloud point (°C)	Pour point (°C)	Flash Point (°C)
IMC	18.5±0.70a	15.5±0.70a	232±2.82a
MC	16.0±1.41a	13.5±2.12a	227±0.70a
OMC	18.0±1.41a	15.0±0.00a	227±0.70a

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference between means ($p<0.05$). IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut.

3.5.2.2 Flash point

Flash point was observed at very high temperature for all VCO samples as shown in Table 16. However, there was no difference amongst the samples ($p>0.05$). Flash point specifies the temperature, at which a flash appears at any point on the surface of the sample. The oil with low flash point can ignite when exposed to

heat easily (Bello *et al*., 2015). The flash points for all VCO samples were very high. This made VCO suitable for deep frying and cooking.

3.5.2.3 Sensory characteristics

There were no differences in color, odor, viscosity, flavor and overall scores amongst different VCO samples ($p>0.05$) (Table 17). All VCO samples were transparent and clear like water. All samples had natural odor and flavor without fermentation smell. It can be inferred that there were no differences in sensory properties of VCO samples with different maturity stages.

Table 17. Difference-from-blind control test scores of VCO with three different maturity stages.

Samples	Color	Odor	Viscosity	Flavor	Overall
IMC	0.32±0.07a	0.94±0.40a	0.47±0.13a	0.61±0.29a	0.85±0.29a
MC	0.33±0.19a	0.99±0.32a	0.56±0.15a	0.51±0.31a	0.86±0.31a
OMC	0.36±0.21a	0.97±0.49a	0.55±0.24a	0.51±0.19a	0.96±0.56a

Values are mean ± standard deviation (n=30). Different lowercase letters in the same column indicate significant difference ($p<0.05$). IMC: Immature coconut, MC: Mature coconut and OMC: Overlay mature coconut

3.6 Conclusions

VCO separated from three different maturity stages of coconut had similar fatty acid composition and other properties. VCO had high contents of MCFAs, especially lauric acid. However, VCO separated using Alcalase showed the highest recovery when coconut milk from OMC was used as starting material. The maturity stages had the influence on oil recovery, in which the OMC rendered the highest yield. Nevertheless, the maturity stages had no influence on properties, oxidative stability and sensory characteristics of resulting VCO.

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CHAPTER 4
CHARACTERISTICS OF ALBUMIN AND GLOBULIN FROM COCONUT MEAT AND THEIR ROLE IN EMULSION STABILITY WITHOUT AND WITH PROTEOLYSIS

4.1 Abstract

Albumin and globulin were fractionated from defatted coconut meat. Characteristics and emulsifying properties of these protein fractions were comparatively studied. Both fractions had protein with MW of 55 kDa as predominant and glutamine/ glutamic acid were the major amino acids. However, differences in the protein pattern and amino acid composition were observed between two fractions. Higher average hydrophobicity was found in globulin fraction, compared with albumin. Additionally, globulin fraction was more hydrolyzed by Alcalase, in comparison with albumin. Coconut milk oil-in-water model emulsion was prepared using albumin and globulin protein fractions and stability of these emulsions was evaluated. Oil droplets with larger size caused by coalescence along with higher polydispersity were observed in albumin stabilized emulsion after 24 h of storage time. Conversely, globulin stabilized emulsion showed smaller oil droplet with low coalescence index and flocculation factor. Thus, emulsion stabilized by globulin fraction was more stable than that containing albumin. However, the higher oil recovery was found in the globulin stabilized emulsion when treated with 1% Alcalase for 90 min, compared with albumin counterpart. This was caused by the higher susceptibility towards hydrolysis of globulin fraction. Therefore, globulin fraction mainly determined coconut milk stability and must be hydrolyzed by protease to release oil for virgin coconut oil production.

4.2 Introduction

Coconuts (*Cocos nucifera)* are economically important and extensively used in many traditional foods of Asian and Pacific regions (DebMandal and Mandal, 2011). Coconut milk, which is a milky white oil-in-water emulsion, is generally obtained from grated coconut meat (Tansakul and Chaisawang, 2006). Apart from using as food ingredient, coconut milk has been used for virgin coconut oil (VCO)

production. Coconut oil is different from other vegetable oils due to high content of medium chain fatty acids, mainly lauric acid (Dayrit, 2014). VCO is rapidly gaining an immense importance due to various health benefits and high stability (Carandang, 2008). Apart from oil, coconuts also provide a potential source of proteins with good nutritive value and relatively well balanced profile. Globulin and albumin, which are soluble in NaCl and water, respectively, were major proteins in coconut meal (Kwon, Park, and Rhee, 1996). Peamprasart and Chiewchan (2006) reported that some proteins present in the aqueous phase of the coconut milk emulsion could act as emulsifier to stabilize fat globules. Basically, coconut milk emulsion can be disrupted via enzymatic treatment, heating and freeze–thawing, etc. (Gunetileke and Laurentius, 1974). Nevertheless, no information on the role of particular proteins in stabilization of coconut milk exists. Also, the collapse of coconut milk model emulsion stabilized by particular proteins caused by enzymatic treatment via protein hydrolysis, has not been studied. The information on coconut proteins and their role in emulsion stabilization could provide the better understanding on enhancing or destabilizing the emulsion of coconut milk. Thus, coconut milk emulsion can be stabilized or collapsed to obtain the desired products. The objectives of this study were to comparatively investigate the impact of albumin and globulin from coconut meat on characteristics of oil-in-water emulsion and to study the effect of proteolysis on the instability of coconut milk model emulsion, in which coconut oil could be released.

4.3 Objective

To fractionate proteins in coconut meat and elucidate their role in emulsion stability.

4.4 Materials and Methods

4.4.1 Chemicals

2,4,6-Trinitrobenzenesulphonic acid (TNBS), sodium azide, boric acid and Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulfate, sodium chloride and isooctane were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, propanol, hydrochloric acid, sulfuric acid

and n-hexane were procured from Lab-Scan (Bangkok, Thailand). Chemicals for electrophoresis were obtained from Biorad (Richmond, VA, USA). Protein molecular weight marker was procured from GE healthcare (Buckinghamshire, UK). Alcalase (2.4L FG) with the activity of 2.4 AU/g was obtained from novozymes (Bagsvaerd, Denmark).

4.4.2 Preparation of defatted coconut meat

Coconuts (matured stage, which is the stage commercially used) were purchased from a plantation site in Yaring district, Pattani province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was collected manually and grated using a rotary wedge cutting machine. Grated meat was frozen in liquid nitrogen and then ground into a fine power with a grinder. The powder was sieved through a siever (0.4 mm mesh). The powder defatted with hexane, using a matter/solvent ratio of 1:10 (w/v) for 1 h. The defatting was performed twice. Defatted coconut meat was freeze-dried using a SCANVAC Cool Safe™ freeze-dryer (Cool-Safe 55, Scan Laf A/S, Lynge, Denmark). Dried powder had protein content of 13% (dry weight basis), as determined by the Kjeldahl method (AOAC, 2000).

4.4.3 Fractionation of proteins

The coconut proteins were sequentially fractionated from defatted coconut meat using five different solvents, including deionized water, 0.5 M NaCl, 70% 2-propanol, 50% glacial acetic acid, and 0.1 M NaOH following the method of Kwon *et al*. (1996). Sample was mixed with solvent, using a powder to solvent ratio of 1:10 (w/v) and the mixture was stirred at 4 °C for 14-16 h. Insoluble residue was removed by centrifugation at 20000g for 30 min using a centrifuge (Beckman Coulter, Allegra™ centrifuge, Brea, CA, USA). Extraction with each solvent was repeated three times, and all supernatants for each solvent were pooled together. Each fraction was then dialyzed against 20 volume of deionized water. Dialysates were then freeze-dried, and the resulting protein fractions were placed in a zip-lock bag and stored at -

20 °C. Water, NaCl, IPA, acetic acid, and NaOH soluble fractions were designated as albumin, globulin, prolamine, glutelin-1, and glutelin-2 fractions, respectively. Protein content of each fraction was determined by the Kjeldahl method using the conversion factor of 6.25. Protein of each fraction was calculated and expressed as the percentage of total proteins in the defatted coconut powder.

4.4.4 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of albumin and globulin fractions were determined by SDS-PAGE according to the method of Laemmli (1970) using 4% stacking gel and 12% separating gel. Samples (0.5 g) were dissolved in 10 mL of 5% SDS and heated at 95°C for 1 h, followed by centrifugation at 7000g for 10 min at 25°C using a centrifuge (Beckman Coulter, Allegra™ centrifuge, CA, USA). The protein concentration of the supernatant was determined by the Biuret method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as a standard. Samples were mixed with the sample buffer containing 2% SDS, 10% glycerol and 0.05% bromophenol blue in 0.5 M Tris-HCl, pH 6.8. β-mercaptoethanol was added to sample buffer to obtain a final concentration of 5%. The mixtures were heated at 95 °C for 3 min prior to loading. The prepared samples (12 µg protein) were loaded onto the gel. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, VA, USA) at a constant voltage of 200 V/plate. The gels were stained with 0.125% Coomassie blue R125 in 25% methanol and 10% acetic acid. The gels were destained with 40% methanol and 10% acetic acid. Relative mobility (R_f) of proteins was calculated and the molecular weight of the proteins was estimated from the plot between R_f and log (MW) of standards.

4.4.5 Amino acid analysis

Amino acid compositions of albumin and globulin fractions were determined using an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan). Samples were hydrolyzed under the reduced pressure in 4 M methane sulfonic acid containing 0.2% 3-2(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were

neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 mL was applied to an amino acid analyzer.

4.4.6 Effect of Alcalase on hydrolysis of coconut protein fractions

Albumin and globulin fractions were dissolved in deionized water and adjusted to pH 8 using 1 M NaOH. The solution was added with Alcalase at a level of 1% (v/v). The hydrolysis was performed at 60 ºC in a shaking water bath (W350, Memmert, Schwabach, Germany). The samples were taken at different times from 0 to 90 min. The enzymatic reaction was terminated by adding 1 volume of hot SDS solution (85 ºC) and the mixture was placed in a water bath at 85 ºC for 15 min. All samples were determined for protein pattern by SDS-PAGE.

Degree of hydrolysis (DH) was also determined according to the method of Benjakul and Morrissey (1997). The hydrolyzed samples with the appropriate dilution (125 μL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino group was expressed in terms of *L*-leucine. The DH was calculated as follows:

$$DH = [(L - L0) / (L\max - L0)] \times 100$$

where L is the amount of α-amino groups of hydrolyzed sample. $L0$ is the amount of α-amino groups in the initial sample. $L\max$ is the total α-amino groups obtained after acid hydrolysis (6 M HCl at 100°C for 24 h).

4.4.7 Preparation of coconut milk model oil-in-water emulsions

Albumin and globulin fractions were firstly dissolved in 5 mM phosphate buffer containing 0.02% sodium azide (pH 6.4) to obtain 1% protein. The mixture was stirred overnight at 4 °C. Emulsions were prepared by mixing virgin coconut oil and prepared protein solutions at a ratio of 1:9 (w/w). The mixtures were

homogenized using a high-speed homogenizer at 11000 rpm for 2 min. These coarse emulsions were passed through high-pressure homogenizer (Microfluidics, Model HC 5000, Stanwood, WA, USA) at 3000psi for two times. The emulsions prepared using albumin and globulin fractions were designated as albumin stabilized emulsion (ASE) and globulin stabilized emulsion (GSE), respectively. These two emulsions were immediately used for further study.

4.4.8 Characterization of coconut milk model oil-in-water emulsions

4.4.8.1 Microstructure determination of oil droplets

The microstructures of ASE and GSE were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan.). The samples were dissolved in Nile blue A solution (1:10) and manually stirred until uniformity was obtained. Fifty microliters of sample solutions were smeared on the microscopy slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis. A magnification of 400x was used.

4.4.8.2 Determination of particle size

Particle size distribution of ASE and GSE was determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani, Belhomme, David-Briand, Guérin-Dubiard, and Anton (2006). Prior to analysis, samples (5 mL) were diluted with 1% (v/v) sodium dodecyl sulphate (SDS) solution (20 mL) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

4.4.8.3 Determination of coalescence and flocculation

ASE and GSE samples were diluted with distilled water in the presence and absence of 1% SDS. The coalescence index (C_i) and flocculation factor (F_f) were calculated using the following equations (Hebishy, Buffa, Guamis, and Trujillo, 2013):

$$F_f = \frac{d_{43} - \text{SDS}}{d_{43} + \text{SDS}}$$

$$C_i = \frac{(d_{43} + \text{SDS, t} - d_{43} + \text{SDS, in})}{d_{43} + \text{SDS, in}} \times 100$$

where d_{43}+SDS and d_{43}-SDS are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 1% SDS, respectively; d_{43}+SDS,in and d_{43}+SDS,t are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 1% SDS at time 0 and the designated storage time (24 h). The determination was conducted at room temperature (28-30°C).

4.4.8.4 Measurements of creaming

Creaming of ASE and GSE samples was measured as per the method of Senphan and Benjakul (2015). Samples (10 mL) were transferred into a test tube (with 15 mm internal diameter and 125 mm height) and tightly sealed with a plastic cap. The total height of the emulsions in the tubes (HE) was recorded. Thereafter, the emulsion samples were left to stand at room temperature for 24 h. The emulsions were separated into an opaque layer (cream) at the top and a slightly turbid or transparent layer (serum) at the bottom. The height of the serum layer (HS) was measured. The creaming index was calculated as follows:

Creaming Index = (HS/HE) × 100

4.4.9 Effect of Alcalase on instability of model emulsions

ASE and GSE samples were adjusted to pH 8 using 1 M NaOH and added with Alcalase at a level of 1% (v/v). The hydrolysis was performed at 60 ºC in a shaking controlled temperature water bath (W350, Memmert, Schwabach, Germany). Samples were taken at different times of incubation (0, 45 and 90 min). The treated samples were determined for microstructure, particle size and distribution of oil droplets. Additionally, the oil recovery was also calculated.

After the designated time, the mixtures were centrifuged at 4900xg for 30 min at room temperature (28 ºC) using a centrifuge. VCO was collected from the upper oil phase. Oil recovery was calculated using the following equation (Mansor *et al*., 2012):

$$\text{Oil recovery (\%)} = \frac{\text{Weight of VCO}}{\text{Weight of total oil in emulsion}} \times 100$$

4.4.10 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.5 Results and discussion

4.5.1 Distribution of proteins in defatted coconut meat

Coconut proteins were fractionated from defatted coconut meat based on their solubility using five different solvents. The contents of protein recovered in different fractions are given in Table 18. The globulin fraction (36%) was predominant, followed by albumin (19%), glutelin-1 (10%), glutelin-2 (4%) and prolamine (2%) fractions. The sum of five protein fractions was 71% of total proteins present in the defatted meat. The result suggested that the sequential extraction using five solvents were unable to completely extract all proteins from defatted coconut meat. Samson, Khaund, Cater, and Mattil (1971) reported that most of the coconut proteins are inside the cell and vigorous grinding is required for recovering proteins from coconuts. Kwon *et al*. (1996) reported that defatted coconut meat had globulin as the major protein fraction (40.1%), followed by albumin (21%), glutelin-1 (14.4%), glutelin-2 (4.8%) and prolamine (3.3%) fractions. The differences in starting material, maturation stage, climate, fertilizer, etc. might lead to the differences in proportion of various proteins in coconut meat.

Table 18. Distribution of proteins in defatted coconut meat

Fractions	Extraction Solvents	Total protein extracted (%)*
Albumin	Deionized water	19±0.84
Globulin	0.5 M NaCl	36±1.70
Prolamine	70% 2-propanol	2±0.94
Glutelin-1	50% acetic acid	10±0.95
Glutelin-2	0.1 M NaOH	4±0.87

Values are mean ± standard deviation (n=3).

*Total protein extracted was expressed, relative to total proteins in the defatted coconut meat, determined by Kjeldahl method (conversion factor of 6.25)

4.5.2 Electrophoretic patterns of albumin and globulin

Protein patterns of albumin and globulin fractions of defatted coconut meat are shown in Figure 6. Both fractions contained protein with MW of 55kDa as the major protein. The major protein (~65%) in coconut endosperm is cocosin having MW of 55 kDa (Garcia, Arocena, Laurena, and Tecson-Mendoza, 2005), which is believed to play a prominent role in governing the stability of coconut milk (Tangsuphoom and Coupland, 2008). Protein with MW of 22 kDa was also found in two fractions. It was noted that albumin had another protein with MW of 13.2 kDa, however it was not detected in globulin fraction. For globulin fraction, proteins with MW lower than 55 kDa including those with MW of 44, 37, 33, 31 and 30 kDa were observed. Additionally, proteins with MW lower than 21 kDa involving proteins with MW of 19, 17, 16, 14 and 12.9 kDa were also found. Nevertheless, those proteins were not detected in albumin fraction. Proteins with varying MWs in both fractions might contribute to varying properties.

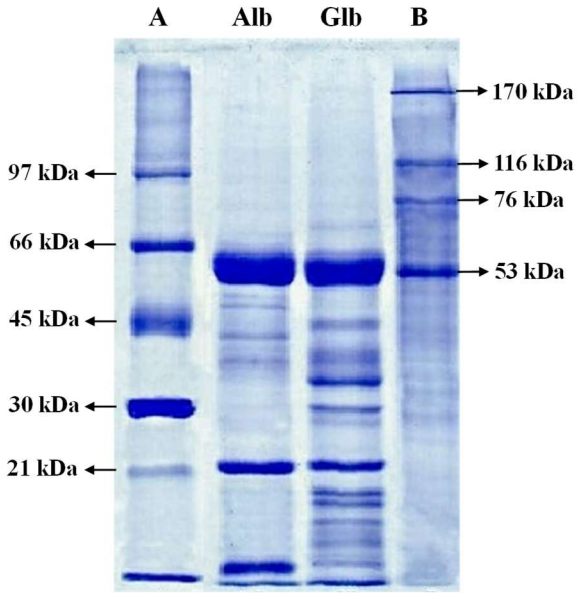

Figure 6. SDS-PAGE pattern of albumin and globulin fractions from defatted coconut meat.

A: low molecular weight standards; B: high molecular weight standards; Alb: albumin; Glb: globulin.

4.5.3 Amino acid compositions of albumin and globulin fractions

Amino acid compositions of albumin and globulin fractions of defatted coconut meat expressed as g/100g are shown in Table 19. For albumin fraction, glutamic acid/glutamine (24.07 g/100g) were the major amino acids. Arginine was found as the second major amino acid (16.53 g/100g), followed by aspartic acid/asparagine (8.02 g/100g). The similar trend was observed for globulin fraction, in which glutamic acid/glutamine (19.66 g/100g) were dominant, followed by arginine (14.88 g/100g) and aspartic acid/asparagine (9.20 g/100g). Similar results were reported by Samson *et al*. (1971) and Kwon *et al*. (1996) in which coconut proteins (albumin and globulin) had high content of glutamic acid, followed by arginine and aspartic acid. It was noted that there was a difference in proportion and distribution of polar and non-polar amino acids between both samples. Albumin fraction showed higher proportion of polar amino acids (uncharged, positively charged and negatively charged side chain) and lower proportion of non-polar amino acids than globulin counterpart. Negligible amount of cysteine was found for both fractions. The amino

acid composition of coconut proteins varies, depending on variety, age, growing environment, cultural practices, method of preparation, and the process conditions used for extraction (Onsaard, Vittayanont, Srigam, and McClements, 2006).

Table 19. Amino acid composition of albumin and globulin fractions from defatted coconut meat (g/100 g protein).

Amino acids	**Fractions**	
	Albumins	**Globulins**
Polar		
Arginine	16.53	14.88
Aspartic acid /asparagine	8.02	9.20
Glutamine /glutamic acid	24.07	19.66
Histidine	2.40	2.82
Lysine	6.26	5.00
Serine	4.76	5.00
Threonine	3.17	3.40
Tyrosine	2.55	3.30
Total	**67.83**	**63.30**
Non-polar		
Alanine	5.99	4.46
Cysteine	0.00	0.01
Glycine	4.42	4.96
Isoleucine	2.44	3.54
Leucine	5.13	7.23
Methionine	1.40	1.94
Phenylalanine	3.45	5.19
Proline	5.45	3.86
Valine	3.89	5.47
Total	**32.17**	**36.70**

According to Bigelow (1967), polar to nonpolar side chain ratio (P), frequency of nonpolar side chains (NPS) and average hydrophobicity (H**Φ**) were calculated from the amino acid compositions of albumin and globulin protein fractions as shown in Table 20. Albumin fraction showed high P value (2.28) but low NPS (0.21) and H**Φ** (780.23) values. The results indicated that albumin fraction had the higher proportion of polar side chains, which were associated with its high-water solubility. Conversely, globulin fraction had higher levels of NPS (0.29) and H**Φ** (879.42), thereby reflecting the higher hydrophobicity. In general, globulin was less soluble in water but was solubilized in solution with high ionic strength. NPS (0.18), H**Φ** (765) and P (3.46) were reported for coconut albumin fraction and NPS (0.29), H**Φ** (965) and P (1.70) were found in globulin fraction (Kwon *et al*., 1996). Amino acid composition of proteins plays an important role in its functional properties. Generally, the hydrophilic nature of proteins influences solubility and water holding capacity. This is related with high proportion of amino acids with positively charged and negatively charged side chains. On other hand, the hydrophobic proteins exhibit high emulsifying properties and fat adsorption capacity, due to high proportion of amino acids with non-polar side chains (Siebert, 2003). The results suggested that difference in properties of albumin and globulin fractions were more likely associated with varying amino acid compositions as well as the difference in proportion and distribution of polar and non-polar amino acids in proteins, particularly on the surface.

Table 20. Bigelow parameters of albumin and globulin protein fractions from defatted coconut meat.

Proteins	**P**	**NPS**	**HΦ**
Albumin	2.28	0.21	780.23
Globulin	1.69	0.29	879.42

P: The ratio of polar volume to nonpolar volume.

NPS: The frequency of nonpolar side chains was calculated according to Waugh's definition by counting the Trp, Ile, Tyr, Phe, Pro, Leu and Val residues and expressing the sum as a function of the total number of residues.

HΦ: Average hydrophobicity.

Amino acid compositions also determine solubility of proteins. Solubility is also governed by pK values of the ionizable residues, and environmental factors such as temperature, pH, and the presence of co-solvents (Schein 1990). Proteins and polypeptides are generally least soluble at pH values near their isoelectric point (pI), where the overall net charge is zero, which generally affect the emulsion stability.

4.5.4 Effect of Alcalase on hydrolysis of coconut albumin and globulin fractions

Alcalase is proteolytic enzyme, which has been widely used for the hydrolysis of proteins. It played an important role in hydrolyzing proteins surrounding oil droplets in coconut milk (Senphan and Benjakul, 2016). Therefore, Alcalase was selected for hydrolysis of coconut protein fractions in the present study.

4.5.4.1 Degree of hydrolysis (DH)

The enzymatic hydrolysis of albumin and globulin fractions as a function of time expressed as free amino group is depicted in Figure 7. Hydrolysis of both albumin and globulin fractions was more pronounced with increasing time. The rapid hydrolysis was observed within the first 10-20 min. The results suggested that the rate of hydrolysis was higher at the initial stage, compared to the final stage. At the same hydrolysis time, higher DH was observed in globulin fraction. After 120 min of hydrolysis, DHs of 31.87 and 35.04 % were obtained for albumin and globulin fractions, respectively. The results suggested that globulin fraction was more susceptible toward hydrolysis by Alcalase than albumin. Alcalase has been known to cleave preferably the hydrophobic amino acid (Charalambous, 2012). This coincided with the higher H**Φ** of globulin (Table 20).

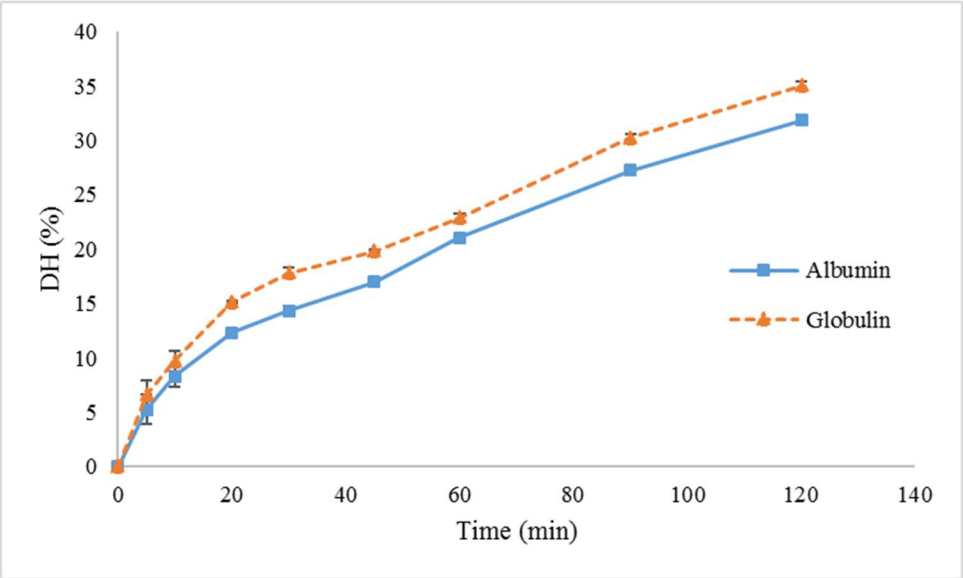

Figure 7. Degree of hydrolysis (DH) of albumin and globulin fractions from defatted coconut meat subjected to hydrolysis using 1% Alcalase for different times. Bars represent standard deviation (n=3).

4.5.4.2 Protein pattern

Degradation of both fractions, albumin and globulin, by Alcalase was comparatively studied as a function of time (Figure 8). Within the first 5 min, the slight hydrolysis was found in albumin fraction, as shown by the slight decrease in protein with MW of 55 kDa, a dominant protein. With increasing hydrolysis time up to 90 min, protein with MW of 55 kDa almost disappeared. For protein with MW of 21 kDa, the second dominant protein in albumin fraction, it was resistant to hydrolysis by Alcalase. Nevertheless, it was degraded to higher extent with the coincidental formation of proteins or peptides with lower MW as the hydrolysis time increased. Protein with MW of 13 kDa was also hydrolyzed by Alcalase to some degree, particularly when the hydrolysis time increased. For globulin fraction, the drastic decrease in protein with MW of 55 kDa was noticeable after 5 min of hydrolysis. Protein band was rarely observed after 45 min of hydrolysis. The hydrolysis of this protein coincided with the increase in band intensity of protein with MW of 33 kDa. It was noted that protein with MW of 21 kDa totally disappeared after 5 min of hydrolysis. Proteins with MW of 19 and 17 kDa became dominant up to 90 min of hydrolysis. This was plausibly due to the high resistance of those proteins against

Alcalase. The result suggested that proteins with MW of 55 kDa in albumin and globulin fractions were different proteins as evidenced by different susceptibility to Alcalase hydrolysis. Degradation of major proteins and the remaining proteins in each fraction might contribute to emulsion stability in different fashions.

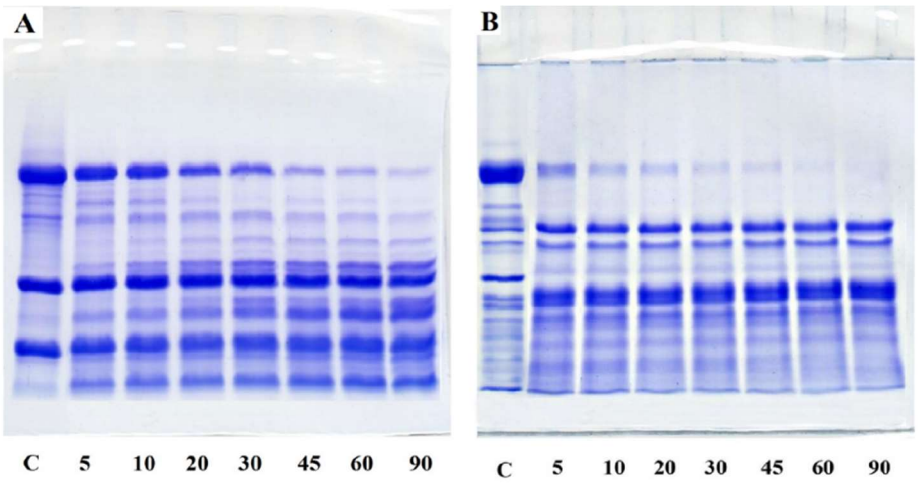

Figure 8. SDS-PAGE pattern of albumins (A) and globulin (B) fractions from defatted coconut meat subjected to hydrolysis using 1% Alcalase for different times. C: control without Alcalase addition. Numbers designate the hydrolysis time (min).

4.5.5 Stability of coconut milk model oil-in-water emulsions containing albumin and globulin fractions

4.5.5.1 Microstructure of oil droplets

Microscopic structure of oil droplets in ASE and GSE visualized by confocal laser scanning microscopy is illustrated in Figure 9. At time 0 h, the oil droplets dispersed in aqueous phase in GSE were smaller in size, when compared to those found in ASE. For GSE, the oil droplets with more uniform shape and size were noticeable. Emulsion having the same size oil droplets is referred as a monodisperse emulsion, whereas that containing a range of droplet sizes is referred as a polydisperse emulsion (McClements, 2015).

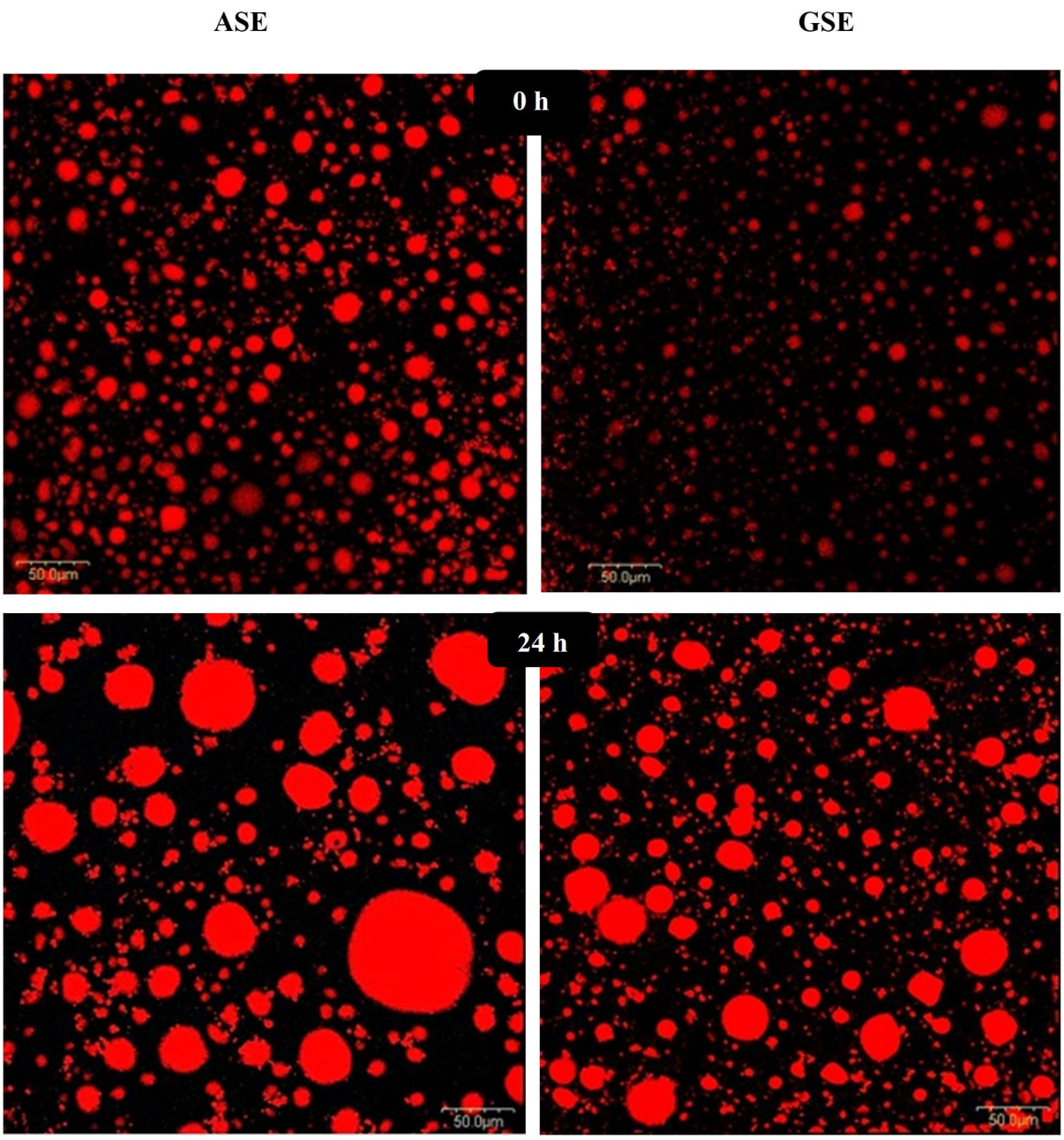

Figure 9. Confocal laser scanning micrographs of ASE and GSE at time 0 and 24 h after emulsification. ASE: Albumin stabilized emulsion; GSE: Globulin stabilized emulsion. Magnification: 400x

The coconut milk is an oil-in-water emulsion naturally stabilized by coconut proteins (Birosel, Gonzalez, and Santos, 1963). The results revealed that globulin fraction was more effective than albumin in generating small droplets after emulsification. More efficient migration and adsorption at oil-water interface of

globulin fraction was postulated, compared with albumin. Polydisperse emulsion with larger sizes of oil droplets was observed. However, the collapse or coalescence was more pronounced in ASE than GSE. The differences in oil droplet size might be related with differences in the emulsifying properties of albumin and globulin fractions. The large size of droplet with a non-uniform distribution was more likely owing to the lower emulsifying property of albumin fraction. The emulsifying property of proteins could be affected by the difference in proportion and distribution of polar and non-polar amino acids (Kinsella, 1982). Albumin fraction had higher proportion of polar side chains (P), while globulin had higher proportion of NPS and H**Φ** (Table 20). Nonpolar side chains or hydrophobic domain of the proteins were able to interact with hydrocarbon chains on fatty acids, thus promoting physical entrapment of oil (Al-Kahtani and Abou-Arab, 1993). The results indicated that globulin fraction was more efficient as an emulsifier in oil-in-water emulsion than albumin and also yielded more stable emulsion.

4.5.5.2 Particle size distribution

Particle size distribution of ASE and GSE expressed as d_{32} and d_{43} is shown in Table 21. GSE had the smaller d_{32} (2.27±0.04 μm) than ASE (2.56±0.00 μm). For ASE, d_{32} was increased from 2.56±0.00 μm to 3.70±0.02 μm after 24 h of storage time, whereas d_{43} increased from 2.95±0.00 μm to 3.98±0.21 μm. The similar trend was also found for GSE, in which d_{32} was increased from 2.27±0.04 μm to 2.52±0.00 μm after 24 h of storage time, whereas d_{43} increased from 2.66±0.18 μm to 2.89±0.00 μm. The d_{32} is related to the average surface area of droplet exposed to the continuous phase per unit volume of the emulsion. The smaller d_{32} means the higher specific surface area (Intarasirisawat, Benjakul, and Visessanguan, 2014). For d_{43}, GSE also showed the low value (2.66±0.08 μm) than ASE (2.95±0.00 μm). After 24 h, d_{43} of GSE and ASE increase to 2.89±0.00 and 3.98±0.21 μm, respectively. The d_{43} is the sum of the volume ratio of droplets in each size class multiplied by the mid-point diameter of size class. The d_{43} can be used as the index of coalescence and flocculation (Hebishy *et al*., 2013). GSE therefore had the smaller particle diameters at the initial time (0 h), when compared with ASE. Emulsions are thermodynamically unstable due to the unfavorable contact between oil and water. As a result, their

physical structures likely to change over time by various mechanisms including coalescence and flocculation. The increases in both d_{32} and d_{43} (Table 21) indicated the coalescence of oil droplets or instability of emulsion. The formation of larger oil droplet of ASE indicated poor emulsion stability. The interactions between oil droplets depend on the quality and quantity of the proteins (Zayas and Lin, 1989). Overall, the lower increases in d_{32} and d_{43} were observed for GSE. When the repulsive forces dominate, the droplets tend to remain as individual entities and form stable emulsion (McClements, 2004). The differences in droplet sizes were also confirmed using CLSM (Figure 9), in which ASE showed the largest oil droplets after the extended storage time (24 h). Proteins in coconut milk have been known to function as emulsifiers, which stabilized the oil droplets in coconut milk (Senphan *et al*., 2015). The results reconfirmed that albumin fraction showed lower emulsifying properties, compared to globulin in the coconut model oil-in-water emulsion.

Table 21. Droplet size and stability of coconut milk model oil-in-water emulsion containing albumin and globulin fractions

Samples	Storage time (h)	d_{32} (µm)	d_{43} (µm)	F_f	C_i	Creaming Index (%)
Albumin	0	2.56±0.00A	2.95±0.00A	1.18±0.03A	-	-
	24	3.70±0.02a	3.98±0.21a	0.77±0.04a	14.5±0.23a	59±0.81a
Globulin	0	2.27±0.04B	2.66±0.08B	0.90±0.07B	-	-
	24	2.52±0.00b	2.89±0.00b	0.78±0.03a	4.55±0.80b	45±1.2b

Values are mean ± standard deviation (n=3).

Different uppercase letters in the same column indicate significant difference ($p<0.05$). Different lowercase letters in the same column indicate significant difference ($p<0.05$).

4.5.5.3 Coalescence and flocculation

Coalescence index (C_i) and flocculation factor (F_f) of ASE and GSE are shown in Table 21. After 24 h, higher C_i was found in ASE than GSE, indicating that the higher coalescence occurred in the former. On the other hand, F_f of both ASE and GSE was decreased, suggesting that individual oil droplets assembled to form

larger oil droplet. As a consequence, the floc was diminished. GSE with lower C_i had the lower rate of changes in d_{32} and d_{43}, compared with ASE. The stabilizing effect of proteins in emulsions results from the protective barrier they form around fat droplets, in which repulsive interactions (e.g. steric and electrostatic) between the oil droplets are generated and prevent their coalescence (McClements, 2004). Albumin fraction more likely exhibited the poor ability in preventing the coalescence than globulin fraction. During storage, the adsorbed proteins might undergo some rearrangements or alteration of layer at the interface, leading to droplet aggregation and thereby facilitating the coalescence (Keerati-u-rai, Wang, and Corredig, 2011; Wang and Corredig, 2011). The differences of emulsifying ability between different proteins maybe caused by their different surface loads, surface activities or adsorption rates (McClements, 2015). The results clearly indicated that the globulin fraction was more effective than albumin in stabilizing the emulsion against the coalescence.

4.5.5.4 Creaming index

ASE and GSE had different creaming index as given in Table 21. ASE and GSE had creaming index of 59 and 45 %, respectively. Creaming index provides the indirect information of droplet aggregation in the emulsion (Onsaard *et al*., 2006). The higher creaming index found in ASE indicated that more droplet aggregation occurred in ASE, in comparison with GSE, during the storage. Emulsions instability can be observed as oil separation or creaming, which may be initiated by flocculation and coalescence (Garrett, 1965). The higher creaming index observed in ASE was in agreement with higher coalescences index, compared with GSE (Table 21). Long-term stability of emulsions depends basically on the thickness and strength of adsorbed protein films at the oil-water interface (Zayas *et al*., 1989). The results suggested that albumin fraction was less effective to form efficient film surrounding the oil droplets, in which coalescences took place at a higher extent. Those aggregated or merged oil droplets consequently led to creaming or floatation of oil phase at the top of emulsion.

4.5.6 Effect of Alcalase on instability of model emulsions

4.5.6.1 Microscopic structure

ASE and GSE hydrolyzed with 1% Alcalase for different hydrolysis time (0, 45 and 90 min) were visualized by confocal laser scanning microscopy (Figure 10). For both samples, oil droplet size was increased with increasing hydrolysis time. However, difference was observed in droplet size of both samples. Although GSE had the lower oil droplet at the beginning, the larger oil droplets were observed after hydrolysis for 45 and 90 min. The largest oil droplets were found in both emulsion, when hydrolysis was performed for 90 min. The more collapse or higher coalescence of GSE as evidenced by larger droplet size were in accordance with higher DH and more degradation of globulin fraction than albumin (Figure 7 and 8). Therefore, the hydrolysis by Alcalase could induce the coalescence or collapse of emulsion, in which oil phase could be separated at a higher extent. Emulsion containing by globulin fraction was more susceptible to destabilization via Alcalase hydrolysis, compared with that containing albumin.

4.5.6.2 Particle size distribution

Particle size distribution of ASE and GSE with 1% Alcalase treatment at various times is shown in Table 22. GSE hydrolyzed by 1% Alcalase for 0, 45 and 90 min showed d_{32} of 2.27±0.04, 4.00±0.01 and 6.30±0.41, respectively. Similar trend was also found for d_{43} in which the values of 2.86±0.18, 9.82±0.17 and 26.27±0.49 were obtained. For ASE, d_{32} was increased from 2.56±0.00 μm to 4.20±0.03 μm, whereas d_{43} increased from 2.95±0.00 μm to 24.83±0.23 μm after 90 min of hydrolysis using Alcalase. Size of oil droplets was much altered upon the hydrolysis time. However, GSE showed higher droplet size (d_{32} and d_{43}) after 90 min of hydrolysis. The obtained results were in agreement with microstructure visualized using confocal laser scanning microscope (Figure 10). The results suggested that globulin fraction proteins surrounding oil droplets were more susceptible to Alcalase hydrolysis, resulting in the larger droplet size of oil droplet in emulsion caused by enhanced coalescence.

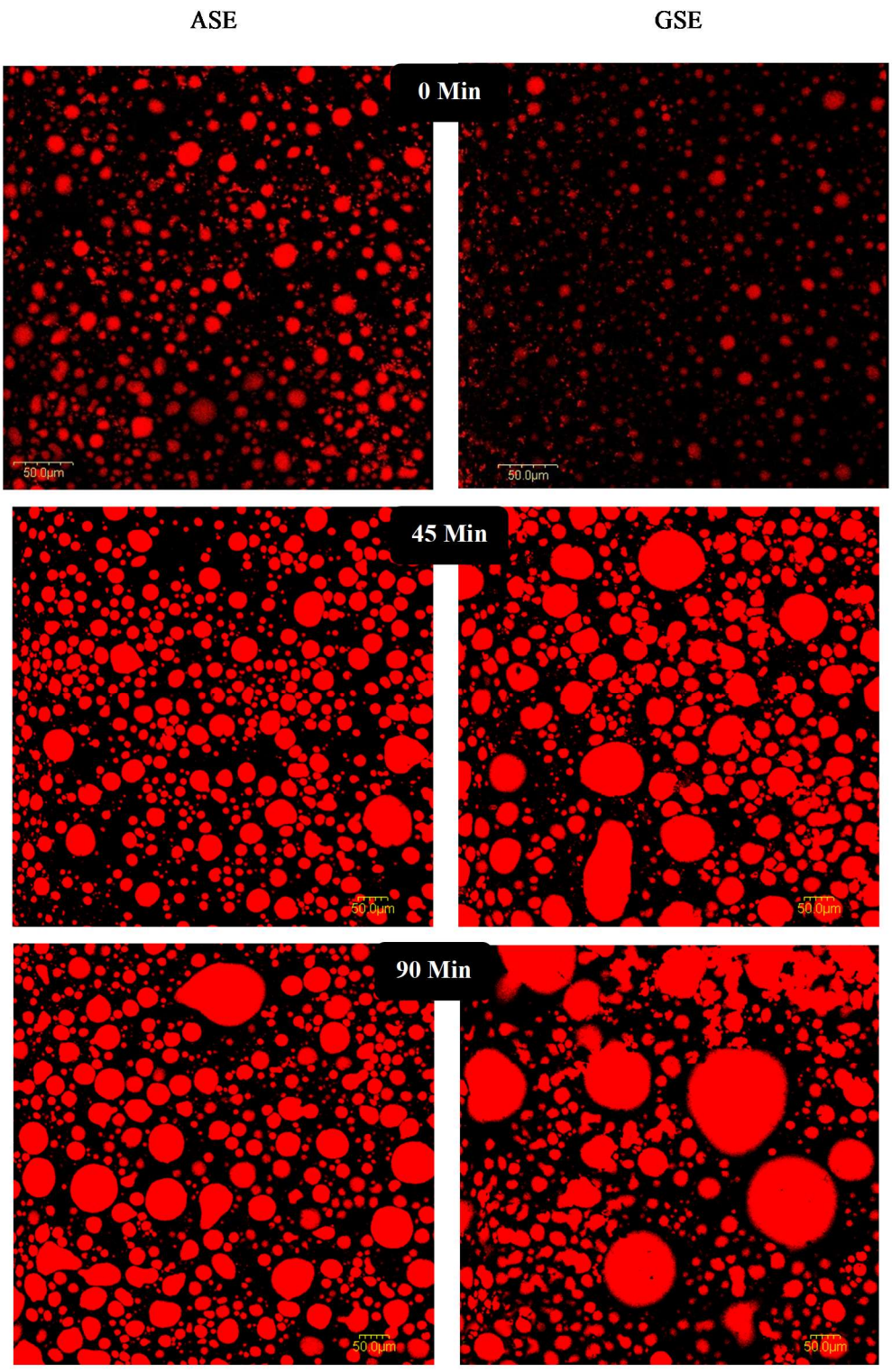

Figure 10. Confocal laser scanning micrographs of ASE and GSE subjected to hydrolysis using 1% Alcalase for different times. ASE: Albumin stabilized emulsion; GSE: Globulin stabilized emulsion. Magnification: 400x.

4.5.6.3 Oil recovery

Oil recovery gives a quantitative measurement of oil separated from the prepared emulsion. The percentages of oil recovery from ASE and GSE are shown in Table 22. For both samples, oil yield was continuously increased as hydrolysis time increased up to 90 min ($p<0.05$). However, difference in oil recovery was observed between both samples. Proteins in globulin or albumin fractions might play a role as the emulsifier in stabilizing the oil droplets in oil-in-water emulsion. After those proteins were hydrolyzed, emulsion was not stable with concomitant release of oil from the emulsion. Senphan *et al*. (2016) found that Alcalase effectively destabilized coconut milk emulsion. The lower oil recovery (12.70%) was obtained for GSE, compared with ASE at time 0 min (without Alcalase treatment). The globulin fraction could stabilize oil droplets in emulsion more effectively and thus oil released was lower as indicated by lower yield. Conversely, the highest oil recovery (77.35%) was found in GSE when sample was hydrolyzed with Alcalase for 90 min. Lower yield (58.75%) was obtained for ASE.

Table 22. Droplet size and oil recovery of coconut milk model oil-in-water emulsion containing albumin and globulin fractions subjected to hydrolysis by 1% Alcalase for different time

Samples	Hydrolysis time (min)	d_{32} (µm)	d_{43} (µm)	Oil recovery (%)
Albumin	0	2.56±0.02C	2.95±0.00C	13.50±0.70C
	45	3.95±0.02B	9.56±0.04B	19.90±0.14B
	90	4.20±0.03A	24.83±0.23A	58.75±0.21A
Globulin	0	2.27±0.04c	2.86±0.18c	12.70±0.14c
	45	4.00±0.01b	9.82±0.17b	24.90±0.14b
	90	6.30±0.41a	26.27±0.49a	77.35±0.21a

Values are mean ± standard deviation (n=3).

Different uppercase letters in the same column indicate significant difference ($p<0.05$).

Different lowercase letters in the same column indicate significant difference ($p<0.05$).

This was attributed to the fact that globulin fraction was more susceptible to hydrolysis than albumin, where the strong protein film surrounding oil droplets was destroyed or disrupted. The results suggested that the yield of oil was related with hydrolysis of proteins. Globulin fraction proteins surrounding oil droplets were more hydrolyzed by Alcalase, compared with albumin, resulting in higher yield of oil liberated from emulsion.

4.6 Conclusion

Albumin and globulin were the predominant protein fractions in defatted coconut meat. Both fractions showed the difference in protein pattern and amino acid composition. The difference in amino acid composition resulted in varying emulsifying property between albumin and globulin fractions. Water-soluble coconut proteins (albumin) exhibited low emulsifying properties, compared to salt-soluble proteins (globulin). Nevertheless, globulin fraction was more susceptible to hydrolysis by Alcalase, leading to the higher collapse of emulsion. This led to the higher oil recovery from emulsion. Thus, emulsion stability in coconut, which was governed by type of proteins could be destabilized by hydrolysis using proteases in which oil could be separated effectively.

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CHAPTER 5
COMPARATIVE STUDY ON EXTRACTION OF VCO WITH THE AID OF PARTIALLY PURIFIED PROTEASE FROM SEABASS PYLORIC CAECA AND COMMERCIAL TRYPSIN

5.1. Abstract

Coconut milk was hydrolyzed with the aid of partially purified protease from seabass pyloric caeca (PPSP) and commercial trypsin (CT) at different levels (5 and 10 unit/g protein) at 60 °C for various hydrolysis times (0-150 min). Varying degrees of hydrolysis (DH) were noticeable as a function of time, depending on the proteases and their levels. At the same hydrolysis time, higher DH was observed in coconut milk hydrolyzed by PPSP, compared to CT. VCO yield was continuously increased as hydrolysis time increased up to 120 min, regardless of enzyme levels used. The highest VCO yield (77.35%) was found when sample was hydrolyzed by PPSP (10 units/g protein) for 150 min. At the same protease level, droplet size of coconut milk hydrolyzed with PPSP was higher than that of coconut milk using CT, as visualized by confocal microscopy. Protein patterns suggested that proteins in coconut milk were more susceptible to hydrolysis by PPSP, compared to CT. No marked difference was observed in fatty acid profile, moisture content, free fatty acid content and oxidative stability of commercial VCO and VCO extracted from coconut milk with the aid of PPSP (10 unit/g protein) for 120 min. Quality of VCO was in the range of APCC standards. Therefore, VCO could be extracted using PPSP under optimal condition, in which the extraction could be achieved in a short time and yielded VCO with characteristics similar to that of commercial VCO.

5.2. Introduction

Virgin coconut oil (VCO) dominantly consists of medium chain fatty acids (MCFAs), mainly lauric acid (Dayrit, 2014). MCFAs are burned up immediately after consumption and is not stored as body fat, causing obesity (Enig, 1996). Lauric acid is converted into very valuable compound known as monolaurin, which possesses bioactivities such as antiviral and antibacterial activity (DebMandal and Mandal, 2011). It is therefore assumed that consumption of VCO may help to protect

the body from infections. Additionally, VCO shows anti-inflammatory, antimicrobial, and antioxidant properties and boosts the immune system (Carandang, 2008). VCO does not undergo any hydrolytic and atmospheric oxidation as confirmed by its low peroxide value as well as very low free fatty acid content (Senphan and Benjakul, 2015; Patil *et al.*, 2016). Because of various health benefits and high stability, VCO has drawn attention for consumer and processor (Carandang, 2008). Wet extraction process is commonly used, based on destabilization of coconut milk emulsion via numerous processes including enzymatic and physical extraction as well as fermentation (Raghavendra and Raghavarao, 2010). Amongst all processes, the enzyme-assisted separation process has been known to be effective and less time consuming. Additionally, high VCO yield could be obtained from coconut milk with the aid of proteases such as Alcalase (Man *et al.*, 1996; Senphan and Benjakul, 2016). Although microbial proteases, are available, the high cost is one of limitations for application, particularly for VCO production. The alternative and cheap protease are of attention, in which the operation cost can be reduced for production of VCO.

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and Pacific (Sae-leaw and Benjakul, 2014). In general, it is sold as whole fish or fillets. During processing or dressing of seabass, viscera generated is considered as waste. Nevertheless, fish viscera is a potential source of enzymes, particularly proteases with some unique properties for industrial applications (Haard, 1992). The most important digestive enzymes are pepsin secreted from gastric mucosa, and trypsin and chymotrypsin secreted from the pancreas, pyloric caeca and intestine (Simpson, 2000). Fish visceral proteases can be recovered with ease and introduced as the aid for VCO extraction. The efficiency of enzyme in extraction of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu *et al.*, 2008). Therefore, the present study aimed to comparatively investigate the uses of partially purified seabass protease (PPSP) and commercial trypsin on extraction efficiency and properties of VCO from coconut milk.

5.3. Objective

To comparative study on extraction of VCO with the aid of partially purified protease from seabass pyloric caeca and commercial trypsin.

5.4. Materials and Methods

5.4.1. Chemicals

Commercial bovine trypsin (CT), sodium hydroxide and Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulphate was obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, propanol, petroleum ether, and hydrochloric acid were procured from Lab-Scan (Bangkok, Thailand). Chemicals for electrophoresis were obtained from Biorad (Richmond, VA, USA), and protein molecular weight marker was procured from GE healthcare (Buckinghamshire, UK). Commercial VCO was procured from Posture Trading, Ltd (Pathumthani, Thailand).

5.4.2. Preparation of partially purified protease from seabass pyloric caeca

Pooled viscera of seabass were purchased from a market in Hat Yai, Songkhla province, Thailand. Viscera were placed in a polyethylene bag, embedded in ice with a matter/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, pyloric caeca were dissected and stored at -18 °C until use.

To prepare the crude extract, pyloric caeca were powdered in liquid nitrogen and subjected to extraction as tailored by Khantaphant and Benjakul (2008) with a slight modification. Pyloric caeca powder was suspended in an extraction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$) with a ratio of 1:10 (w/v). The mixture was homogenized for 2 min using a homogenizer (Model T25B, IKA labortechnik, Selangor, Malaysia) at 11,000 rpm. The homogenate was then continuously stirred for 30 min at 4 °C and centrifuged at 8000xg for 30 min at 4 °C using a refrigerated centrifuge (Becman Coulter Model Avant J-E, Becman Coulter, Inc., CA, USA). The supernatant was filtered through a Whatman filter paper No. 1

(Schleicher & Schuell, Maidstone, England) and the filtrate obtained was named as 'pyloric caeca extract, PCE'.

PCE was subjected to 40-60% saturated ammonium sulfate precipitation, as per the method of Khantaphant and Benjakul (2008). After the addition of ammonium sulfate, the mixture was stirred gradually at 4 °C for 30 min. Subsequently, the mixture was centrifuged at 8000 xg for 30 min at 4 °C and the pellet obtained was dissolved in the minimum volume of 50 mM Tris–HCl buffer, pH 8.0. The solution was dialyzed against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'partially purified seabass trypsin, PPSP'.

5.4.2.1. Trypsin activity assay

Trypsin activity of PPSP and CT was measured using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008). A 200 µL of sample was mixed with 200 µL of distilled water and 1000 µL of reaction buffer (50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$). The reaction was initiated by adding 200 µL of 2 mg/mL BAPNA to the reaction mixture. After incubation for 20 min at 60 °C, 200 µL of 30% acetic acid (v/v) was added to terminate the reaction. Production of *ρ*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). A blank was conducted in the same manner except that sample was added after addition of 30% acetic acid. Trypsin activity was calculated using the following equation:

$$Trypsin\ activity\ = \frac{\left((A - A_0)\ \times\ mixture\ volume\ (ml) \times 1000\right)}{8800\ \times\ reaction\ time\ (min) \times 0.2}$$

where 8800 ($cm^{-1}\ M^{-1}$) is the extinction coefficient of *ρ* -nitroaniline; A and A_0 are A_{410} of sample and blank, respectively. One unit of activity is defined as that releasing 1 nmol of *ρ*-nitroaniline per min.

5.4.3. Preparation of coconut milk

Mature coconuts (11-12 months old from pollination) were purchased from a plantation site in Yaring district, Pattani province, Thailand and transported to

the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was removed manually and grated using a rotary wedge cutter machine. Coconut milk was then prepared from grated kernel without water addition using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand).

5.4.4. Effect of PPSP and CT at different levels on hydrolysis of coconut milk and oil recovery

5.4.4.1. Hydrolysis of coconut milk with PPTS and CT as a function of time

Hydrolysis of VCO from coconut milk was performed according to the method of Senphan and Benjakul (2016) with a slight modification. The coconut milk (100 mL) in a 250 mL-Erlenmeyer flask was adjusted to pH 8 using 2 M NaOH. Thereafter, PPTS or CT was added into coconut milk to obtain different protease levels (5 and 10 units/g protein). The hydrolysis was allowed to proceed at an optimal temperature of PPTS and CT (60 °C) for various times (30-150 min). The treated samples were determined for degree of hydrolysis (DH), oil recovery, microstructure and protein pattern.

5.4.4.1.1. Degree of hydrolysis (DH)

DH was determined according to the method of Benjakul and Morrissey (1997). The samples with the appropriate dilution (125 µL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino group was expressed in terms of *L*-leucine. The DH was calculated as follows:

$$DH = [(L - L_0) / (L_{max} - L_0)] \times 100$$

where, L is the amount of α-amino groups of hydrolyzed sample. L_0 is the amount of α-amino groups in the initial sample. L_{max} is the total α-amino groups obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

5.4.4.1.2. Oil recovery

After the designated hydrolysis, the mixtures were centrifuged at 8000xg for 30 min at room temperature (28 °C) using AllegraTM 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). VCO was collected from the upper oil phase. Oil recovery was calculated using the following equation (Mansor *et al.*, 2012):

$$\text{Oil recovery (\%)} = \frac{\text{Weight of VCO}}{\text{Weight of total oil in emulsion}} \times 100$$

Total oil content was determined by Bligh and Dyer method (Bligh and Dyer, 1959).

5.4.4.1.3. Confocal laser scanning microscopy (CLSM)

The microstructures of coconut milk samples without and with hydrolysis using PPSP and CT at the level of 5 and 10 unit/g proteins for hydrolysis time of 120 min were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan.). The samples were dissolved in Nile blue A solution (1:10) and manually stirred until uniformity was obtained. Fifty µl of sample solutions were smeared on the microscope slide. The CLMS was operated in the fluorescence mode at excitation and emission wavelengths of 533 and 630 nm, respectively, using a Helium Neon Red laser (HeNe-R) for lipid analysis. A magnification of 400x was used.

5.4.4.1.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of coconut milk hydrolyzed with the aid of PPSP and CT at level of 10 unit/g protein as function of hydrolysis time were determined by SDS-PAGE according to the method of Laemmli (1970) using 4% stacking gel and

12% separating gel. Samples were dissolved in 5% SDS at the ratio of 1:1 and heated at 95 °C for 1 h, followed by centrifugation at 7000g for 10 min at 25 °C using a centrifuge (Beckman Coulter, Allegra™ centrifuge, CA, USA). The protein concentration of the supernatant was determined by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin (BSA) as a standard. Samples were mixed with the sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue in 0.5 M Tris-HCl, pH 6.8. The mixtures were heated at 95 °C for 3 min prior to loading. The prepared samples (12 µg protein) were loaded onto the gel. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, VA, USA) at a constant voltage of 200 V/plate. The gels were stained with 0.125% Coomassie blue R125 in 25% methanol and 10% acetic acid. The gels were destained with 40% methanol and 10% acetic acid. Relative mobility (Rf) of proteins was calculated and the molecular weight of the proteins was estimated from the plot between Rf and log (MW) of standards.

5.4.5. Characterization of VCO

Commercial VCO (VCO-COM) and VCO extracted with the aid of PPSP (10 unit/g protein) using hydrolysis time of 120 min (VCO-PPSP) were subjected to analyses. Data were compared with quality parameters issued by Asian Pacific Coconut Community (APCC) (APCC, 2009)

5.4.5.1. Determination of fatty acid profile

Fatty acid profile of VCO samples was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared as per the method of Muhammed *et al.* (2015). The prepared FAMEs were injected to the gas chromatography (7890B GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (100 m × 0.25 mm × 0.20 µm), coated with bonded polysiloxane, was used. The analytical conditions were as follows: injection port temperature of 250 ºC and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention time of FAME standards was used to

identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

5.4.5.2. Determination of moisture content, free fatty acid content (FFA), peroxide value (PV) and ρ-anisidine value (AnV)

Moisture content, FFA content, PV and AnV of VCO samples were analyzed as described by Patil *et al.* (2016).

5.4.6. Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was conducted by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.5. Results and discussion

5.5.1. Effect of PPSP and CT at different levels on hydrolysis of coconut milk and oil recovery

5.5.1.1. Degree of hydrolysis (DH)

Enzymatic hydrolysis of coconut milk using PPSP and CT at different levels (5 and 10 unit/g protein) as a function of time is depicted in Figure 11. Proteases from seabass pyloric caeca were partially purified by ammonium sulfate precipitation (40-60% saturation). After being precipitated by ammonium sulfate, the obtained PPSP fraction had the increase in purity by 8-fold and a yield of 11% was obtained. Different DHs were noticeable as a function of time, depending on the proteases and their levels. DH of coconut milk was increased with increasing hydrolysis time and protease levels. The rapid hydrolysis was observed within the first 30 min, followed by a slower hydrolysis rate. The results suggested that a larger number of peptide bonds were hydrolyzed at the initial stage (Shahidi *et al.*, 1995). With increasing hydrolysis time, the hydrolysis rate was lowered. This was postulated to be related

with a decrease in the available hydrolysis sites, enzyme auto-digestion and/or product inhibition (Kristinsson and Rasco, 2000). At the same hydrolysis time and protease level, higher DH was observed in coconut milk hydrolyzed by PPSP than that of sample hydrolyzed by CT. Results indicated that PPSP was more effective in hydrolysis of coconut milk proteins, compared to CT. Thus, PPSP at the level of 10 units/g protein could be used to hydrolyze proteins in coconut milk, particularly those surrounding oil droplets. This could help disrupt the emulsion.

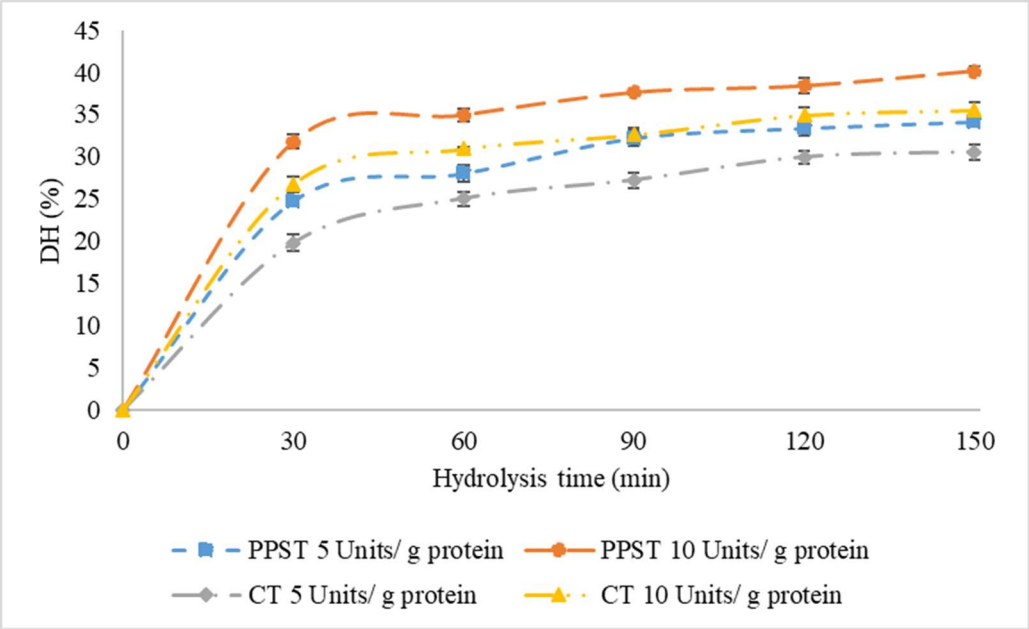

Figure 11. Degree of hydrolysis of coconut milk hydrolyzed with PPSP and CT at different levels as a function of hydrolysis time. Hydrolysis was conducted at 60 °C. PPSP: Partially purified seabass protease, CT: Commercial trypsin. Bars represent SD (n=3).

5.5.1.2. Oil recovery

Oil recovery from coconut milk without and with hydrolysis by PPSP and CT, incubated at 60 °C for various times (30, 60, 90, 120 and 150 min) is depicted in Figure 12. For all samples, the VCO yield was continuously increased as hydrolysis time increased up to 120 min, especially when protease at high level (10 units/g protein) were used. However, difference in VCO yield was observed for different samples. Enzymatic hydrolysis, mediated by proteases, effectively

destabilized the coconut emulsion and released the oil (Rahayu *et al.*, 2008). The highest VCO yield (77.35%) was found when sample was hydrolyzed by PPSP (10 units/g protein) for 120 or 150 min. No difference in yield was observed between 120 and 150 min of hydrolysis ($p>0.05$). Enzymatic pre-treatment has been known as a potential means to obtain the high yield of oil (Marina *et al.*, 2009). At same hydrolysis time and protease level, PPSP showed higher VCO yield, compared to CT. The results suggested that PPSP was able to hydrolyze coconut milk proteins more effectively than CT. On the other hand, the lowest VCO yield was obtained when coconut milk was not hydrolyzed by proteases.

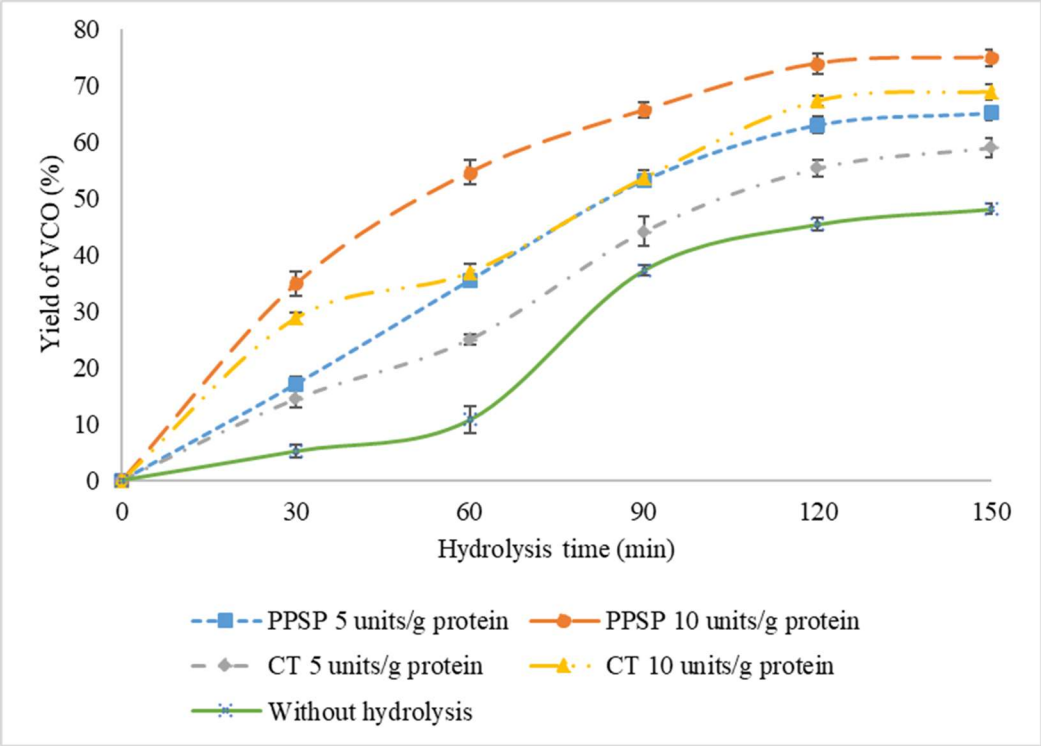

Figure 12. Yield of virgin coconut oil extracted from coconut milk with the aid of PPSP and CT at different levels as function of hydrolysis time. Hydrolysis was conducted at 60 °C. PPSP: Partially purified seabass protease, CT: Commercial trypsin. Bars represent SD (n=3).

The use of proteases could shorten the extraction time of VCO. Proteins surrounding oil droplets were hydrolyzed by protease, resulting in the liberation of oil. Those proteins (mainly globulin and albumin) played a role as the emulsifier in stabilizing the oil droplets in oil-in-water emulsion (Patil and Benjakul,

2017). When those proteins were hydrolyzed, emulsion was not stable with concomitant release of oil from the emulsion. The results were in line with Senphan and Benjakul (2016) who documented that protease from hetapatopancrease of Pacifice white shrimp effectively destabilized coconut milk emulsion, thereby liberating free oil. Without hydrolysis, VCO was liberated gradually after 60 min and the highest yield was obtained when the coconut milk was allowed to stand at 60 °C for 150 min ($p<0.05$). This typical curve was also reported by Senphan and Benjakul (2015) for extraction of VCO from coconut milk at 60 °C without protease hydrolysis. During the incubation of coconut milk at 60 °C for sufficient time, the heat more likely induced the denaturation of proteins in coconut milk, which functioned as an emulsifier. As a result, the emulsion was collapsed as indicated by the increased amount of oil liberated. The results suggested that PPSP, particularly at the level of 10 units/g protein could hydrolyze the proteins surrounding oil droplets in coconut milk to high extent, providing higher VCO yield, compared to other samples. Thus, VCO could be extracted from coconut milk with the aid of PPSP at an appropriate level (10 unit/g protein) at 60 °C for 120 min.

5.5.1.3. Microscopic structure

Microstructures of coconut milk without and with hydrolysis using PPSP and CT (5 units/g protein and 10 units/g protein) at 60 °C for 120 min were visualized by confocal laser scanning microscopy (Figure 13). Differences in droplet size between samples were observed. The largest oil droplets were found in coconut milk hydrolyzed using proteases, when compared to those of samples without hydrolysis. At the same protease level, droplet size of PPSP hydrolyzed sample was higher, compared to that of CT hydrolyzed counterpart. Moreover, droplet size was increased with increasing protease level. The more collapse or higher coalescence of PPSP treated sample was evidenced by larger droplet size, higher DH and VCO yield (Figure 11 and 12).

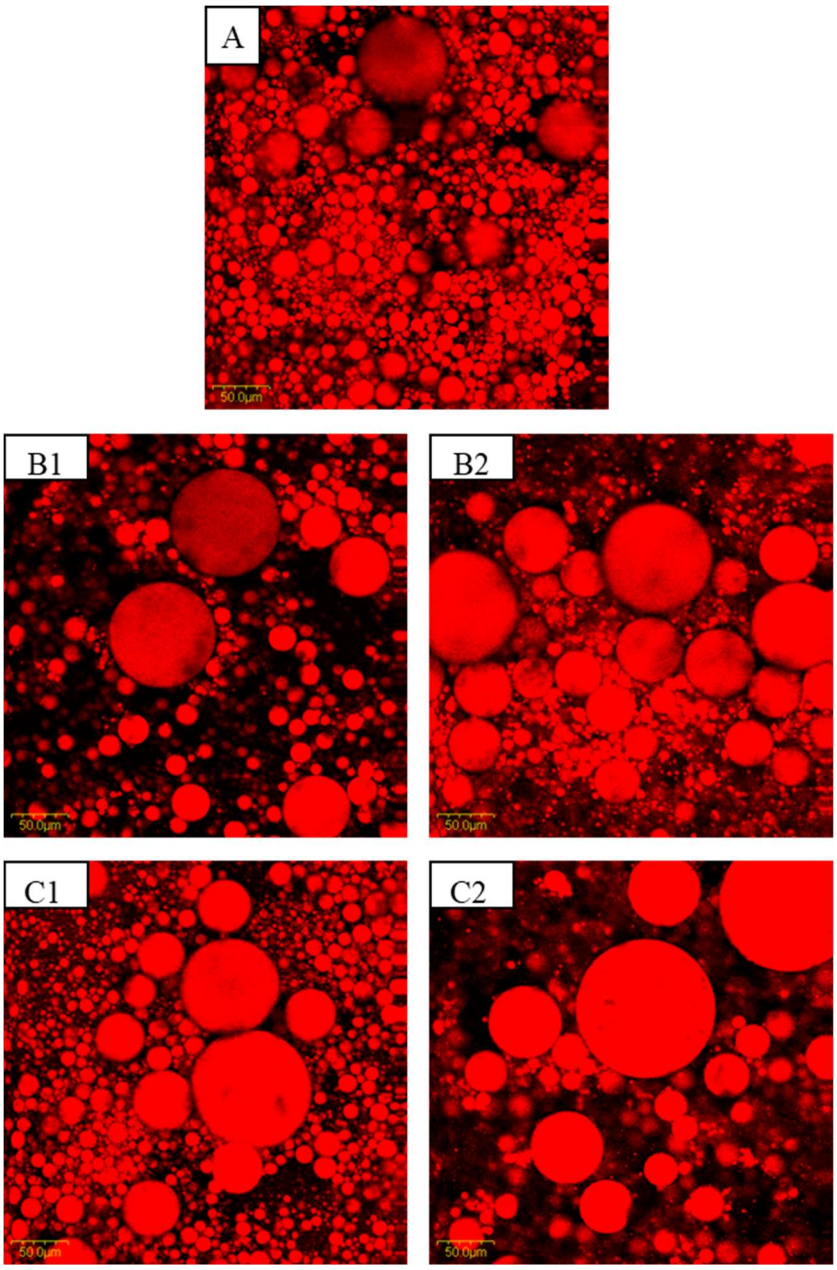

Figure 13. Confocal laser scanning micrographs of coconut milk hydrolyzed with the aid of PPSP and CT at different levels at 60 ºC for 120 min.

(A) Control (without protease);

(B) CT (B1: 5unit/g protein) and (B2: 10 unit/g protein);

(C) PPSP (C1: 5 unit/g protein) and (C2: 10 unit/g protein).

PPSP: Partially purified seabass protease, CT: Commercial trypsin.

Magnification: 400×. Scale bar = 50 µm.

Highest droplet size was observed for coconut milk hydrolyzed with PPSP at a level 10 units/ g protein for 120 min. Conversely, the lowest droplets size was observed for sample without hydrolysis. Therefore, the hydrolysis by PPSP could induce the coalescence or collapse of emulsion, in which VCO could be separated at a higher extent.

5.5.1.4. SDS-PAGE

Protein patterns of coconut milk hydrolyzed with PPSP and CT at level of 10 units/ g proteins as a function of time under reducing condition are shown in Figure 14. For the control (without protease), proteins with MW lower than 55 kDa including those with MW of 44, 34, 32 and 30 kDa were observed. Additionally, proteins with MW lower than 24 kDa involving proteins with MW of 22, 20 and 18 kDa were also attained. Protein patterns for coconut milk hydrolyzed by PPSP and CT were found to be different after hydrolysis at different times. For both samples within the first 5 min, several proteins were degraded with the coincidental formation of proteins or peptides with lower MW. Protein degradation was more pronounced as the hydrolysis time increased. Proteins were degraded to higher extent in coconut milk hydrolyzed with PPSP, compared to CT. The obtained results were in accordance with DH (Figure 11). After 5 min of hydrolysis with PPSP, the drastic decrease was noticeable in protein with MW of 55 kDa, a dominant protein. With increasing hydrolysis time up to 150 min, it almost disappeared. When the sample was hydrolyzed with CT, no drastic degradation of protein with MW of 55 kDa was observed up to 150 min of hydrolysis. The hydrolysis of 55 kDa protein coincided with the increase in band intensity of protein with MW of 34 kDa. The major protein (~65%) in coconut endosperm is cocosin with MW 55 kDa (Garcia *et al.*, 2005). It was believed to play a prominent role in stabilization of coconut milk emulsion (Tangsuphoom and Coupland, 2008; Patil and Benjakul, 2017). Protein with MW of 31 kDa was observed after 5 min of hydrolysis with PPSP and disappeared after 20 min. However, protein with MW of 31 kDa was not observed in coconut milk hydrolyzed by CT. Protein with MW of 44 kDa disappeared in both sample after 5 min of hydrolysis. Conversely, protein with MW of 32 and 22 kDa was retained in both samples, regardless of hydrolysis time. However, band intensity of protein with

MW of 32 was lower in coconut milk hydrolyzed by CT. After 5 min of hydrolysis with CT, protein having MW of 24 kDa totally disappeared. Drastic decrease in the aforementioned protein was noticeable after 5 min of hydrolysis by PPSP and disappearance was more obvious with increasing hydrolysis time up to 150 min. There were several bands with MW below 20 kDa in both sample. Patil and Benjakul (2017) reported that degradation of major proteins and the remaining proteins most likely affected emulsion stability in different fashions. It can be inferred that coconut milk proteins were more susceptible to hydrolysis by PPSP than CT.

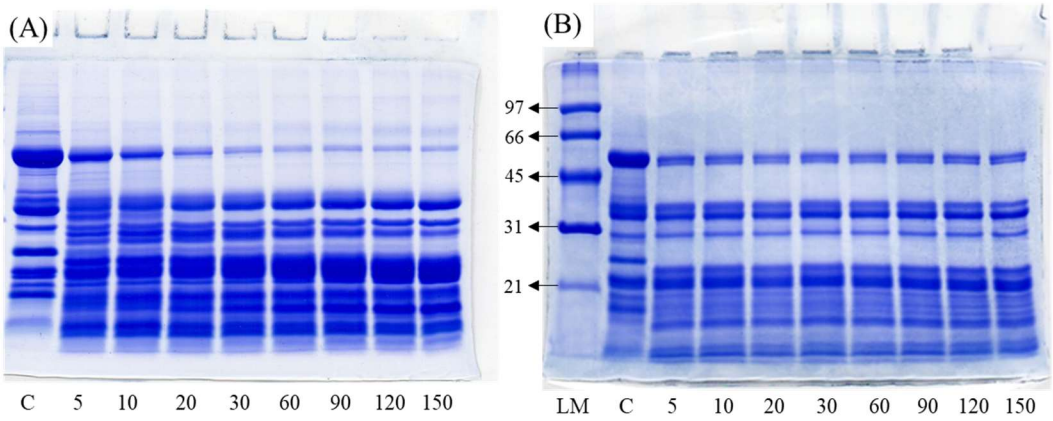

Figure 14. Protein pattern of coconut milk hydrolyzed with PPSP (A) and CT (B) at level of 10 units/g protein for different hydrolysis times.
LM: low molecular weight, C: Control without enzyme addition (time:0). Numbers designate the hydrolysis time (min). PPSP: Partially purified seabass protease, CT: Commercial trypsin.

5.5.2. Characterization of VCO

5.5.2.1. Fatty acid profile

Fatty acid profiles of commercial VCO (VCO-COM) and VCO extracted from coconut milk with the aid of PPSP (VCO-PPSP) are shown in Table 23. Both VCO samples prevalently contained MCFAs, mainly lauric acid. The second major fatty acid was myristic acid. VCO-PPSP had higher lauric acid (48.84%) compared to VCO-COM ($p<0.05$). The most abundant MCFA present in coconut oil is lauric acid, which is responsible for health benefits (Enig, 1996). On the other hand,

VCO-COM had higher myristic acid (19.75%). MCFAs having 6 to 12 carbons include caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids (Papamandjaris *et al.*, 1998). Palmitic acid (9.09% and 9.61%) was found in VCO-PPSP and VCO-COM, respectively. Caprylic acid and capric acid constituted at 6.30% and 5.61% in VCO-PPSP, respectively. Conversely, lower caprylic acid and capric acid contents were found in VCO-COM. In general, there was no marked difference in fatty acid profiles between both samples. Furthermore, the fatty acid compositions were comparable to those guided by Asian and Pacific Coconut Community (APCC) standards (APCC, 2009).

Table 23. Fatty acid profile of VCO-COM and VCO-PPSP.

Fatty acids (g/100 g oil)	**VCO-PPSP**	**VCO-COM**	**APCC Standards**
Caproic acid (C6:0)	0.43±0.00b	0.44±0.00a	0.10 – 0.95
Caprylic acid (C8:0)	6.30±0.00a	6.17±0.00b	4 – 10
Capric acid (C10:0)	5.61±0.00a	5.50±0.04b	4 – 8
Lauric acid (C12:0)	48.84±0.03a	47.46±0.04b	45 – 56
Myristic acid (C14:0)	18.86±0.02b	19.75±0.08a	16 – 21
Palmitic acid (C16:0)	9.09±0.01b	9.61±0.10a	7.5 – 10.2
Stearic acid (C18:0)	3.63±0.00a	3.59±0.02b	2 – 4
Cis-9-Octadecanoic acid (C18:1 n-9)	6.02±0.00b	6.10±0.32a	4.5 - 10
Cis-9,12-Octadecadienoic acid (C18:2 n-6)	0.83±0.00b	1.14±0.02a	0.7 – 2.5
Arachidic acid (C20:0)	0.10±0.00a	0.10±0.00a	-
Unidentified peak	0.29±0.00a	0.14±0.00b	-

Values are mean ± standard deviation (n=3). Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

VCO-COM: commercial VCO and VCO-PPSP: VCO extracted with the aid of PPSP (10 unit/g protein) for 120 min at 60 °C.

5.5.2.2. Moisture content

Moisture content of both VCO samples was negligible (~0.1%) (Table 24). According to APCC standard, VCO must have moisture content below 0.5% (APCC, 2009). With high moisture content, VCO could be susceptible to hydrolysis process, releasing free fatty acids. Free fatty acid underwent oxidation with ease (Raghavendra and Raghavarao, 2010). Both VCO samples had very low moisture content, thereby assuring their long shelf-life. There was no difference in moisture content between VCO-COM and VCO-PPSP. Therefore, VCO could be extracted from coconut milk effectively using PPSP as a processing aid.

Table 24. Properties of VCO-COM and VCO-PPSP.

Sr. No.	Parameters	VCO-PPSP	VCO-COM	APCC Standards
1.	Moisture (%)	0.1±0.03a	0.07±0.02a	Max 0.5
2.	Free fatty acid (%)	0.20±0.03a	0.09±0.02b	Max 0.5
3.	Peroxide Value (meq O_2/kg)	2.65±0.57a	2.06±0.86a	Max 3
4.	*ρ*-anisidine	0.85±0.15a	0.91±0.28a	-

The mean ± standard deviation, n=3. Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

VCO-COM: commercial VCO and VCO-PPSP: VCO extracted with the aid of PPSP (10 unit/g protein) for 120 min at 60 °C.

5.5.2.3. Free fatty acid (FFA)

FFA contents of VCO-COM and VCO-PPSP are shown in Table 24. Low FFA content was observed in both VCO samples. The result indicated that ester bonds of triglycerides were cleaved to low degree. Similar results were reported by Senphan and Benjakul (2016), in which VCO extracted using proteases from hepatopancreas of Pacific white shrimp showed low FFA contents. However, slight difference was observed between both samples. This might be related to different extraction process of VCO. FFAs are responsible for unacceptable taste and aroma in

oils and fats. VCO produced by enzymatic process contained low FFA with a good smell and long lifetime (Rahayu *et al.*, 2008). Marina *et al.* (2009) found that FFAs were high in coconut oils having high moisture content. FFA contents of both VCO samples were below APCC standard (APCC, 2009), indicating their good initial quality.

5.5.2.4. Peroxide value (PV)

Low PV was found in both VCO samples (Table 24). PV is used to determine the primary oxidation products of oil (Choe and Min, 2006). Low PV in VCO indicated that low lipid oxidation took place in both samples. VCO was rich in saturated fatty acids (Table 23), which were resistant to oxidation. When comparing PV between both samples, the similar PV was observed, suggesting the similar oxidative stability of VCO-COM and VCO-PPSP. PV of both VCO samples were within the standard range (max 3 meq O_2/kg) guided by to APCC standard (APCC, 2009).

5.5.2.5. Anisidine value (AnV)

Low AnV was observed in both VCO samples (Table 24). No difference in AnV was found between both samples ($p>0.05$). Rossell (1983) documented that oils with an AnV below 10 were considered as good quality. AnV is used to determine the secondary oxidation products of oil. The primary oxidation products i.e. peroxides are not stable and decomposed to the secondary products such as aldehydes, which are responsible for rancid odor and taste. High AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Low AnV of both samples was in line with low PV (Table 24). The results reconfirmed high oxidative stability of both VCO samples.

5.6. Conclusion

PPSP could be used to destabilize coconut milk emulsion for production of VCO with the increased yield. The extraction of VCO using PPSP (10 unit/g protein) at 60 °C for 120 min increased the yield without negative effect on quality, particularly fatty acid composition. VCO had high contents of MCFAs,

especially lauric acid and myristic acid. Thus, the use of PPSP at an appropriate concentration could shorten the extraction time and increase the efficiency in VCO production without the detrimental impact on quality.

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CHAPTER 6
PRODUCTION OF VIRGIN COCONUT OIL WITH AID OF PROTEASE FROM SEABASS PYLORIC CAECA IN COMBINATION WITH DIFFERENT TREATMENTS

6.1. Abstract

Virgin coconut oil (VCO) was extracted from coconut milk emulsion with the aid of partially purified protease from seabass pyloric caeca (PPSP) in combination with different treatments including micro-fluidization, chill-thawing and freeze-thawing. Lowest VCO yield was obtained for coconut milk homogenized at 4000 psi, followed by hydrolysis using PPSP at the level of 5 units/ g proteins. Conversely, the highest VCO yield was obtained from non-homogenized and unhydrolyzed coconut milk, compared to homogenized and hydrolyzed counterpart ($p<0.05$). Coconut milk emulsion hydrolyzed by PPSP at 10 units/g protein, followed by freeze-thawing showed the highest yield among all the samples. VCO yield increased as chill-thawing cycles of hydrolyzed coconut milk increased up to 5 cycles ($p<0.05$). Similar trend was found in combined treatment of hydrolysis, followed by chill-thawing. Hydrolysis by PPSP, followed by freeze-thawing of 5 cycle, yielded the highest yield of VCO (98.6 %). No marked difference was observed in fatty acid profile, moisture content, free fatty acid content and oxidative stability of all VCO obtained, regardless of subsequent chill-thawing and freeze-thawing were applied. All VCO samples were in the range of APCC standards. Thus, combined treatment including hydrolysis using PPSP followed by freeze-thawing (5 cycles) was the powerful method for extraction of VCO, in which high yield and prime quality could be gained.

6.2. Introduction

Coconut (*Cocos nucifera* L.) well-known products are coconut water, coconut meat, coconut milk and coconut oil. Coconut milk is generally extracted from grated coconut meat after pressing or squeezing with or without addition of water. It is milky white oil-in-water emulsion. It has been used as a starting material for production of virgin coconut oil (VCO), in which collapse of coconut milk emulsion

is required (Patil and Benjakul, 2018). Stability of coconut milk emulsion is generally governed by some proteins present in aqueous phase, mainly albumin and globulin (Peamprasart and Chiewchan, 2006; Patil and Benjakul, 2017). To maximize the yield of VCO, the coconut milk emulsion must be collapsed to a high degree, in which oil can be released and separated effectively. VCO consists of medium chain fatty acids (MCFAs), mainly lauric acid. This is not similar to other vegetable oils because of its high MCFAs content (Dayrit, 2014). Due to of high stability and various health benefits, VCO is rapidly getting an enormous importance (Carandang, 2008). To obtain VCO from wet extraction process, destabilization of coconut milk emulsion has been implemented via several processes such as physical extraction, fermentation and enzymatic extraction (Raghavendra and Raghavarao, 2010).

Proteases, especially from microorganism, have been widely employed for hydrolysis. Despite their availability, the cost is still one of the major concern for application, particularly for VCO production. Thus, the proteases from cheap sources, especially fish processing byproducts, e.g. seabass pyloric caeca, can be alterative and promising for production of VCO. Additionally, the cost of commercial protease can be reduced. Yield is generally one of prime parameters in VCO production. To fulfil the extraction of VCO with prime quality and high yield, the combined methods should be developed, in which the shorter processing time and lower cost could be achieved. Therefore, the main objectives of the present work were to recover proteases from seabass pyloric caeca and to study different treatments for destabilization of coconut milk emulsion to obtain VCO.

6.3. Objective

To study the production of virgin coconut oil with aid of protease from seabass pyloric caeca in combination with different treatments.

6.4. Materials and Methods

6.4.1. Chemicals

Sodium hydroxide was purchased from Sigma (St. Louis. MO, USA). Methanol, ethanol, acetic acid, propanol, petroleum ether, hydrochloric acid, and n-hexane were procured from Lab-Scan (Bangkok, Thailand).

6.4.2. Preparation of coconut milk

Mature coconuts (11-12 months old from pollination) were purchased from a plantation site in Yaring district, Pattani province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was removed manually and grated using a rotary wedge cutter machine. Coconut milk was then prepared from grated kernel without water addition using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand).

6.4.3. Preparation of partially purified trypsin from seabass pyloric caeca

Pooled viscera of seabass were purchased from a market in Hat Yai, Songkhla province, Thailand. Viscera was placed in a polyethylene bag, kept in ice with a matter/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, pyloric caeca were dissected and stored at -18 °C until use.

To prepare the crude extract, pyloric caeca were powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2008) with a slight modification. Pyloric caeca powder was suspended in an extraction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$) with a ratio of 1:10 (w/v). The mixture was homogenized for 2 min using a homogenizer (Model T25B, IKA labortechnik, Selangor, Malaysia) at 11,000 rpm. The homogenate was then continuously stirred for 30 min at 4 °C and centrifuged at 8000xg for 30 min at 4 °C using a refrigerated centrifuge (Becman Coulter Model

Avant J-E, Becman Coulter, Inc., CA, USA). The supernatant was filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The filtrate obtained was referred to as 'pyloric caeca extract, PCE'.

PCE was subjected to 40-60% saturated ammonium sulfate precipitation, according to the method of Khantaphant and Benjakul (2008) with a slight modification. After the addition of ammonium sulfate, the mixture was stirred gradually at 4 °C for 30 min. Subsequently, the mixture was centrifuged at 8000xg for 30 min at 4 °C and the pellet obtained was dissolved in the minimum volume of 50 mM Tris–HCl buffer, pH 8.0. The solution was dialyzed against 20 volumes of the extraction buffer /overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'partially purified seabass protease, PPSP'.

6.4.3.1. Trypsin activity assay

Trypsin activity of PPSP was measured using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008). A 200 µL of sample was mixed with 200 µL of distilled water and 1000 µL of reaction buffer (50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$). The reaction was initiated by adding 200 µL of 2 mg/mL BAPNA to the reaction mixture. After incubation for 20 min at 60 °C, 200 µL of 30% acetic acid (v/v) was added to terminate the reaction. Production of *ρ*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). A blank was conducted in the same manner except that sample was added after addition of 30% acetic acid. Trypsin activity was calculated using the following equation:

$$Trypsin\ activity\ = \frac{\left((A - A_0)\ \times\ mixture\ volume\ (ml) \times 1000\right)}{8800\ \times\ reaction\ time\ (min) \times 0.2}$$

where 8800 ($cm^{-1}\ M^{-1}$) is the extinction coefficient of *ρ* -nitroaniline; A and A_0 are A_{410} of sample and blank, respectively. One unit of activity is defined as that releasing 1 nmol of *ρ*-nitroaniline per min.

6.4.4. Effect of combined methods on destabilization of coconut milk emulsion

6.4.4.1. Enzymatic hydrolysis of coconut milk without and with prior micro-fluidization by PPSP

The coconut milk was subjected to a high-speed homogenizer at 11000 rpm for 2 min. These coarse emulsions were passed through high-pressure homogenizer (Microfluidics, Model HC 5000, Stanwood, WA, USA) at 2000 and 4000 psi for two times. All coconut milk samples were subjected to enzymatic hydrolysis.

6.4.5. Hydrolysis of coconut milk with PPSP as a function of time

Enzymatic extraction of VCO from homogenized coconut milk was performed according to the method of Senphan and Benjakul (2016) with a slight modification. The coconut milk (100 mL) in a 250 mL-Erlenmeyer flask was adjusted to pH 8 using 2 M NaOH. Thereafter, PPSP was added into coconut milk to obtain different protease levels (5 and 10 units/g protein). The hydrolysis was allowed to proceed at an optimal temperature of PPSP (60 °C) for different times (30, 60, 90, 120 and 150 min). Control was prepared with same manner without homogenization and PPSP addition. All samples were used for oil recovery.

6.4.5.1. Oil recovery

After the designated time, the mixtures were centrifuged at 8000 xg for 30 min at room temperature (28 °C) using AllegraTM 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). VCO was collected from the upper oil phase. Oil recovery was calculated using the following equation (Mansor *et al.*, 2012):

$$\text{Oil recovery (\%)} = \frac{\text{Weight of VCO}}{\text{Weight of total oil in emulsion}} \times 100$$

Total oil content was determined by Bligh and Dyer method (Bligh and Dyer, 1959).

6.4.6. Enzymatic hydrolysis followed by different methods

Coconut milk (without homogenization) was hydrolyzed with PPSP (5 and 10 units/g protein) as a function of different time (30, 60, 90, 120 and 150 min) at 60 °C as described previously. Thereafter, centrifugation at low speed (3585xg) for 10 min was conducted to obtain coconut cream and aqueous phase. Control was prepared in the same manner without homogenization and PPSP addition. The obtained cream (without oil separations) was further subjected to chill-thawing and freeze-thawing.

6.4.6.1. PPSP hydrolysis followed by chill-thawing

The obtained cream was chilled at 4 °C for 6 h and then thawed at room temperature (30±2 °C) for 1 h. Finally, cream was centrifuged at high speed (8000xg) to obtain oil as described previously.

To study the impact of chill-thawing cycles, coconut milk was firstly hydrolyzed with PPSP (10 units/ g protein) at 60 °C for 60 min followed by low speed centrifugation (3585xg) to obtain cream. The cream was then subjected to chill-thawing with different cycles (1, 2, 3, 4 and 5). Control was prepared in the same manner without PPSP addition. This combined extraction method was named as 'PPSP-CT process'. Oil recovery was calculated as described previously.

6.4.6.2. PPSP hydrolysis followed by freeze-thawing

The cream was frozen at -20 °C for 6 h and then thawed at room temperature (30±2 °C) for 1 h. Finally, cream was centrifuged at high speed (8000xg) to obtain oil as described above.

To investigate the impact of freeze-thawing cycles, the cream was prepared as detailed in section 6.4.6. The cream was then subjected to freeze-thawing with different cycles (1, 2, 3, 4 and 5). Control was prepared in the same manner without PPSP addition. This combined extraction method was termed as 'PPSP-FT process'. Oil recovery was then calculated.

6.4.7. Characterization of VCO

VCO samples extracted from PPSP-CT and PPSP-FT processes with five cycles of chill-thawing, respectively were subjected to analyses.

6.4.7.1. Determination of fatty acid profile

Fatty acid profile of VCO sample was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared as per the method of Muhammed *et al.* (2015). The prepared FAMEs were injected to the gas chromatography (7890B GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (100 m × 0.25 mm × 0.20 µm), coated with bonded polysiloxane, was used. The analytical conditions were as follows: injection port temperature of 250 ºC and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention time of FAME standards was used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

6.4.7.2. Determination of moisture content, free fatty acid content (FFA), peroxide value (PV) and ρ-anisidine value (AnV)

Moisture content, FFA content, PV and AnV of VCO samples were analyzed according to the method described by Patil *et al.* (2016).

6.4.8. Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.5. Results and discussion

6.5.1. Effect of combined methods on destabilization of coconut milk emulsion

6.5.1.1. Enzymatic hydrolysis of coconut milk without and with prior micro-fluidization by PPSP

Effect of micro-fluidization of coconut milk followed by enzymatic hydrolysis with aid of PPSP on VCO yield is depicted in Figure 15. Different homogenization pressures (2000 psi and 4000 psi) and levels of PPSP (5 units/g protein and 10 units/g protein) had different impact on VCO yield. Highest VCO yield was obtained for non-homogenized coconut milk hydrolyzed by PPSP (10 units/ g protein), compared to other samples ($p<0.05$). Proteins (mainly globulin and albumin) played a role as the emulsifiers in stabilizing the oil droplets in coconut milk (Patil and Benjakul, 2017). When those proteins were hydrolyzed, emulsion was not stable with concomitant release of oil from the emulsion. For non-homogenized and without hydrolysis sample, VCO was liberated gradually after 60 min and the highest yield was obtained when the coconut milk was allowed to stand at 60 °C for 150 min ($p<0.05$). During the incubation of coconut milk at 60 °C for long time (150 min), the heat might induce the denaturation of proteins surrounding oil droplets in coconut milk. Thermal treatment increases the degree of flocculation, probably because of hydrophobic-hydrophobic interactions between denatured domains. This led to an increased effective particle size (Tangsuphoom and Coupland, 2005). When force during centrifugation was applied, destabilization of emulsion was enhanced, resulting in the separation of two phases, oil and aqueous phase.

On the other hand, the lowest VCO yield was obtained for sample homogenized at 4000 psi followed by hydrolysis using PPSP (5 units/g protein) ($p<0.05$). It was noticed that VCO yield was decreased when homogenization pressure was increased. During the homogenization, the high shear forces could break down droplet in dispersed phase (Floury *et al.*, 2002). Increasing homogenization pressure marginally decreased the droplet size. As a result, small oil droplets were generated at higher homogenizing pressures. The homogenized coconut milk samples

were more viscous than the non-homogenized counterpart. Smaller droplets were closely packed or extensively interconnected to form network. Moreover, proteins surrounding oil droplets might increase the emulsion stability. Therefore, low VCO yield was obtained even after hydrolysis of coconut milk at 60 °C and further application of centrifugation force. Strong interconnected network of small oil droplets prevented them against coalescence. Nevertheless, PPSP treatment, especially at higher level of non-homogenized coconut milk, rendered the VCO with the higher yield, compared with others. Hydrolysis of proteins surrounding oil droplets in coconut milk (non-homogenized) was able to increase the collapse of emulsion. Therefore, homogenization followed by hydrolysis had negative impact on VCO recovery from coconut milk.

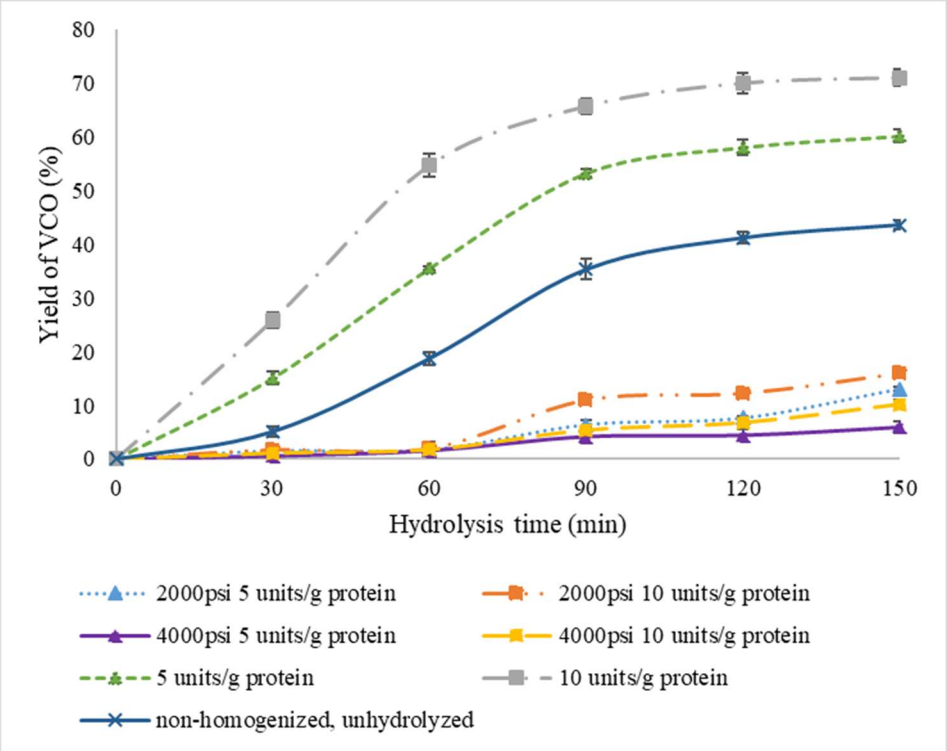

Figure 15. Yield of VCO extracted by combined treatments as a function of hydrolysis time. Micro-fluidization at different pressures followed by hydrolysis with the aid of PPSP at different enzyme levels was used. Hydrolysis was conducted at 60 °C.

PPSP: Partially purified seabass protease. Bars represent SD (n=3).

6.5.1.2. Hydrolysis followed by chill-thawing

6.5.1.2.1. Impact of hydrolysis time

The effect of hydrolysis of coconut milk (non-homogenized) with the aid of PPSP (5 and 10 units/ g protein) followed by chill-thawing on VCO yield is shown in Figure 16. High VCO yield was observed within the first 30 min ($p<0.05$), followed by a slower oil recovery rate from 60 to 150 min. The highest oil recovery was observed when sample hydrolyzed with PPSP (10 units/ g protein) for 150 min, followed by chill-thawing ($p<0.05$). However, no difference was observed between sample hydrolyzed with PPSP (10 units/ g protein) for 60 and 150 min ($p>0.05$). At the same hydrolysis time, the yield of VCO was increased with increasing levels of PPSP ($p<0.05$). The efficiency of enzyme in extraction of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu *et al.*, 2008). PPSP more likely hydrolyzed proteins surrounding oil droplets. As a consequence, degraded proteins might be less effective to form a film surrounding oil droplets. Thereafter, centrifugation at 3585xg for 10 min allowed closely packaging of the oil droplets. Subsequently at low temperature, solidification of oil and conformational changes of some proteins plausibly occurred. Chilling and thawing is important to enhance destabilization of the enzyme treated emulsion. Packing of fat globules during chilling is necessary to facilitate oil separation (Raghavendra and Raghavarao, 2010). Upon thawing, closely packed oil droplets loose their spherical structure and finally the close contact among large droplets was enhanced by applied force during centrifugation. This led to destabilization of coconut milk emulsion, resulting in the phase separation and formation of oil and aqueous layers. The results suggested that interfacial film surrounding oil droplet was hydrolyzed by PPSP and was not efficient to protect oil droplets against coalescence. On the other hand, unhydrolyzed sample showed low oil recovery, compared to hydrolyzed counterpart ($p<0.05$). Yield of VCO from non-homogenized coconut milk was increased slowly until 150 min. During chilling, a variety of different physicochemical processes might occur, including fat solidification, ice formation, interfacial phase transitions and conformational changes of proteins (McClements, 2004). Those phenomena might play a role in destabilizing

the emulsion. As a result, the emulsion was collapsed as indicated by an increased amount of VCO liberated. The results indicated that enzymatic hydrolysis prior to chill-thawing led to the high destabilization of coconut milk emulsion, liberating VCO to higher extent.

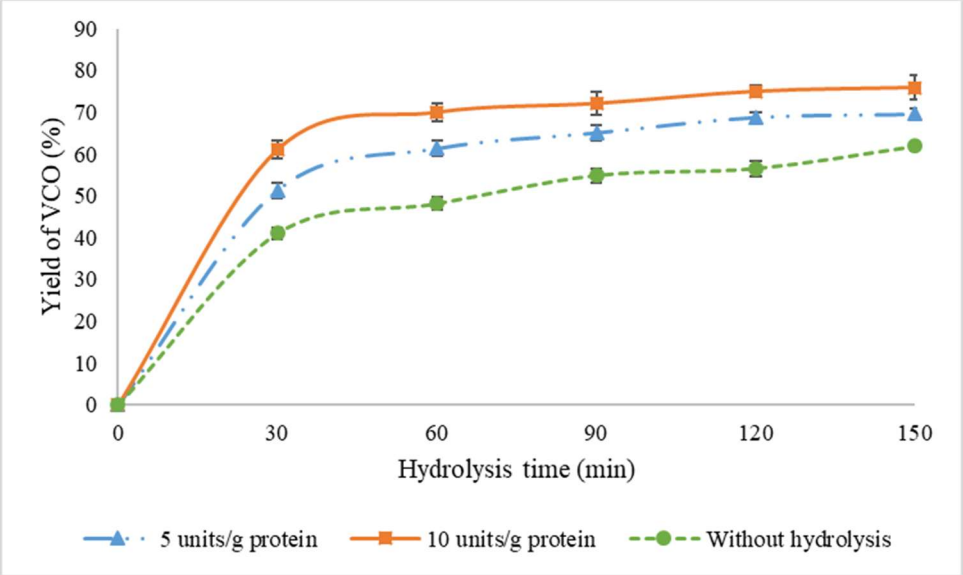

Figure 16. Yield of VCO extracted by combined treatment, hydrolysis with the aid of PPSP at different levels as a function of hydrolysis time followed by chill-thawing.

Hydrolysis was conducted at 60 °C, Chill-thawing: 4 °C for 6 h, followed by 37 °C for 1 h. PPSP: Partially purified seabass protease. Bars represent SD (n=3).

6.5.1.2.2. Impact of chill-thawing cycles

Yield of VCO as influenced by PPSP-CT process with repeated cycles is shown in Figure 17. For both samples, with increasing chill-thawing cycles from 1 to 5, yield was increased from 70% to 91% and from 45.55% to 67.1%, for hydrolyzed and unhydrolyzed sample, respectively. Increase in chill-thawing cycles plausibly caused the additional conformational changes in proteins surrounding oil droplets, which might lead to denaturation and lowering emulsifying properties of protein. Therefore, after thawing, proteins might not be able to protect oil droplets against coalescence. With the same chill-thawing cycles, the yield of VCO was higher in hydrolyzed sample, compared to unhydrolyzed sample ($p<0.05$). Highest oil

recovery (91%) was obtained when chill-thawing cycles was used for coconut milk hydrolyzed with PPSP (10 units/ g protein). The results suggested that interfacial film might be affected by action of combined treatments, hydrolysis followed by chill-thawing. For unhydrolyzed sample at the fifth chill-thawing cycle, incomplete separation of oil from cream was observed after the centrifugation. Some proteins might be unaffected by chill-thawing. As a result, oil droplets were still protected against coalescence. In general, stability of hydrolyzed and unhydrolyzed coconut milk emulsion was induced by chill-thawing cycles.

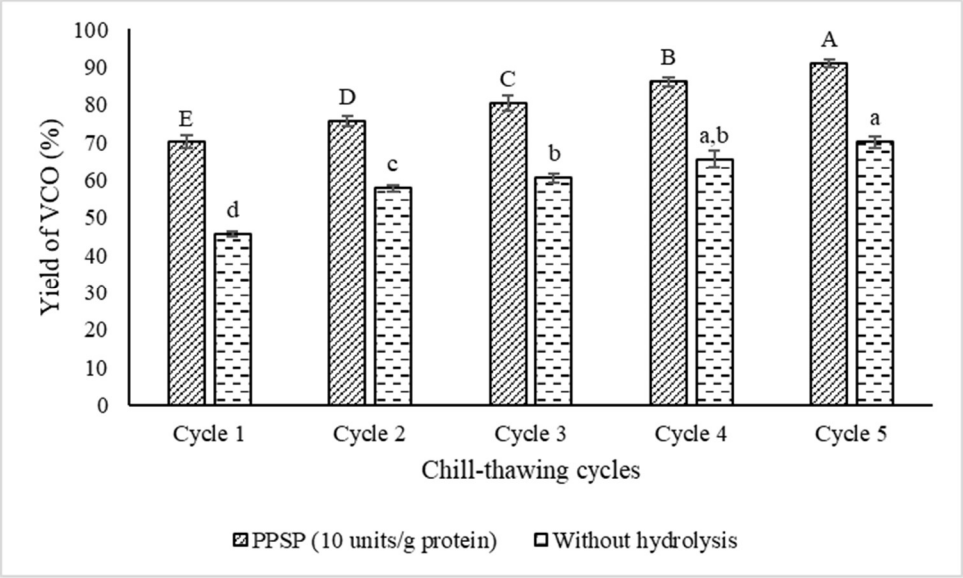

Figure 17. Yield of VCO extracted by PPSP-CT process with different cycles.

PPSP-CT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h at 60 °C, followed by chill-thawing with various cycles.

PPSP: Partially purified seabass protease. Bars represent SD (n=3).

6.5.1.3. Hydrolysis followed by freeze-thawing

6.5.1.3.1. Impact of hydrolysis time

The effect of hydrolysis of coconut milk with the aid of PPSP (5 and 10 units/ g protein) followed by freeze-thawing (-20 °C for 6 h, then thawing at 30 °C for 1 h) on VCO yield is shown in Figure 18. Similar trend was observed with enzymatic hydrolysis followed by chill-thawing (Figure 16). High oil yield was

observed for all samples within the first 30 min, followed by a slower oil recovery rate from 60 min to 150. The highest oil recovery was observed when sample was hydrolyzed with PPSP (10 units/ g protein) for 150 min, followed by freeze-thawing ($p<0.05$). During freeze-thawing, crystallization of oil and denaturation of some proteins took place. As a result of lowering temperature to -20 °C, ice crystal formed might induce the disarrangement of the emulsion. The liquid/semisolid droplets constantly collided with the adjacent solid or liquid droplets and then coalesced or partially coalesced during freezing (Lin *et al.*, 2008). Furthermore, coconut milk is oil-in-water emulsion. Water is the continuous phase, which was transformed to ice with volume expansion. The oil-water interface plausibly compressed/penetrated and coalescence of oil droplet occurred upon thawing. Coalesced or partially coalesced droplets fused into large drops and phase separation took place. The results suggested that proteins surrounding oil droplet were hydrolyzed/denatured and were unable to protect oil droplets against coalescence.

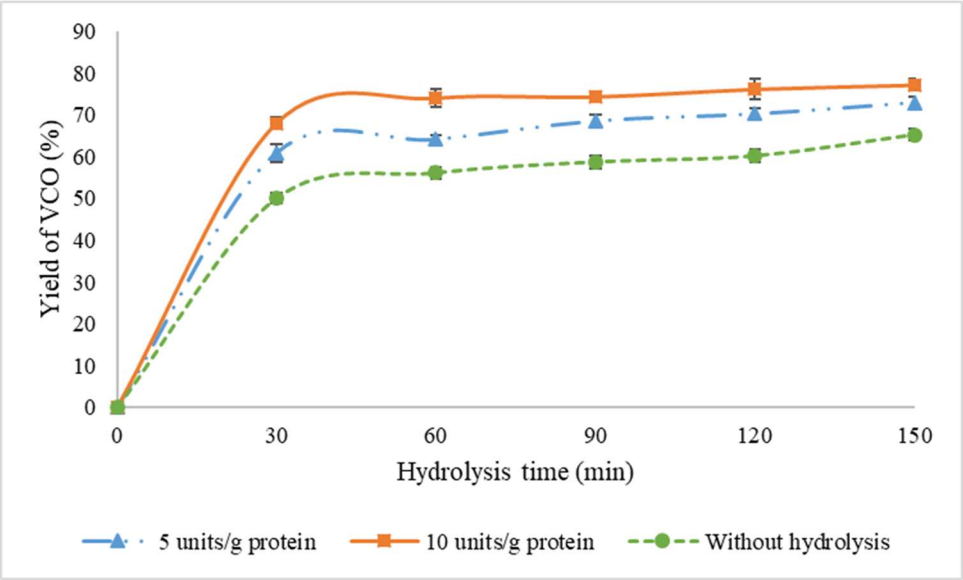

Figure 18. Yield of VCO extracted by combined treatment, hydrolysis with the aid of PPSP at different levels as a function of hydrolysis time followed by freeze-thawing.

Hydrolysis was conducted at 60 °C, freeze-thawing: -20 °C for 6 h, followed by 37 °C for 1 h. PPSP: Partially purified seabass protease. Bars represent SD (n=3).

Unhydrolyzed sample showed low oil recovery, compared with hydrolyzed samples. Thus, prior hydrolysis of coconut milk, following by freeze-thawing was a potential method for VCO production with increased yield.

6.5.1.3.2. Impact of freeze-thawing cycles

Yield of VCO as influenced by PPSP-FT process with various cycles is shown in Figure 19. For all the samples, as freeze-thawing cycles increased from 1 to 5, yield was increased from 74% to 98.6% and from 56.1% to 81.45%, for hydrolyzed and unhydrolyzed sample, respectively. Extensive droplet flocculation was observed after only one freeze-thawing cycle, which led to rapid droplet creaming. Obvious oil separation was obtained after third freeze-thawing cycle, suggesting that extensive coalescence occurred. Difference in VCO yield was noticed for both PPSP-CT and PPSP-FT process. At the same cycle, PPSP-FT process showed higher VCO yield, compared to PPSP-CT process.

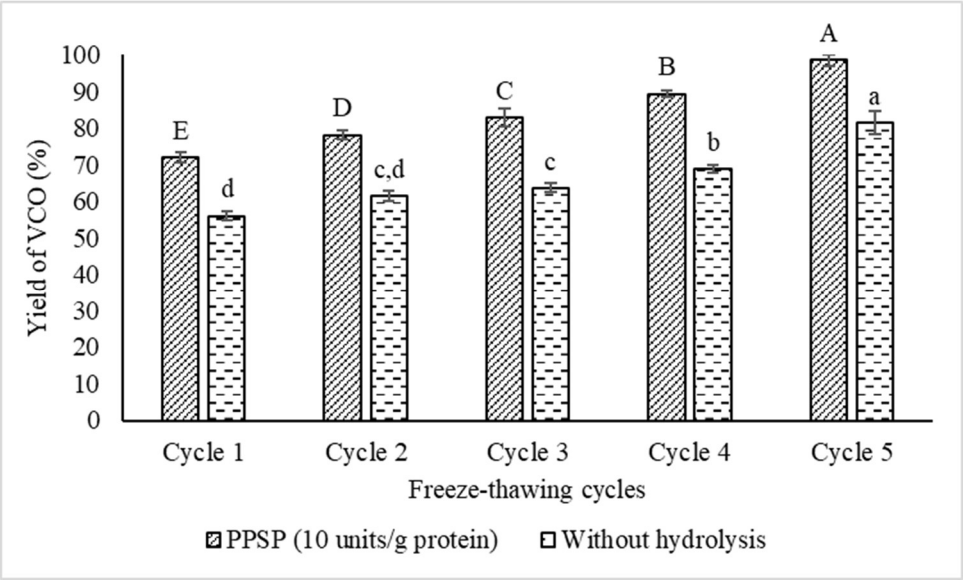

Figure 19. Yield of VCO extracted by PPSP-FT process with different cycles.

PPSP-FT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h at 60 °C, followed by freeze-thawing with various cycles.

PPSP: Partially purified seabass protease. Bars represent SD (n=3).

This might be ascribed to their different de-emulsification processes and mechanisms caused by certain differences in the physical properties between their oil and aqueous phases. The physical properties including freezing point, specific heat capacity, thermal conductivity, density, and viscosity, probably affected the destabilization of emulsion induced by chill-thawing and freeze-thawing (Lin *et al.*, 2008). It was suggested that combined treatment, enzymatic hydrolysis followed by freeze-thawing for 5 cycles, was the most promising destabilization process for coconut milk emulsion.

6.5.2. Characterization of VCO

6.5.2.1. Fatty acid profile

Fatty acid profiles of VCO extracted from PPSP-CT and PPSP-FT process with 5 cycles are shown in Table 25. VCO samples dominantly contained MCFAs, mainly lauric acid. The second major fatty acid was myristic acid. The VCO from PPSP-FT process had the higher lauric acid (49.60%) and myristic acid (19.80%), compared to that from PPSP-CT process ($p<0.05$). The most abundant MCFA present in coconut oil is lauric acid, which is responsible for health benefits (Enig, 1996). MCFAs have 6 to 12 carbons, including caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids (Papamandjaris *et al.*, 1998). Palmitic acid (9.5% and 10.29%) was found in VCO extracted by PPSP-FT and PPSP-CT processes, respectively. Caprylic acid and capric acid constituted at 5.90% and 5.19 in VCO from PPSP-CT process, respectively. Conversely, lower caprylic acid and capric acid contents were found in VCO extracted from PPSP-FT process that those of PPSP-FT process. In general, there was no marked difference in fatty acid profiles between VCO extracted using both processes. Furthermore, the fatty acid composition was comparable to those guided by APCC standards.

6.5.2.2. Moisture content

Moisture content of both VCO samples was negligible (~0.1%) (Table 26). As per APCC standard, VCO must have moisture in the limited range (max 0.1%) (APCC, 2009). High moisture content indicated that VCO could be prone to

hydrolysis process, leading to the enhanced rancidity (Raghavendra and Raghavarao, 2010). Both VCO samples had very low moisture content, thereby assuring their long shelf-life. There was no difference in moisture content among VCOs obtained from both processes. Results suggested that VCO could be extracted from coconut milk effectively using the combined treatments.

Table 25. Fatty acid profile of VCO extracted using PPSP-CT and PPSP-FT processes with 5 cycles.

Fatty acids (g/100 g oil)	**PPSP-CT**	**PPSP-FT**	**APCC Standards**
Caproic acid (C6:0)	0.40±0.00a	0.20±0.03b	0.10 – 0.95
Caprylic acid (C8:0)	5.90±0.00a	4.68±0.42b	4 – 10
Capric acid (C10:0)	5.19±0.00a	5.14±0.26a	4 – 8
Lauric acid (C12:0)	45.29±0.05b	49.60±0.48a	45 – 56
Myristic acid (C14:0)	18.72±0.03b	19.80±0.41a	16 – 21
Palmitic acid (C16:0)	10.19±0.02a	9.50±0.35b	7.5 – 10.2
Stearic acid (C18:0)	3.78±0.00a	3.74±0.17a	2 – 4
Cis-9-Octadecanoic acid (C18:1 n-9)	8.13±0.01a	6.13±0.24b	4.5 - 10
Cis-9,12-Octadecadienoic acid (C18:2 n-6)	1.56±0.00a	0.85±0.02b	0.7 – 2.5
Arachidic acid (C20:0)	0.11±0.00a	0.11±0.00a	-
Unidentified peak	0.73±0.00a	0.25±0.00b	-

Values are mean ± standard deviation (n=3). Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

PPSP-CT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h at 60 °C, followed by chill-thawing for 5 cycles.

PPSP-FT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h min at 60 °C, followed by freeze-thawing for 5 cycles.

6.5.2.3. Free fatty acid (FFA) content

FFA contents of VCOs separated from of PPSP-CT and PPSP-FT process with 5 cycles are shown in Table 26. Both VCO samples had the low FFA content. The result indicated that ester bonds of triglycerides were cleaved to low degree. FFAs are responsible for unacceptable taste and aroma in oils and fats. It was reported that VCO produced by enzymatic process contained low FFA with a good smell and long lifetime (Rahayu *et al.*, 2008). Marina *et al.* (2009) found that FFAs were high in coconut oils having high moisture content. There was no difference in FFA content in VCOs produced by both processes. FFA contents of both VCO samples were below APCC standard (APCC, 2009), indicating their good initial quality.

Table 26. Properties of VCO extracted using PPSP-CT and PPSP-FT processes with 5 cycles.

Sr. No.	Parameters	PPSP-CT	PPSP-FT	APCC Standards
1.	Moisture (%)	0.1±0.04a	0.1±0.03a	Max 0.1
2.	Free fatty acid (%)	0.18±0.06a	0.16±0.09a	Max 0.2
3.	Peroxide Value (meq O_2/kg)	2.09±0.46a	1.96±0.57a	Max 3
4.	*ρ*-anisidine	0.75±0.20a	0.97±0.25a	-

The mean ± standard deviation, n=3. Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).

PPSP-CT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h at 60 °C, followed by chill-thawing for 5 cycles.

PPSP-FT process: enzymatic hydrolysis of coconut milk using PPSP (10 units/g protein) for 1 h at 60 °C, followed by freeze-thawing for 5 cycles.

6.5.2.4. Peroxide value (PV)

Low PV was found in both VCO samples (Table 26). PV of VCO samples were within the standard range (max 3 meq O_2/kg) guided by to APCC standard (APCC, 2009). PV is used to determine the primary oxidation products of oil

(Choe and Min, 2006). Low PV in VCO indicated that lipid oxidation took place at low degree in both samples. This was more likely because saturated fatty acids were prevalent in VCO (Table 25). This made VCO more stable to oxidation. When comparing PV between both samples, the similar PV was observed, suggesting the similar oxidative stability of VCO extracted by both process, PPSP-CT and PPSP-FT process.

6.5.2.5. Anisidine value (AnV)

Low AnV was observed in both VCO samples (Table 26). There were no differences in AnV between both samples ($p>0.05$). According to Rossell (1983), oils with an AnV below 10 were considered as good quality. AnV is used to determine the secondary oxidation products of oil. The primary oxidation products i.e. peroxides are not stable and decomposed to the secondary products such as aldehydes, which are responsible for rancid odor and taste. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Low AnV of both samples was in agreement with low PV, regardless of extraction processes. The results reconfirmed high oxidative stability of both VCO samples.

6.6. Conclusion

Coconut milk emulsion was more stable after the micro-fluidization, in which further enzymatic hydrolysis using PPSP had negative impact on oil recovery. On the other hand, coconut milk emulsion was highly unstable when PPSP-FT process with 5 cycles was implemented. Properties and oxidative stability of VCO obtained by PPSP-FT process yielded VCO with prime quality and high oil recovery.

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CHAPTER 7
OXIDATIVE STABILITY OF MAYONNAISE PREPARED USING VIRGIN COCONUT OIL/FISH OIL BLEND

7.1 Abstract

Chemical changes in mayonnaise prepared using virgin coconut oil (VCO)/ fish oil (FO) blends at different ratios (95:5, 90:10, 85:15, v/v) were monitored during the storage of 30 days at room temperature in comparison with the mayonnaise prepared using soybean oil (SO). Free fatty acid (FFA) contents in all mayonnaise were increased after the storage of 30 days. Peroxide value (PV), thiobarbituric acid reactive substances (TBARS), *ρ*-anisidine value (AnV) and Totox values of mayonnaise prepared using VCO were lowest during the storage, indicating the highest oxidative stability among all samples. Lipid oxidation was increased with increasing storage time and level of FO added in mayonnaise. Highest lipid oxidation took place in mayonnaise containing SO after the storage of 30 days. At day 0, linoleic acid (50.07%) was the dominant fatty acid in SO containing mayonnaise, whereas lauric acid (47.05%) was predominant in VCO containing mayonnaise. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found in mayonnaise prepared using VCO/FO blends. Lauric acid, myristic acid, EPA and DHA were decreased in all samples after the storage of 30 days. Volatile compounds, mainly hexanal, was observed after storage of 30 days. For mayonnaise prepared from VCO/FO (90:10) blend, which had no differences in sensorial property with that containing SO. Thus, VCO/FO (90:10) blend could be used to prepare mayonnaise with health benefit and the increased oxidative stability.

7.2 Introduction

Mayonnaise is one of the most favorite sauces in the world today (Huang *et al.*, 2016). It is oil-in-water emulsion with acidic pH, comprising three different components: vinegar/water (continuous phase), 70-80% oil (dispersed phase) and egg yolk as an emulsifier localized at the interface (Li *et al.*, 2014). Mayonnaise is a high oil containing product, in which soybean oil is commonly used. Basically, soybean oil containing unsaturated fatty acids are highly susceptible to oxidation,

resulting in the formation of undesirable components such as free radicals and reactive aldehydes (Gorji *et al.*, 2016). To conquer the problem, natural or synthetic antioxidants have been widely used in mayonnaise to prevent oxidation (Meyer and Jacobsen, 1996; Jacobsen *et al.*, 2001). However, synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), and ethylene diamine tetraacetic acid (EDTA) have a negative impression due to their toxicity and carcinogenic effects, particularly when high concentrations are used (Martinez-Tome *et al.*, 2001).

Fish oil (FO) has been well known for health-promoting benefits. Therefore, health advisories recommend a higher intake of fish oil rich in polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hartvigsen *et al.*, 2000). Incorporation of PUFAs into foods can be of consumers' health benefit (Gorji *et al.*, 2016). Susceptibility of lipids towards oxidation is determined by the number and location of the double bonds (McClements and Decker, 2000). Generally, saturated lipids are more stable to oxidation than unsaturated counterpart.

Virgin coconut oil (VCO) consists of medium chain fatty acids (MCFAs), mainly lauric acid (Dayrit, 2014). Because of high stability and various health benefits, VCO has gained the interest for consumer and processor (Carandang, 2008). Incorporation of VCO in combination with FO could be a means to prepare a functional mayonnaise. The balance between saturated and unsaturated oil would achieve both targets: health promotion and oxidative stability of resulting mayonnaise. Nevertheless, different types of oil in mayonnaise can provide different physical and sensory characteristics. Therefore, this work was undertaken to incorporate VCO and FO at different ratios into mayonnaise. Oxidative stability and physical properties of resulting mayonnaises were examined during the storage of 30 days in comparison with that prepared using soybean oil (SO).

7.3 Objective

To study the oxidative stability of mayonnaise prepared using virgin coconut oil/fish oil blend.

7.4 Materials and Methods

7.4.1 Chemicals

Sodium chloride, *ρ*-anisidine, ammonium thiocyanate, sodium hydroxide, and Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulphate, trichloroacetic acid and isooctane were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, chloroform, acetic acid, propanol, petroleum ether, hydrochloric acid, and *n*-hexane were procured from Lab-Scan (Bangkok, Thailand). Soybean oil (SO) and eggs were purchased from a local supermarket in Hat Yai, Songkhla, Thailand.

7.4.2 Preparation of VCO

VCO was prepared as described in part 6 (section 6.4.6.2).

7.4.3 Preparation of fish oil from seabass viscera

Whole viscera of seabass were purchased from a local market in Hat Yai. Samples were packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon arrival, depot fat was dissected from other internal organs including pyloric caeca, intestine, liver and stomach. The obtained depot fat was cut into small pieces using a knife and ground with a blender (National, MX-T2GN, Taipei, Taiwan). The ground sample was used for oil extraction.

7.4.3.1 Extraction of oil from depot fat

Visceral depot fat (100 g) was transferred into a round bottom flask equipped with a rotary evaporator (EYELA, N-1000, Tokyo Rikakikai, Co.,Ltd., Tokyo, Japan). The extraction was performed at 70 °C for 20 min under vacuum. After extraction, oil was placed in an Erlenmeyer flask containing approximately 2–5 g of anhydrous sodium sulfate, shaken well and decanted into a centrifuge tube through a Whatman No. 4 filter paper. The mixture was centrifuged at 10,000 ×g for 20 min at 4 °C using a refrigerated centrifuge (CR22N, Hitachi, Hitachi Koki Co.,

Ltd., Tokyo, Japan). The oil was collected using a Pasteur pipette. The oil sample was transferred into the amber vials and purged with N_2 gas. The vials were capped tightly and kept at −40 °C until further use.

7.4.4 Preparation of mayonnaise

Mayonnaise was prepared following the method of Chotphruethipong and Benjakul (2017). Formulation (% on weight basis) included 8% fresh egg yolk, 4% vinegar, 1% salt, 14% sugar, 3% distilled water and 70% oil. For oil samples, VCO (100%) or VCO/FO blends with different VCO: FO ratios (95:5, 90:10, 85:15, v/v) were used. Soybean oil (100%) was used as the reference. Mayonnaise samples were designated as S: soybean oil (100%), V: VCO (100%), V-F5: VCO (95%) + FO (5%), V-F10: VCO (90%) + FO (10%), V-F15: VCO (85%) + FO (15%). The resulting mayonnaise samples were subjected to analysis.

7.4.5 Sensory evaluation

Samples were subjected to sensory evaluation using 9 – point hedonic scale, where 9 is the most likeness and 1 is the most dislikeness (Meilgaard *et al.*, 2006). Fifty panelists were recruited for sensory evaluation. They were the students and staffs from the Department of Food Technology, who were acquainted with mayonnaise. The samples were served at room temperature with freshly made bread. Appearance, color, odor, flavor, texture, and overall likeness were evaluated. Between the samples, panelists were asked to rinse their mouth using mineral water.

7.4.6 Chemical changes of mayonnaise during the storage of 30 days

Mayonnaise samples containing SO, VCO and VCO/FO blends at different ratios was analyzed for 30 days. Oil from mayonnaise samples was extracted every 5 days during the storage of 30 days for analysis. All mayonnaise samples were kept in zip lock bag and stored at room temperature (30-32 °C) in the incubator (Memmert, Schwabach, Germany).

7.4.6.1 Extraction of oil from mayonnaise

The oil was extracted from mayonnaise sample using the method of Bligh and Dyer method (Bligh and Dyer, 1959). Sample (25 g) was homogenized with 200 ml of a chloroform: methanol: distilled water mixture (50:100:50, v/v/v) at a speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 °C. The homogenate was added with 50 ml of chloroform and homogenized at 9500 rpm for another 1 min. Thereafter, 25 ml of distilled water were added and homogenized at the same speed for 30 s. The homogenate was centrifuged at 3000g at 4 °C for 15 min and transferred into a separating flask. The chloroform phase was drained off into the 125 ml Erlenmeyer flask containing 2–5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 25 °C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. Oil was transferred into a vial, flushed with nitrogen gas and used for further analysis.

7.4.6.2 Determination of free fatty acid content (FFA)

Free fatty acid content (FFA) was determined following the method as described by Patil *et al.* (2016).

7.4.6.3 Measurement of lipid oxidation products

Lipid oxidation products in mayonnaise samples were determined. Peroxide values (PV), *ρ*-anisidine values (AnV) and thiobarbituric acid reactive substances (TBARS) were measured following the method tailored by Takeungwongtrakul *et al.* (2012). Totox vaule was calculated by a formula: Totox value = 2 PV + AnV (Shahidi and Wanasundara, 2002).

7.4.6.4 Analysis of fatty acid profiles

Oil samples extracted from mayonnaise at day 0 and day 30 were determined for fatty acid profile. Firstly, fatty acid methyl esters (FAMEs) were

prepared as per the method of Muhammed *et al.* (2015). The prepared FAMEs were injected to the gas chromatography (7890B GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (100 m × 0.25 mm × 0.20 µm), coated with bonded polysiloxane, was used. The analytical conditions were as follows: injection port temperature of 250 ºC and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention time of FAME standards was used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

7.4.7 Determination of volatile compounds

Mayonnaise sample rich in both medium chain fatty acid and PUFA, with the likeness score equivalent to that of reference sample (S) was collected at day 0 and 30 for analysis of volatiles.

7.4.7.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, the samples (3 g) were homogenized at a speed of 13,500 rpm for 2 min with 8 ml of distilled water. The mixture was centrifuged at 2000xg for 10 min at 4 °C. The supernatant (6 ml) was heated at 60 °C with equilibrium time of 1 h in a 20 mL headspace vial. Finally, the SPME fiber (75 µm Carboxen^{TM}/PDMS StableFlex^{TM}; (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fiber for 1 h. The volatile compounds were then desorbed in the GC injector port for 10 min at 250 °C.

7.4.7.2 GC-MS analysis

GC-MS analysis was performed using a GC mass spectrometer consisting of an Agilent 7890B gas chromatography equipped with a 7000D triple-quadrupole mass spectrometry detector (Agilent Technologies, Wilmington, DE, USA). Compounds were separated on VF-WAXms capillary column (30 m x 0.25

mm ID; 0.25 μm film thickness, Agilent, USA). Helium was used as the carrier gas, with a constant flow of 1.5 ml/min and the injection was performed in the split less mode. The GC oven temperature program was: 35 °C for 3 min, followed by an increase of 3 °C /min to 70 °C, then an increase of 10 °C /min to 200 °C and hold for 5 min and finally an increase of 20 °C /min to a final temperature of 250 °C and hold for 5 min. Transfer line temperature was maintained at 250 °C. Mass spectrometer conditions were: electronic ionization (EI) mode at 70 eV; source temperature: 230 °C; quad temperature: 150 °C; scanning rate 0.220s/scan; mass range: 10-200 amu.

7.4.7.3 Analysis of volatile compounds

Identification of volatile compounds was performed, based on their retention times, compared with the mass spectra of reference compounds in the Wiley Mass Spectral Libraries (version 9). Selected volatile compounds identified related to lipid oxidation, included aldehydes, alcohols, ketones, etc., and were expressed as abundance of each identified compound.

7.4.8 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.5 Results and discussion

7.5.1 Sensory characteristics

Likeness score of mayonnaise prepared with SO, VCO and VCO/FO blends with different ratios is presented in Table 27. High likeness scores for appearance and color were obtained for S sample ($p<0.05$). However, appearance, color, and texture likeness scores were decreased by incorporation of VCO and VCO/FO blends. For odor likeness score, no difference was observed between S, V,

V-F5 and V-F10 samples ($p>0.05$). Nonetheless, V-F15 the showed lower score for odor likeness ($p<0.05$). The results suggested that VCO might be able to mask fishy odor when FO at level up to 10% was used. At high concentration of FO (15%), panelists could detect the fishy odor in the mayonnaise sample. Therefore, low likeness score was obtained. Lowest flavor likeness score was also gained for V-F15 sample ($p<0.05$). It was noticed that there was no difference between S and V-F10 ($p>0.05$). VCO has natural distinctive coconut odor and flavor (Patil *et al.*, 2016). Results suggested that incorporation of VCO was able to improve the flavor of mayonnaise containing FO at low content but it was unable to mask the flavor of fish oil when highest level of FO (15%) was used. For texture likeness, the highest score was found for S sample ($p<0.05$). No difference between mayonnaise containing VCO and VCO/FO blends was attained ($p>0.05$). The difference in texture between S sample and other samples might be governed by different fatty acid compositions. VCO rich in saturated fatty acids (Patil *et al.*, 2016) became solidified. This might increase the viscosity or consistency of samples. The overall likeness was decreased when FO added was at levels more than 10% ($p<0.05$). Nevertheless, no difference was observed among the rest of samples ($p>0.05$).

Table 27. Likeness score of mayonnaise containing different oils.

Samples	Appearance	Color	Odor	Flavor	Texture	Overall
S	8.47±0.51a	8.50±0.63a	6.90±1.30a	7.00±0.79a	7.87±0.63a	7.57±0.97a
V	7.00±0.79b	7.07±0.98b	7.37±0.67a	7.73±0.94a	6.83±0.99b	7.43±0.89a
V-F5	7.57±0.73b	7.33±1.03b	6.87±0.94a	7.67±1.06a	7.00±0.91b	7.10±0.61a
V-F10	7.47±0.82b	7.60±1.07b	6.87±0.90a	7.07±0.94a	7.13±0.90b	7.07±0.53a
V-F15	7.73±0.98b	7.50±1.22b	5.57±0.57b	6.00±0.95b	7.30±0.95b	5.77±0.97b

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

Values are mean ± standard deviation (n=50). Different lowercase letters in the same column indicate significant difference ($p<0.05$)

The results suggested that VCO could be used instead of SO for the production of mayonnaise. Additionally, FO could be added up to 10% in VCO/FO blend (V-F10) for mayonnaise preparation. To increase the level of FO, the masking agent or potential removal of off-odor, particularly fishy odor should be implemented prior to make the blend for mayonnaise preparation.

7.5.2 Free fatty acid (FFA)

FFA contents of mayonnaise prepared using SO, VCO and different VCO/FO blends during 30 days of storage at room temperature are presented in Figure 20. At day 0, all mayonnaise samples showed different FFA content. The lowest FFA content was found in V sample ($p<0.05$). Generally, VCO has low FFA content (Patil *et al.*, 2016). FFA content was increased with increasing FO level in the blends. Some FFA were presented in FO, used for the preparation of blends for mayonnaise. Therefore, increasing concentration of FO eventually increased FFA content of the resulting mayonnaise. After 5 days of storage, gradual increase in FFA content was observed in all the samples ($p<0.05$). During the storage, all samples showed the similar pattern, in which no drastic change was observed. FFA content of V sample was lowest amongst all samples throughout the storage of 30 days ($p<0.05$). This result indicated that the hydrolysis of ester bonds of triglyceride occurred at lower rate. However, significant difference was observed between day 0 and day 30 for all the samples ($p<0.05$), more likely attributed to the activity of acid tolerant microorganisms such as lactic acid bacteria, which might be present in the aqueous phase in mayonnaise (Karas *et al.*, 2002). Also, these increases were probably due to the activity of hydrolytic enzymes present in eggs (Abu-Salem and Abou-Arab, 2008). The results suggested that type of oil used for preparation of mayonnaise could affect the FFA content of resulting mayonnaise at the beginning and after the storage.

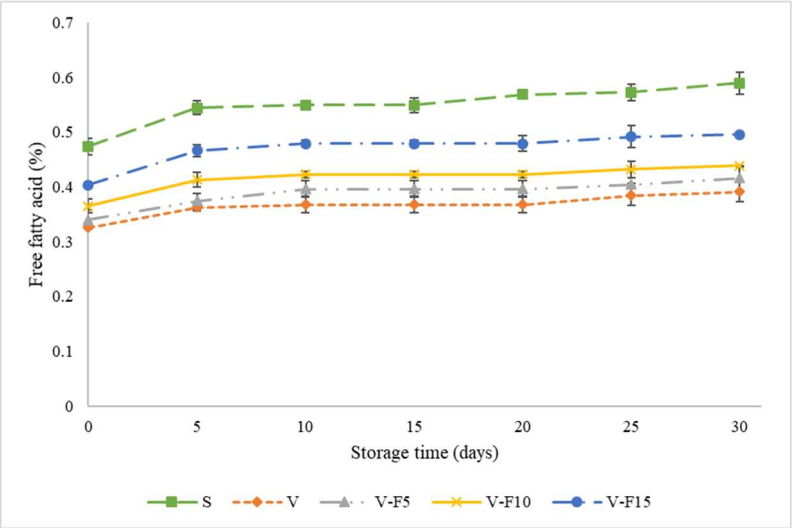

Figure 20. Free fatty acid (FFA) content of mayonnaise containing different oils during the storage of 30 days at room temperature. Bars represent standard deviations (n=3).

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

7.5.3 Oxidative stability of mayonnaise during storage

7.5.3.1 Peroxide value (PV)

Changes in PV of mayonnaise prepared using SO, VCO and different VCO: FO blends during the storage of 30 days at room temperature are depicted in Figure 21 (A). At day 0, all mayonnaise samples showed different PV. It was lowest in V sample ($p<0.05$). The highest PV was observed for S sample, compared to other samples ($p<0.05$). PV is typically used for measurement of the concentration of hydroperoxides formed at the initial stage of lipid oxidation. PVs were continuously increased for all the samples with increasing storage time ($p<0.05$). The increase in PV of all samples indicated that the samples were in propagation stage of lipid oxidation with a lower rate of decomposition of hydroperoxides formed. Among all the samples, S sample had the highest PV throughout the storage than those containing VCO or VCO/FO blends ($p<0.05$). In general, mayonnaise is susceptible

to lipid oxidation due to their large surface area that facilities the interactions between the oil, water and air (Gorji *et al.*, 2016). Lipid oxidation is generally initiated at the interface between the oil and water, where pro-oxidants (transition metals) in the continuous phase are able to contact with the hydroperoxides located at the droplet surface (McClements and Decker, 2000). Moreover, the pH is the main factor affecting lipid oxidation in mayonnaise. In mayonnaise, egg yolk, used as the emulsifier, contained a large amount of iron (734 μM) (Jacobsen, 1999). The iron forms cation bridges between the protein phosvitin and other components at pH 6. At the low pH (3.8-4) found in the mayonnaise, the iron bridges between phosvitin, lipovilelin and low-density lipoprotein (LDL) are destroyed and the iron is released, leading to the increased lipid oxidation (Jacobsen, 1999). When comparing PV of all samples, the lowest increase in PV was found in the V sample ($p<0.05$), indicating the VCO is less prone to lipid oxidation. This was more likely due to the low content of unsaturated fatty acids (Patil *et al.*, 2016). However, PV was increased with increasing concentration of FO in VCO/FO blend. This might be caused by the increase in unsaturated fatty acids, which were prone to oxidation. The results suggested that differences in PV between mayonnaises with different oils possibly resulted from differences in their chemical structure and fatty acid compositions.

7.5.3.2 TBARS

TBARS values of mayonnaise prepared using SO, VCO and VCO/FO blends at different ratios during 30 days of storage are shown in Figure 21 (B). TBARS values of all samples were continuously increased with increasing storage time ($p<0.05$). The S sample showed the higher TBARS values throughout 30 days of storage, compared to all other samples ($p<0.05$). The increase in TBARS value of lipids indicated the formation of the secondary lipid oxidation products. TBARS value is an index of decomposition of hydroperoxides into the secondary oxidation products in the later stages of lipid oxidation (Sae-leaw and Benjakul, 2017). Hydroperoxides are decomposed to malonaldehyde, which contributes to off-flavor of oxidized lipids (Zhang *et al.*, 2013). Some prooxidants could accelerate lipid oxidation to a higher extent as the storage time increased.

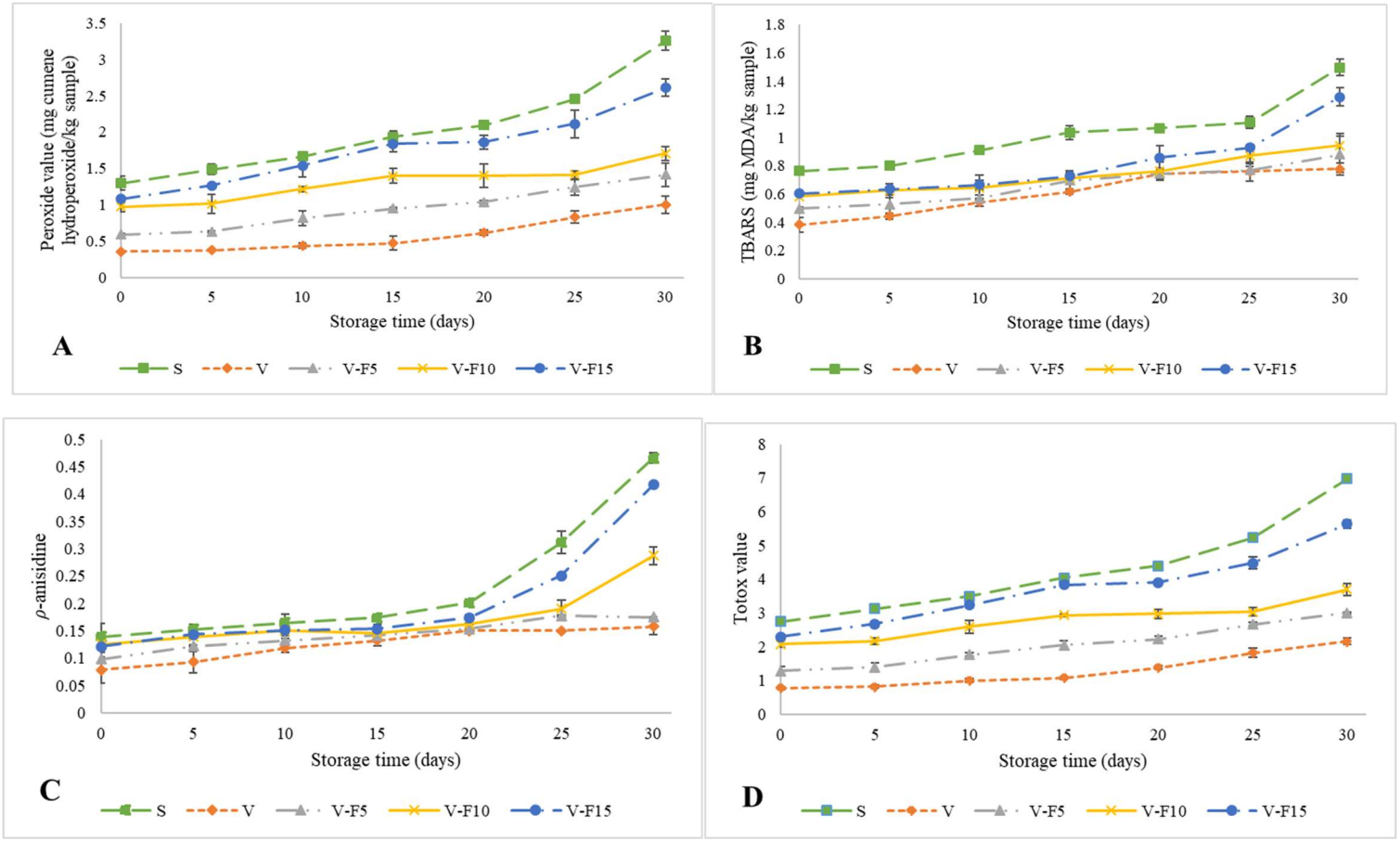

Figure 21. Peroxide value (PV) (A), thiobarbituric acid reactive substances (TBARS) (B), *ρ*-anisidine value (AnV) (C) and totox value (D) of mayonnaise containing different oils during the storage of 30 days at room temperature. Bars represent standard deviations (n=3). S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

On the other hand, the lowest TBARS values were observed for V sample throughout 30 days. The results reconfirmed that VCO in mayonnaise was less prone to lipid oxidation. When comparing TBARS values between the samples added with FO, the slight difference was observed until day 25. However, the sharp increase in TBARS was noticeable in V-F15 at day 30 ($p<0.05$). Overall, PVs were much lower than that reported by Chotphruethipong and Benjakul (2017) for mayonnaise enriched with fish oil. The result suggested that the rate of lipid oxidation depended on the type of oil used for the preparation of mayonnaise.

7.5.3.1 *ρ*-anisidine values (AnV)

AnV of mayonnaise prepared with SO, VCO and VCO/FO blends during the storage of 30 days at room temperature is shown in Figure 21 (C). All samples had slight increase in AnV up to day 20 of storage ($p<0.05$). Subsequently, S, V-F15 and V-F10 samples had a marked increase in AnV up to the end of storage ($p<0.05$). The S sample had the highest AnV, as compared to other samples throughout 30 days of storage ($p<0.05$). The increase in AnV indicated the formation of the secondary lipid oxidation products, mainly non-volatile compounds (principally 2-alkenals and 2,4-alkadienals) in lipids (Choe and Min, 2006). When comparing AnV of all the samples at day 0, the lowest AnV was observed for the V sample, whereas the highest AnV was found in the S sample ($p<0.05$). The results suggested that SO might contain some oxidative products at the beginning or during mayonnaise preparation. Overall, AnVs were much lower than that reported by Chotphruethipong and Benjakul (2017) for mayonnaise enriched with fish oil. The difference was most likely caused by the difference in oils used. Chotphruethipong and Benjakul (2017) prepared mayonnaise using SO blended with FO at ratio of SO:FO (90:10 v/v). The secondary oxidation products are important in food products for human consumption, because they are generally odor-active, whereas primary oxidation products are colorless and flavorless (Osborn and Akoh, 2004). Lipid oxidation of oil is determined by its chemical structure, in particular, the number and location of the double bonds (McClements and Decker, 2000). The results suggested that VCO used for mayonnaise was able to retard lipid oxidation of mayonnaise.

7.5.3.2 Totox value

Total oxidation value (Totox value) measures both hydroperoxides and their breakdown products and provides a better estimation of the progressive oxidative deterioration of fats and oils (Shahidi and Zhong, 2005). It was noticed that the Totox value was increased with increasing storage time for all the samples ($p<0.05$). The highest increase in Totox value was observed in S sample ($p<0.05$). Samples contained VCO showed the lowest totox value throughout 30 days of storage, compared to others ($p<0.05$). Overall, a similar trend was observed in comparison with PVs. Totox value was calculated from PV and AnV. Primary oxidation products and secondary oxidation products, together with free radicals, constitute the basis for measuring the oxidative deterioration of mayonnaise (Shahidi and Zhong, 2005). Lipid oxidation in mayonnaise leads to the development of potentially toxic reaction products (Coupland and McClements, 1996), undesirable off-flavors and consequently decreases the shelf-life of mayonnaise (Alemán *et al.*, 2015). In general, mayonnaise showed high oxidative stability when VCO was used, while FO at higher ratio increased oxidation.

7.5.4 Fatty acid composition

Fatty acid compositions of mayonnaise prepared with SO, VCO and VCO/FO blends at day 0 and day 30 are given in Table 28. At day 0, fatty acid composition of S sample was observed to be different from V sample and those containing VCO/FO blend. In S sample, linoleic acid with 50.07% was the dominant fatty acid, followed by oleic acid (24.18%) and palmitic acid (11.16%). The results were in agreement with previous report for fatty acid composition of mayonnaise prepared using SO, in which, linoleic acid (50.4%) was the dominant fatty acid, followed by oleic acid (26.1%) and palmitic acid (11.6%) (Enig *et al.*, 1983). For V sample, lauric acid (47.05%) was predominant fatty acid followed, by myristic acid (19.57%) and palmitic acid (9.55%). Patil *et al.* (2016) documented that VCO dominantly contained medium chain fatty acids (MCFA), especially lauric acid (49.74-51.18%) and the second major fatty acid was myristic acid (18.70-19.84%). MCFA present in coconut oil is lauric acid, which is responsible for health benefits.

Table 28. Fatty acid profile of mayonnaise containing different oils at different storage time.

Fatty acids (g/100 g oil)	S		V		V-F5		V-F10		V-F15	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
C6:0 (Caproic)	ND	ND	0.45±0.00a	0.38±0.00b	0.41±0.00a	0.37±0.00b	0.38±0.00a	0.35±0.00b	0.36±0.00a	0.33±0.00b
C8:0 (Caprylic)	ND	ND	6.34±0.00a	5.91±0.00b	5.91±0.01a	5.69±0.00b	5.53±0.00a	5.35±0.01b	5.18±0.00a	5.05±0.00b
C10:0 (Capric)	ND	ND	5.49±0.01a	5.32±0.00b	5.15±0.00a	5.09±0.00b	4.83±0.00a	4.78±0.00b	4.52±0.00a	4.48±0.00b
C12:0 (Lauric acid)	0.30±0.00a	0.07±0.00b	47.05±0.10a	43.09±0.03b	44.14±0.02a	41.62±0.20b	41.62±0.02a	39.15±0.01b	38.81±0.02a	36.44±0.01b
C14:0 (Myristic acid)	0.23±0.00a	0.1±0.00b	19.57±0.05a	16.66±0.00b	18.61±0.01a	16.65±0.01b	17.80±0.01a	15.90±0.02b	16.81±0.00a	14.99±0.01b
C15:0 (Pentadecanoic)	ND	ND	ND	ND	ND	0.04±0.00	0.06±0.00b	0.07±0.00a	0.09±0.00a	0.09±0.00a
C16:0 (Palmitic)	11.16±0.00a	10.63±0.00b	9.55±0.01b	10.00±0.00a	10.49±0.01b	10.93±0.01a	11.15±0.00b	11.66±0.01a	11.88±0.00b	12.37±0.02a
C16:1 (Palmitoleic)	0.24±0.00a	0.08±0.00b	ND	0.20±0.00	0.37±0.00b	0.51±0.00a	0.64±0.00b	0.82±0.00a	0.95±0.00b	1.09±0.00a
C17:0 (Heptadecanoic)	0.10±0.00a	0.09±0.00b	ND	ND	ND	ND	0.062±0.00b	0.07±0.00a	0.09±0.00b	0.10±0.00
C18:0 (Stearic)	4.17±0.00a	4.12±0.00b	3.55±0.00a	3.45±0.00b	3.70±0.00a	3.58±0.00b	3.80±0.00a	3.69±0.00a	3.91±0.00a	3.85±0.00b
C18:1 cis 9 (Oleic)	23.47±0.00b	24.18±0.00a	5.78±0.00b	9.25±0.00a	7.26±0.2b	10.01±0.10a	8.54±0.00b	11.02±0.02a	9.68±0.00b	11.98±0.00a
C18:2 cis 9,12 (Linoleic)	50.07±0.00b	52.90±0.00a	1.13±0.00b	4.07±0.01a	1.85±0.00b	3.08±0.00a	2.38±0.00b	3.42±0.00a	2.99±0.00b	3.99±0.00a
C20:0 (Arachidic)	0.32±0.00b	0.34±0.00a	0.10±0.00a	0.09±0.00b	0.11±0.00a	0.10±0.00b	0.13±0.00a	0.10±0.00b	0.13±0.00a	0.12±0.00b
C18:3 cis 6,9,12 gamma (gramma-Linolenic)	0.49±0.00b	0.53±0.00a	ND	ND	ND	ND	0.06±0.00b	0.07±0.00a	0.10±0.00a	0.10±0.00a
C20:1 cis 11 (cis-11-Eicosenoic)	0.47±0.00b	0.50±0.00a	ND	ND	0.092±0.00a	0.09±0.00b	0.06±0.00a	0.05±0.00b	0.08±0.00a	0.08±0.00a
C18:3 cis 9,12,15 alpha (alpha-Linolenic)	5.28±0.00b	5.58±0.00a	ND	0.24±0.00	0.09±0.00b	0.13±0.00a	0.16±0.00b	0.19±0.00a	0.23±0.00b	0.26±0.00a
C20:0 (Docosanoic)	0.36±0.00b	0.38±0.00a	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 cis 11,14 (cis-11,14-Eicosadienoic)	ND	ND	ND	ND	ND	ND	0.11±0.00b	0.12±0.00a	0.16±0.00b	0.17±0.00a
C20:3 cis 8,11,14 (cis-8,11,14-Eicosatrienoic)	ND	ND	ND	ND	ND	ND	ND	ND	0.06±0.00a	0.06±0.00a
C22:1 cis 13 (Erucanoic)	ND	ND	ND	ND	ND	ND	0.06±0.00a	0.06±0.00a	0.09±0.00a	0.09±0.00a
C23:0 (Tricosanoic)	ND	ND	ND	ND	0.06±0.00b	0.10±0.00a	0.12±0.00b	0.16±0.00a	0.18±0.00b	0.23±0.00a
C24:0 (Lignoceric)	0.11±0.00b	0.12±0.00a	ND	ND	ND	ND	ND	ND	ND	ND
C20:5 cis 5,8,11,14,17 EPA (cis-5,8,11,14,17-Eicosatrienoic)	ND	ND	ND	ND	0.21±0.00a	0.19±0.00b	0.39±0.00a	0.38±0.00b	0.59±0.00a	0.57±0.00b
C22:6 cis 4,710,13,16,19 DHA (cis-4,710,13,16,19-Docosahexaenoic)	ND	ND	ND	ND	0.42±0.00a	0.38±0.00b	0.75±0.00a	0.73±0.00	1.13±0.00a	1.10±0.00b

ND: not detected. S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

Values are mean ± standard deviation (n=3). Different lowercase letters in the same row under the same sample indicate significant difference ($p<0.05$).

FO extracted from seabass visceral depot blended with VCO showed the marked difference in fatty acid composition in the resulting mayonnaise. Sae-leaw and Benjakul (2017) reported that oil extracted from seabass visceral depot fat contained oleic acid (25.49%), palmitic acid (21.8%), linoleic acid (13.84 %), DHA (6.91%) and EPA (2.09%). Saturated fatty acid, mainly lauric acid and myristic acid, were decreased in V-F5, V-F10 and V-F15 samples with increasing FO concentration ($p<0.05$). Conversely, palmitic acid was increased with increasing FO concentration. On the other hand, unsaturated fatty acid, mainly eicosatrienoic (EPA) and docosahexaenoic (DHA) was significantly increased in mayonnaise prepared with VCO/FO blends as the FO ratio increased.

After storage of 30 days, the different fatty acid profiles were observed in all samples, compared to those found at day 0. Fatty acids, mainly lauric acid, myristic acid, EPA and DHA were decreased after the storage of 30 days ($p<0.05$). Conversely, linoleic acid, oleic acid, palmitic acid were increased after the storage of 30 days ($p<0.05$). The changes in fatty acid profile might be due to the activity of microorganisms or hydrolytic and oxidative enzymes present in eggs (Karas *et al.*, 2002). It was postulated that hydrolytic action of enzyme was able to cleave ester bond from glycerol back bone and release free fatty acids. This result was in agreement with FFA content, which significantly increased after 30 days of storage for all the samples (Figure 20). Moreover, lipid oxidation also contributes to the changes in fatty acid composition. Changes in fatty acid profile of unsaturated fatty acids were observed after the 30 days of storage. This was coincidental with the increased lipid oxidation after 30 days of storage as monitored by the increases in PV, TBARS, AnV and totox value of all samples (Figure 21 A, B, C, D). The results suggested that changes in fatty acid compositions were mainly governed by hydrolysis and oxidation process during the extended storage.

7.5.5 Volatile compounds

Selected volatile compounds in V-F10 sample at day 0 and day 30 of storage at room temperature are presented in Table 29. In general, volatile compounds detected in mayonnaise at day 0 were lower in abundance than those found at 30 days.

At day 0 of storage, hexanal was found as the major compounds in the sample. Hexanal has been reported as a good indicator of lipid oxidation (Fuller *et al.*, 1992). The result indicated that oxidation took place before or during mayonnaise preparation. Other aldehydes including 3-methyl-butanal, pentanal, benzaldehyde, heptenal, propanal, (*E, E*)-2,4-heptadienal and octanal were also detected at the low levels. Hexanal and heptenal were major compounds, which contributed to rancid odor and fishy odor (Yarnpakdee *et al.*, 2012). Moreover, volatile alcohols (1-pentanol and 1-cyclobutylcyclopropanol) and furans (2-ethyl-furan and 2-pentyl-furan) were found at low abundance at day 0. However, ketones were not detected at day 0 and 30.

After storage of 30 days, higher formation of volatile compounds was noticeable. Aldehydes were the most prominent volatiles detected in the samples. Aldehydes, ketones and alcohols have been known to be associated with lipid oxidation (Sae-leaw and Benjakul, 2014), which might occur during storage. Furans were increased after 30 days of storage. Furans and their derivatives such as 2-ethyl-furan and 2-pentyl-furan are formed by the decomposition of hydroperoxide of EPA and DHA (Maqsood and Benjakul, 2011). The results coincided with the increases in PV and decreases in PUFA in V-F10 sample. EPA and DHA are susceptible to oxidation and may cause off-flavor in mayonnaise samples owing to low sensory threshold values of oxidation products (Depree and Savage, 2001).

Table 29. Volatile compounds in mayonnaise containing VCO/FO (90:10) blend at day 0 and day 30 of storage at room temperature.

Volatile compounds	Peak area (Abundance) x10^5	
	day 0	**day 30**
Furans		
2-Ethyl-furan	8.15	183.69
2-Pentyl-furan	5.70	79.53
Aldehydes		
Propanal	4.39	7.52
2-Methyl-butanal	ND	ND
3-Methyl-butanal	11.15	63.31
Pentanal	7.81	283.20
Hexanal	113.16	682.89
(E)-2-Hexenal	ND	ND
Heptenal	4.78	68.49
(E, E)-2,4-heptadienal	2.37	11.06
Octanal	1.51	11.15
(E)-2-decenal	ND	ND
Benzaldehyde	6.38	35.49
Alcohols		
1-Cyclobutylcyclopropanol	3.40	ND
1-Pentanol	10.59	3.68
(Z)-2-pentenol	ND	ND
(E)-2-hexenol	ND	ND
1-Methyl-4-(1-methylethenyl)-cyclohexanol	ND	ND
1-Octen-3-ol	1.70	ND
(Z)-1,5-octadien-3-ol	ND	ND

ND: not detectable.

7.6 Conclusion

VCO could be used instead of SO for the production of mayonnaise. Addition of FO up to 10% in VCO/FO blend could yield the mayonnaise with sensorial acceptability. However, masking agent or potential removal of off-odor is still required when FO higher than 10% was added. Type of oil used for preparation of mayonnaise affected FFA content of resulting mayonnaise. Oxidative stability varied with mayonnaises containing different oils. Mayonnaise sample with VCO was less prone to lipid oxidation throughout storage of 30 days. Overall, VCO in combination with FO at an appropriate ratio could be used instead of SO to prepare a functional mayonnaise with increased oxidative stability.

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CHAPTER 8
PHYSICAL AND RHEOLOGICAL PROPERTIES OF MAYONNAISE PREPARED USING VIRGIN COCONUT OIL/FISH OIL BLEND

8.1 Abstract

Physical and rheological properties of mayonnaise prepared using virgin coconut oil (VCO)/fish oil (FO) blends at different ratios were studied in comparison with the mayonnaise prepared using soybean oil (SO) as affected by storage time (30 days). At day 0, sample prepared with SO showed the highest *L*, a*,* and *b** values among all the samples, whereas the lowest values were noticeable for VCO containing sample. At day 30 of storage, decreases in *L**, and *b** values of all mayonnaise samples were observed ($p<0.05$). However, *a** values were increased at day 30 of storage ($p<0.05$). For texture analysis, highest firmness, consistency and cohesiveness were obtained for the sample containing SO. These values were increased with increasing levels of FO in VCO/FO sample. For all the samples, G′ values were higher than loss modulus (G″). After 30 days of storage, all samples demonstrated a slight decrease in G′ and viscosity than freshly prepared mayonnaise (day 0). When the sample containing VCO/FO (90:10) blend was further characterized, slight difference was observed in microscopic structure and droplet size distribution before and after storage of 30 days. Increase in droplet size was noticeable due to coalescence after the storage. Overall, type of oil used for preparation of mayonnaise as well as storage time affected the physical and rheological properties of mayonnaise.

8.2 Introduction

Virgin coconut oil (VCO) consists of medium chain fatty acids (MCFAs), mainly lauric acid (Dayrit, 2014). Because of high stability and various health benefits, VCO has drawn attention for consumer and processor (Carandang, 2008). MCFAs are burned up immediately after consumption and therefore the body uses it instantly to make energy, instead of storing it as body fat (Enig, 1996). Lauric acid is converted into very valuable compound known as monolaurin, which has antiviral and antibacterial properties (DebMandal and Mandal, 2011). It is therefore

assumed that consumption of VCO may help to protect the body from infections. VCO does not undergo any hydrolytic and atmospheric oxidation ascertained by its low peroxide value as well as very low free fatty acid content (Senphan and Benjakul, 2015; Patil *et al.*, 2016). VCO possesses anti-inflammatory, antimicrobial, and antioxidant properties and boosts the immune system (Carandang, 2008).

Fish oil (FO) has been well known for health-promoting benefits. Therefore, health advisories recommend a higher intake of fish oil rich in polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hartvigsen *et al.*, 2000). Incorporation of PUFAs into foods can be of consumers' health benefit (Gorji *et al.*, 2016). Mayonnaise, as an oil in water emulsion, is a highly desirable salad dressing and widely consumed because of its desired flavor and texture (Rahmati *et al.*, 2015). Therefore, incorporation of VCO in combination with FO could be a means to prepare a functional mayonnaise for health benefits. The balance between saturated and unsaturated oil would achieve health promoting target. Different types of oil in mayonnaise can provide different physical characteristics and rheological property, which might be changed over the storage time. Therefore, the present work was undertaken to incorporate VCO or VCO/FO blends at various ratios into mayonnaise. Physical and rheological properties of resulting mayonnaises were examined at day 0 and day 30 in comparison with those prepared using soybean oil (SO).

8.3 Objective

To study the physical and rheological properties of mayonnaise prepared using virgin coconut oil/fish oil blend.

8.4 Materials and Methods

8.4.1 Chemicals

Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulphate was obtained from Merck (Darmstadt, Germany). Soybean

oil, salt, vinegar, sugar and eggs were purchased from a local supermarket in Hat Yai, Songkhla, Thailand.

8.4.2 Preparation of VCO

VCO was prepared as described in part 6 (section 6.4.6.2).

8.4.3 Preparation of fish oil from seabass viscera

Whole viscera of seabass were purchased from a local market in Hat Yai. Samples were packaged in polyethylene bag, kept in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon arrival, depot fat was dissected from other internal organs including pyloric caeca, intestine, liver and stomach. The obtained depot fat was cut into small pieces using a knife and ground with a blender (National, MX-T2GN, Taipei, Taiwan). The ground sample was used for oil extraction.

8.4.3.1 Extraction of oil from depot fat

Visceral depot fat (100 g) was placed in a round bottom flask equipped with a rotary evaporator (EYELA, N-1000, Tokyo Rikakikai, Co.,Ltd., Tokyo, Japan). The extraction was performed at 70 °C for 20 min under vacuum. The obtained oil was placed in Erlenmeyer flask containing approximately 2–5g of anhydrous sodium sulfate, shaken well and decanted into a centrifuge tube through a Whatman No. 4 filter paper. The mixture was centrifuged at 10,000 ×g for 20 min at 4 °C using a refrigerated centrifuge (CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan). The oil was collected using a Pasteur pipette. The oil sample was transferred into the amber vials and purged with N_2 gas. The vials were capped tightly and stored at −40 °C until further use.

8.4.4 Preparation and storage of mayonnaise

Mayonnaise was prepared following the method of Chotphruethipong and Benjakul (2017). Formulation (% on weight basis) included 8% fresh egg yolk, 4% vinegar, 1% salt, 14% sugar, 3% distilled water and 70% oil. For oil samples,

VCO or VCO/FO blends with various VCO: FO ratios (95:5, 90:10, 85:15, v/v) were used. Soybean oil (SO) was used as the reference. Mayonnaise samples were designated as S: soybean oil (100%), V: VCO (100%), V-F5: VCO (95%) + FO (5%), V-F10: VCO (90%) + FO (10%), V-F15: VCO (85%) + FO (15%). All mayonnaise samples were kept in zip lock bag and stored at room temperature (30-32 °C) in the incubator (Memmert, Schwabach, Germany).

8.4.5 Study on physical and rheological properties of mayonnaise containing different oils at day 0 and day 30 of storage

All the mayonnaise samples were subjected to analyses after preparation. Another portion was kept at room temperature (30-32 °C) for 30 days and analyzed.

8.4.5.1 Color determination

Color of mayonnaise samples was measured using a colorimeter (HunterLab, Model colourFlex, VA, USA). The color was reported as *L*, a*, b** values, indicating lightness, redness/greenness, and yellowness/blueness, respectively. Total difference in color (ΔE*) and the difference in chroma (ΔC*) were also calculated using following equations:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample at day 0 and that of day 30.

$$\Delta C^* = C^*_{day\,0} - C^*_{day\,30}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

8.4.5.2 Texture analysis

Firmness, consistency and cohesiveness of samples were evaluated using a texture analyzer TA-XT2 (Stable Micro Systems, UK) at 30 °C and back extrusion software according to the method of Fernandesa and Salas-Mellado (2018).

The samples were carefully poured into 150 mL cylindrical containers (60 mm internal diameter and 80 mm height), up to the 125 mL mark. Compression was done with a disc of 35 mm in diameter (Stable Micro Systems, UK). The applied cycle consisted of constant speed (1 mm/s), until reaching the depth of 40 mm. From the force-time curve, firmness, consistency and cohesiveness were calculated.

8.4.5.3 Measurement of rheological properties

The rheological property of mayonnaise samples was determined using a controlled stress rheometer (RheoStress RS 1, HAAKE, Karlsruhe, Germany) with parallel geometry (60 mm diameter, and 1 mm gap) as per the method of Huang *et al.* (2016). The linear viscoelastic range was determined with strain sweep of 0.1-100% at a fixed frequency of 1.0 Hz. A dynamic frequency sweep was conducted by applying a constant strain of 0.5%, which was within the linear region, over a frequency range between 0.1 and 100 Hz. Samples were examined at room temperature (30 ºC). The storage modulus (G′) and loss modulus (G″) as a function of frequency were obtained (Huang *et al.*, 2016). Thereafter, frequency sweep test was carried out in a frequency range varying from log 0.1 to log 100 Hz inside the linear viscoelastic region at a constant strain of 0.5%. The temperature was kept at 30 °C during the measurement. Flow curves were determined using an increasing shear rate i.e., 1–100 s^{-1} within 2 min.

8.4.6 Characterization of mayonnaise containing VCO/FO (90:10) blend at day 0 and day 30 of storage

Mayonnaise was prepared using VCO/FO blend (90:10), named ‘V-F10’, which showed the sensorial property equivalent to S sample. The prepared sample was stored at room temperature (28-30 °C) for 30 days. Both samples, freshly prepared and stored for 30 days were characterized.

8.4.6.1 Microstructure analysis

The microstructures were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan.). The samples were

mixed with Nile blue A solution and manually stirred until uniformity was obtained. The mixture was smeared on the microscopy slide. The CLMS in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm was used. a Helium Neon Red laser (HeNe-R) for lipid analysis was applied.

8.4.6.2 Determination of particle size

Particle size distributions were determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Patil and Benjakul (2017). Prior to analysis, samples (1 g) were diluted with 1% (v/v) sodium dodecyl sulfate (SDS) solution (20 mL) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

8.4.6.3 Determination of coalescence and flocculation

Samples were firstly diluted with distilled water in the presence and absence of 1% SDS. The coalescence index (C_i) and flocculation factor (F_f) were calculated using the following equations (Hebishy *et al.*, 2013):

$$F_f = \frac{d_{43} - \mathrm{SDS}}{d_{43} + \mathrm{SDS}}$$

$$C_i = \frac{(d_{43} + \mathrm{SDS,t} - d_{43} + \mathrm{SDS,in})}{d_{43} + \mathrm{SDS,in}} \times 100$$

where d_{43}+SDS and d_{43}-SDS are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 1% SDS, respectively; d_{43}+SDS,in and d_{43}+SDS,t are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 1% SDS at day 0 and the designated storage time (30 day). The determination was conducted at room temperature.

8.4.7 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

8.5 Results and discussion

8.5.1 Physical and rheological properties of mayonnaise containing different oils at day 0 and day 30 of storage

8.5.1.1 Color

Lightness (L^*), redness (a^*), yellowness (b^*), total color difference (ΔE^*) and total chroma difference (ΔC^*) of mayonnaises prepared using SO, VCO and VCO/FO blends at day 0 and day 30 are shown in Table 30. At day 0, L^* and b^* values of all mayonnaise samples were different ($P<0.05$). S sample showed the highest L^*, a^* and b^* value, whereas the lowest values were noticeable for V sample ($p<0.05$). Differences in lightness might be due to varying light scattering among samples. Light scattering and absorption depend on the size, concentration, refractive index and dispersion of the droplets, as well as the presence of chromophoric materials (McClements, 2002). Scattering is largely responsible for the turbidity, opacity, or lightness of an emulsion, whereas absorption is largely responsible for chromaticness (blueness, greenness, redness, etc.) (McClements, 2002). The results suggested that different lightness among mayonnaise samples at day 0 was most likely due to the differences in particles size and dispersion of droplets, which alter the light segregation. Egg yolk color was attributed to yellowish fat-soluble carotenoids (Li-Chan and Kim, 2008). VCO is generally colorless and clear like water (Patil *et al.*, 2016), whereas SO and FO had pale yellow color. Therefore, SO and FO possessed some indigenous colored pigments as indicated by higher b^* value (yellowness) for mayonnaise prepared from SO and FO, compared to VCO containing mayonnaise

sample. When comparing b^* values among the samples containing VCO/FO blends, the difference was observed ($p<0.05$). The b^* values were increased with increasing FO level in the blends. The results indicated that the presence of colorants in the SO, FO and egg yolk had the impact on the color of all mayonnaise samples.

After 30 days of storage, the decreases in L^* and b^* values of all mayonnaise samples were observed. On the other hand, a^* values were increased after the storage. Decreases in lightness (L^*) values were probably owing to the increase in oil droplet sizes of the mayonnaise samples after the storage of 30 days. As a result, the scattering efficiency of the droplets was decreased. The light beam could penetrate further into the emulsion and was absorbed to a greater extent (Chantrapornchai *et al.*, 1999). The b^* values (yellowness) for all the mayonnaise samples were decreased, probably because of the oxidation of pigments, mainly carotenoids. This led to the paler color. Lennersten and Lingnert (2000) reported that β-carotene in low-fat mayonnaise was degraded by exposure to light at different wavelengths. Similar results were reported by Kupongsak and Sathitvorapojjana (2017), in which L^* and b^* values of mayonnaise prepared using rice bran oil/coconut oil blend were decreased during the storage of 4 weeks at 30 °C. On the other hand, the increase in a^* values (redness) plausibly was associated with lipid oxidation products generated during storage, which might serve as the source of carbonyl compounds for non-enzymatic browning (Bharate and Bharate, 2014). These reflected the development of the browner color. The secondary oxidation products (e.g., aldehydes) were involved in browning reactions. The results were in agreement with PV, TBARS, AnV, Toxox value, which showed the increase in oxidation of oil in mayonnaise after the storage of 30 days (Chapter 7). Total differences in color (ΔE^*) and the difference in chroma (ΔC^*) were observed after the storage of 30 days in all the mayonnaise samples. Coloring compounds were possibly degraded or generated during the storage of 30 days. Lowest ΔE^* values were observed for VCO containing mayonnaise sample ($p<0.05$). For ΔC^*, no difference was observed in between mayonnaise samples containing VCO and VCO/FO blends ($p>0.05$). The results suggested that type of oil used for preparation of mayonnaise could affect the color before and after storage of 30 days at room temperature.

Table 30. Color of mayonnaise containing different oils at day 0 and day 30 of storage.

Sample	*L**		*a**		*b**		ΔE*	ΔC*
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30		
S	66.52±0.34Aa	60.26±0.22Ba	2.36±0.14Ba	2.91±0.11Aa	23.63±0.75Aa	21.56±0.25Ba	6.65±0.48b	2.01±0.93b
V	41.31±0.22Ae	36.26±0.88Be	0.77±0.55Bc	1.27±0.20Ac	13.65±0.31Ae	10.48±0.29Be	6.00±0.59c	3.12±0.43a
V-F5	45.72±0.42Ad	39.24±0.69Bd	1.11±0.23Bc	1.74±0.17Ab	16.08±0.41Ad	12.51±0.55Bd	7.46±0.50ab	3.49±0.84a
V-F10	47.02±0.39Ac	40.24±1.08Bc	1.55±0.21Bb	2.03±0.27Ab	19.91±0.67Ac	16.48±0.29Bc	7.59±0.92ab	3.35±0.72a
V-F15	48.63±0.35Ab	42.12±0.57Bb	2.13±0.16Ba	2.77±0.42Aa	21.94±0.29Ab	18.11±0.42Bb	7.64±0.67a	3.72±0.38a

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil, V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

Values are mean ± standard deviation (n=3). Different uppercase letters in the same row within the same parameter tested indicated significant difference ($p<0.05$). Different lowercase letters in the same column indicate significant difference ($p<0.05$).

ΔE*: total difference in color expressed the magnitude of difference between day 0 and day 30 storage samples.

ΔC*: difference in chroma expressed the magnitude of difference between day 0 and day 30 storage samples.

8.5.1.2 Textural property

Textural properties of mayonnaise prepared using SO, VCO and VCO/FO blends at day 0 and day 30 of storage are shown in Table 31. At day 0, mayonnaise containing VCO showed the lowest firmness value when compared to other samples ($p<0.05$). Firmness is an indicator of resistance to penetration by a probe and will be greater as the force required for penetration increases (Fernandesa and de las Mercedes Salas-Mellado, 2018). Firmness values were increased with increasing levels of FO in VCO/FO blends. V-F15 showed higher value, compared to V-F5 and V-F10 ($p<0.05$). Kupongsak and Sathitvorapojjana (2017) also reported that firmness values were increased with increasing level of rice bran oil (unsaturated fatty acids) in mayonnaise prepared using different ratios of coconut oil/ rice bran oil blends (0:100, 10:90, 20:80, 30:70 and 40:60, v/v). No difference was observed between the S and V-F15 samples ($p>0.05$). Oil droplets surrounded by proteins became joined together into a network-structure. This probably increased the firmness of mayonnaise. Composition and physicochemical properties of oil in the dispersed phase generally influence the size of the droplets produce during homogenization (McClements, 2015). The consistency showed the similar tendencies to the firmness. Among all mayonnaise samples, the highest consistency was observed for S samples. S sample exhibited the highest negative value, compared to other samples ($p<0.05$). More negative cohesiveness value indicated the more sticky sample (Liu *et al.*, 2007). In general, variations in the type of oil can alter the viscosity ratio between the dispersed and the continuous phase, which further determines the minimum size of droplet that can be produced under steady-state conditions. However, the viscosity of the samples can partially but not totally reflects the texture parameters (Liu *et al.*, 2007).

The firmness and consistency of all mayonnaise samples decreased after storage of 30 days ($p<0.05$) (Table 31). Cohesiveness had the less negative value. As the storage time increased, the oil droplets more likely coalesced and turned to the larger droplets. An increase in the droplet size results in a decrease in the number of droplets per unit volume of the emulsion, and thus the average separation distance between the droplets increases (McClements, 2005).

Table 31. Texture properties of mayonnaise containing different oils at day 0 and day 30 of storage.

Sample	Firmness (g)		Consistency (g.sec)		Cohesiveness (g)	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
S	116.23±7.23Aa	94.79±5.25Bab	3136.08±15.25Aa	2549.51±11.29Ba	-187.95±9.31Bc	-134.48±5.61Ac
V	76.67±5.47Ac	61.37±3.26Bc	2484.86±22.04Ae	1799.85±23.27Be	-113.24±0.33Ba	-102.92±1.92Aa
V-F5	103.79±0.17Ab	68.59±4.00Bc	2654.8±18.48Ad	1977.71±20.97Bd	-141.24±5.06Bb	-108.73±1.46Aab
V-F10	105.47±6.99Ab	86.69±6.59Bb	2773.41±12.25Ac	2113.67±24.71Bc	-151.27±1.80Bb	-113.30±5.07Ab
V-F15	115.56±3.06Aa	103.83±3.46Ba	3005.34±14.26Ab	2445.39±19.90Bb	-145.80±3.33Bb	-127.11±3.49Ac

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil, V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

Values are mean ± standard deviation (n=3). Different uppercase letters in the same row within the same parameter tested indicated significant difference ($p<0.05$). Different lowercase letters in the same column indicate significant difference ($p<0.05$).

The droplets become accordingly more mobile and less resistant to flow (Hayati *et al.*, 2007). Different types of oil used for preparation of mayonnaise could therefore affect the overall textural properties of resulting mayonnaise.

8.5.1.3 Viscoelastic properties

Viscoelastic properties of the mayonnaises prepared using SO, VCO and VCO/FO blends with various ratios expressed as storage modulus (G′) and loss modulus (G″) are shown Figure 22. All mayonnaise samples showed a linear viscoelastic response. For all the samples, G′ values were higher than loss modulus (G″), indicating a predominant elastic behavior than viscous behavior. The experimental linearity of the moduli suggests that mayonnaise may be considered as gel-like network in the frequency range of 0.1-100 Hz (Mancini *et al.*, 2002). This is a typical behavior for concentrated emulsion as previously reported for commercial or model mayonnaise (Fomuso *et al.*, 2001; Moros *et al.*, 2002). Mayonnaise is protein-stabilized emulsion and the plateau region is normally attributed to the formation of a pseudo-gel network due to entanglements among protein segments adsorbed at the oil–water interface of the neighboring droplets (Diftis *et al.*, 2005). The G′ values for all mayonnaise samples were increased with increasing frequency. The G′ values are increased at high frequency due to strong interactions among the droplets that contributes to the elastic modulus, which needs a long time to relax (Turgeon *et al.*, 1996). At day 0, slight differences in G′ values were observed among all mayonnaise samples (Figure 22 A). S sample showed slightly higher G′ than the others. Higher G′ implies that higher stresses are needed to cause the emulsion to flow (Mancini *et al.*, 2002). This might be due to strong droplet–droplet interactions in mayonnaise. G′ values were increased in VCO/FO blends samples with increasing level of FO in mayonnaise. The chain length and the saturation degree of the oil phase partly influenced the interfacial tension (Granger *et al.*, 2003).

After 30 days of storage, all mayonnaise samples showed a slight decrease in G′ value than freshly prepared mayonnaise (day 0) (Figure 22 C). This suggested that the mayonnaise samples were less elastic after 30 days of storage at room temperature.

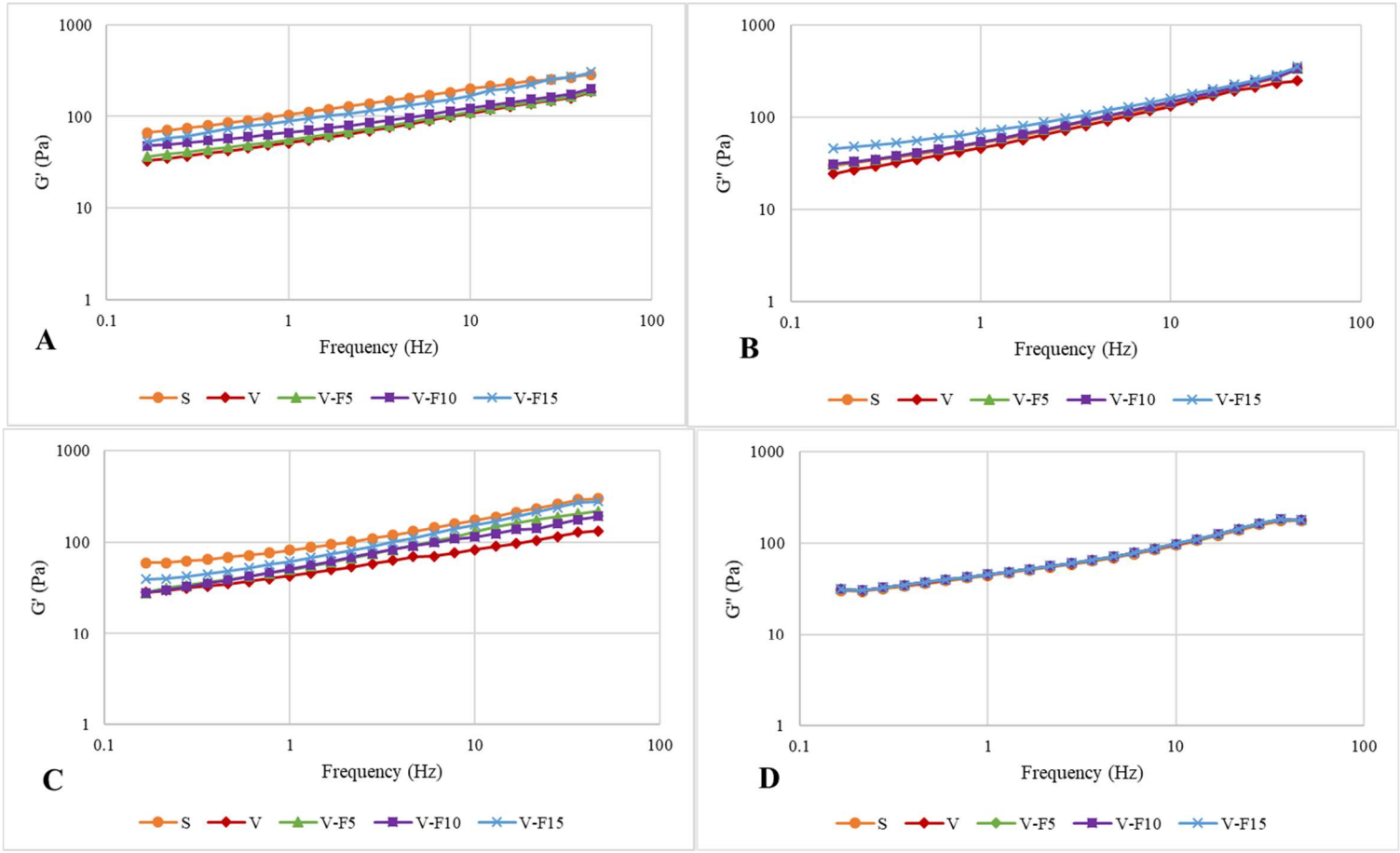

Figure 22. Storage modulus (G′) (A, C) and loss modulus (G″) (B, D) values (in log) of frequency sweep of mayonnaise containing different oils at day 0 (A, B) and day 30 (C, D) of storage.

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil, V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

In general, low G′ implies that low stresses are necessary for the sample to flow, reflecting a less compact structure and a more liquid-like behavior (Langton *et al.*, 1999). The decrease in rheological properties might be the result of structural rearrangement of oil droplets after 30 days of storage, in which a weaker network between the droplets was formed. The structural rearrangement of oil droplets resulted in slight increase in their droplet sizes. Oil droplet coalescence could occur after the droplets had been in contact for a long period (McClements, 2015). The results were in agreement with texture properties, where the firmness and consistency were decreased. S sample showed highest G′ with the highest textural properties. Oil used affected rheological property of all mayonnaise samples before and after storage of 30 days.

8.5.1.4 Viscosity

Viscosity of mayonnaise samples as a function of the shear rate is shown in Figure 23. The viscosity of all emulsions decreased as the applied shear rate increased, indicating a similar shear-thinning behavior. The droplets are very close to one another and thus tend to flocculate through hydrodynamic interactions, colloidal interactions, as well as entanglement (McClements, 2015). During shearing, flocs are deformed and eventually disrupted, resulting in reduction in the viscosity. After a sharp reduction, the viscosity change is flattened at high shear rates. All samples showed similar pattern.

At day 0, slight difference was observed in viscosity properties of all mayonnaise samples (Figure 23A). Higher viscosity was observed for S sample, whereas V sample showed the lower value. Viscosity of mayonnaises is strictly related to the close packing of the droplets as they interact with one another in the matrix (Depree and Savage, 2001). The closer the droplets, the higher the viscosity is attained as a consequence of the higher droplet-droplet interaction (Dickinson, 1998). The composition and physicochemical properties of oil in the dispersed phase have the influence on the size of the droplets produced during homogenization (McClements, 2015). The results coincided with textural and viscoelastic properties, in which S sample had the highest firmness, consistency, cohesiveness and storage

modulus (G′). Conversely, the lowest values were observed for V sample. Viscosity of mayonnaise prepared using soybean oil/palm kernel olein (PKO) blends was reported by Hayati *et al.* (2007). Higher concentration of saturated PKO yielded the resultant mayonnaise with the lower viscosity.

At day 30, the viscosity of all mayonnaise samples was decreased when compared to that found at day 0 (Figure 23B).

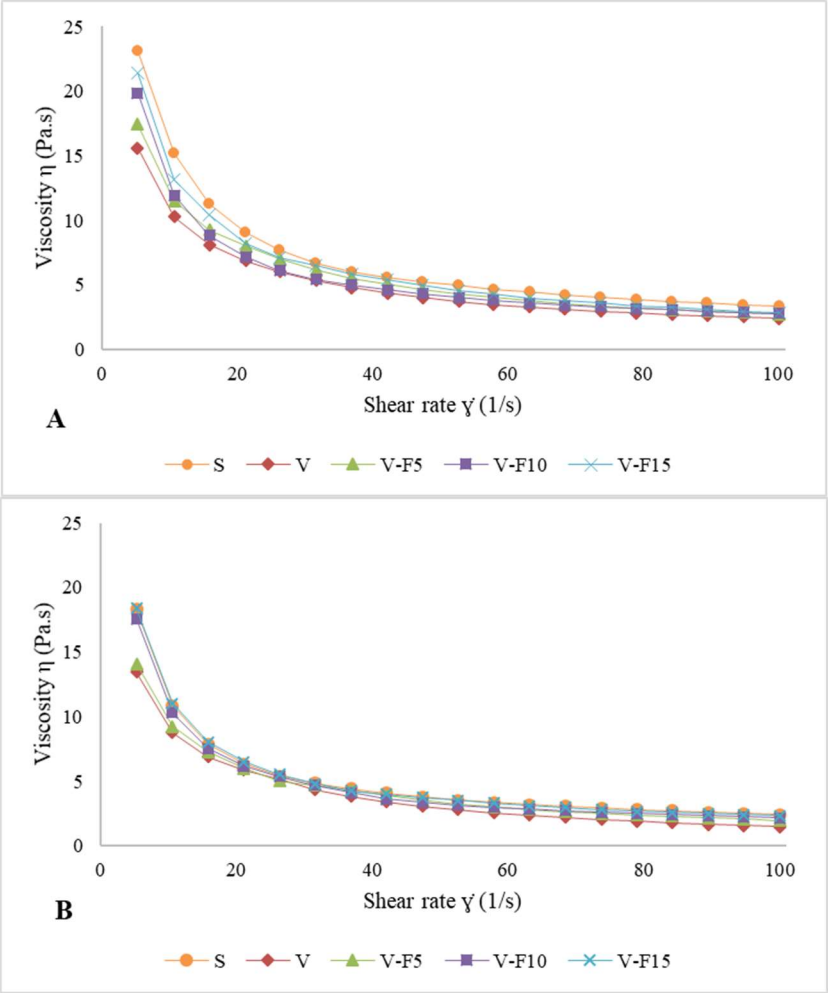

Figure 23. Viscosity versus shear rate rheograms of mayonnaise containing different oils at day 0 (A) and day 30 (B) of storage.

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil, V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

The viscosity of concentrated emulsions greatly depends on the droplet size as well as the degree of polydispersity (Pal, 1997). As droplet size increases, the number of the droplet per unit volume of the emulsion decreases and the average distance of separation between the droplets increases. Therefore, the droplets become more mobile and show less resistance to flow as evidenced by the decreased viscosity.

8.5.1.5 Flow curve

The flow curves of mayonnaise prepared using SO, VCO and VCO/FO blends at day 0 and day 30 are presented in Figure 24. For all the mayonnaise samples, shear stress was increased with increasing shear rate. Shear stress was dependent on shear rate. Thus, all mayonnaise showed non-Newtonian behavior. The results were in agreement with flow curve of low-fat mayonnaise with different fat mimetics reported by Liu *et al.* (2007). Shear stress was increased with increasing shear rate. At day 0, the difference was observed in all mayonnaise samples (Figure 24A). S sample showed higher shear stress, compared to other samples, regardless of shear rate. Viscosity is a ratio between shear stress and shear rate (Izidoro *et al.*, 2007). The higher shear stress of S sample might be due to the higher viscosity (Figure 23A). Increase in shear stress was observed with increasing level of FO in VCO/FO blend. On the other hand, V sample showed the lowest shear stress associated with low viscosity of sample.

At day 30, flow curves of all mayonnaise samples were decreased when compared to those found at the day 0 (Figure 24B). The results indicated that the viscosity of all mayonnaise sample might be decreased after 30 days of storage. The results were in agreement with viscosity measurement (Figure 23B), in which viscosity of all mayonnaise samples was decreased after the storage. The reduction in viscosity provides processing advantage during high-shear processing operations such as pumping and filling, whereas the high viscosity during mastication provides a desirable mouth feel upon consumption (Pyle *et al.*, 2012). Thus, flow properties of all mayonnaise samples were affected by their viscosity as well as storage time.

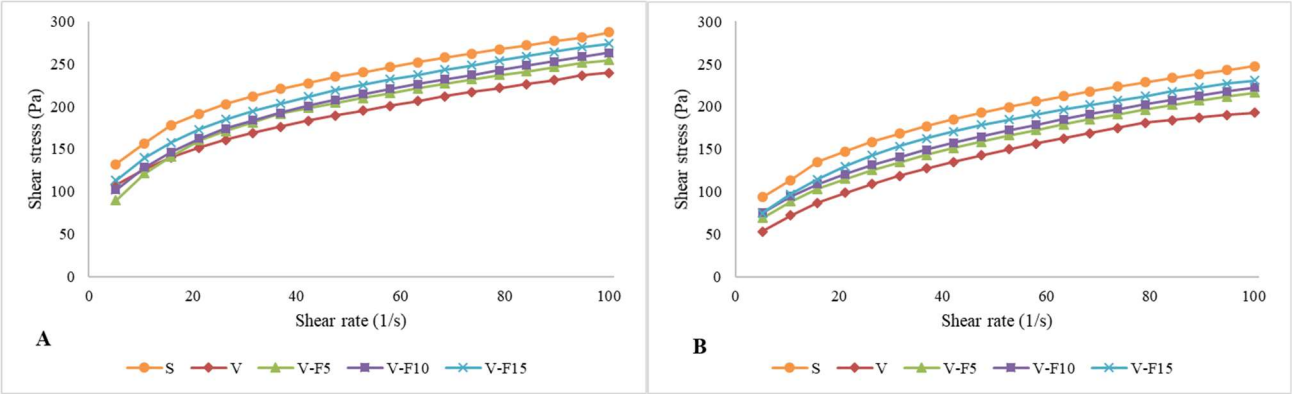

Figure 24. Flow curve of mayonnaise containing different oils at day 0 (A) and day 30 (B) of storage.

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil, V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

8.5.2 Characteristics of mayonnaise prepared using VCO/FO (90:10) blend at day 0 and day 30 of storage.

8.5.2.1 Microstructure

Microstructures of mayonnaise V-F10 sample were visualized by confocal laser scanning microscopy at day 0 and day 30 of storage at room temperature (Figure 25). Mayonnaise consists of oil droplets dispersed in an aqueous medium (Mun *et al.*, 2009). Samples at day 0 and day 30 appeared to have similar microstructure, in which uniform dispersion with close packing of oil droplets was observed. However, slight difference was noted in droplet size of both samples. Smaller oil droplets were observed at day 0, whereas the slightly larger oil droplets were found after storage for 30 days. In a highly concentrated close packing emulsion such as the mayonnaise, an increase in droplet size was possibly due to oil droplet coalescence, which occurred after the droplets aligned close together for a long period (McClements, 2015). Microstructure of mayonnaise prepared using soybean oil/palm kernel olein blends was not different in droplet size after storage for 30 days (Hayati

et al., 2007). The results indicated that microstructure of mayonnaise V-F10 sample was altered with increasing storage time.

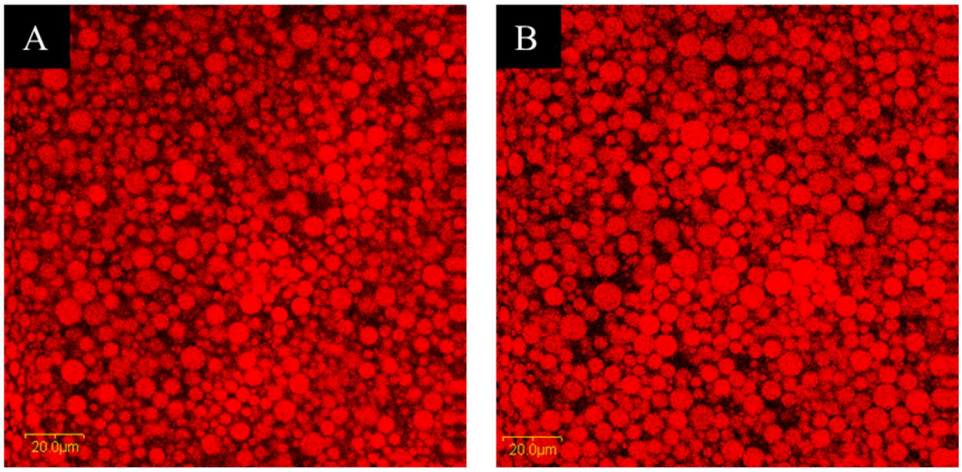

Figure 25. Confocal laser scanning micrographs of mayonnaise prepared using VCO/FO (90:10) blend at day 0 (A) and day 30 (B) of storage. Magnification: 400×. Scale bar = 20 µm.

8.5.2.2 Particle size

Particle size distribution of V-F10 mayonnaise sample at day 0 and day 30 is shown in Table 32. At day 0, V-F10 sample showed d_{32} of 3.56±0.04 and d_{43} of 5.87±0.09. The stabilization of the oil–water interface in mayonnaise is mainly due to the granular micro-particles formed from the phosphoprotein and coalesced low-density lipoprotein constituents of egg yolk. For high-lipid product, the granules keep the oil droplets well separated and prevent coalescence (Langton *et al.*, 1999). Mayonnaise is traditionally made from egg yolk, which act as an emulsifying agent (Gorji *et al.*, 2016). d_{32} was increased from 3.56±0.04 µm to 3.74±0.15 µm, whereas d_{43} increased from 5.87±0.09 µm to 6.48±0.15 µm after 30 days of storage. Size of oil droplets was altered after the storage of 30 days. The obtained results were in line with microstructure visualized using confocal laser scanning microscope (Figure 25). The larger size of oil droplet was observed after 30 days of storage. In mayonnaise, oil droplets are surrounded by a membrane of emulsifier molecules that provides physical stability of the emulsion.

8.5.2.3 Coalescence and flocculation

Coalescence index (C_i) and flocculation factor (F_f) of V-F10 at day 0 and day 30 are shown in Table 32. After the storage of 30 days, C_i was 10.08±1.50, indicating that the low coalescence occurred in the sample to some extent. On the other hand, F_f was decreased after the 30 days of storage, suggesting that individual oil droplets assembled to form larger oil droplet. The results were in accordance with droplet size distribution (Table 32), where d_{32} and d_{43} were increased as the storage time increased. The stabilizing effect of proteins in emulsions results from the protective barrier formed around fat droplets. As a result, the repulsive interactions (e.g. steric and electrostatic) between the oil droplets are generated and prevent their coalescence (McClements, 2004). With increasing storage time, coalescence was augmented, leading to the larger oil droplet. This was related with the changes in viscosity and rheological property of mayonnaise after the storage of 30 days.

Table 32. Droplet size and stability of mayonnaise prepared using VCO/FO (90:10) blend at day 0 and day 30 of storage.

Storage Time (day)	d_{32} (µm)	d_{43} (µm)	F_f	C_i
0	3.56±0.04b	5.87±0.09b	0.99±0.02a	-
30	3.74±0.15a	6.48±0.15a	0.95±0.02b	10.08±1.50

F_f: Flocculation factor, C_i: Coalescence index Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicated significant difference ($p<0.05$).

8.6 Conclusion

Type of oil used for preparation of mayonnaise and storage time affected the color and textural property of resulting mayonnaise. Highest firmness, consistency and cohesiveness were obtained when SO was used for mayonnaise preparation. Rheological properties and viscosity decreased with storage time and governed by type of oil used. However, mayonnaise containing VCO/FO (90:10) blend also showed the property equivalent to that prepared using SO. After 30 days of storage, size of droplets slightly increased associated with coalescence. Thus,

mayonnaise prepared using VCO/FO (90:10) blend could be prepared and had slight change after storage of 30 days at room temperature.

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CHAPTER 9

CONCLUSION AND SUGGESTION

9.1 Conclusions

1. Maturity stages of coconut including IMC, MC and OMC had influence on physicochemical properties, emulsion stability and oil recovery of coconut milk. Cocosin with MW of 55 kDa was the major protein in coconut milk.
2. All VCO samples contained lauric acid as a major fatty acid, regardless of maturity stages. Myristic acid was the second dominant fatty acid in VCO.
3. Water-soluble coconut proteins (albumin) exhibited low emulsifying properties, compared to salt-soluble proteins (globulin). Nevertheless, globulin fraction was more susceptible to hydrolysis by Alcalase, leading to the higher collapse of emulsion.
4. Partially purified protease from seabass pyloric caeca (PPSP) was more effective in hydrolysis of coconut milk proteins, compared to commercial trypsin (CT). PPSP at appropriate level (10 unit/g protein) at 60 °C for 120 min could be used to hydrolyze proteins in coconut milk, particularly those surrounding oil droplets. As a result, emulsion was disrupted, rendering the higher yield of VCO.
5. Combined treatment including hydrolysis using PPSP (10 unit/g protein) at 60 °C for 60 min, followed by freeze-thawing (5 cycles) was the powerful method for extraction of VCO, in which high yield (98.6%) and prime quality could be gained.
6. No marked difference was observed in fatty acid profile, moisture content, free fatty acid content and oxidative stability of all VCO obtained, regardless of all extraction processes used.
7. VCO in combination with FO at an appropriate ratio (90:10 v/v) could be used instead of soybean oil to prepare a functional mayonnaise with increased oxidative stability.

9.2 Suggestions

1. Enzyme from seabass pyloric caeca should be further purified and characterized.
2. The purification and characterization of protein with MW of 55 kDa from albumin and globulin fraction should be carried out.
3. Hydrolysis effect of PPSP on coconut protein fractions should be investigated.
4. Masking agent or potential removal procedure for deodorization of fish oil should be used for VCO based mayonnaise containing FO higher than 10%.
5. VCO should be incorporated in some food products and their properties should be investigated.

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