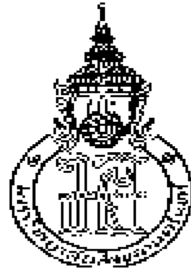

Lipids from Cephalothorax of Pacific White Shrimp (*Litopenaeus vannamei*) using Ultrasonic Assisted Extraction Process: Pre-treatment, Enhanced Oxidative Stability and Applications

Saqib Gulzar

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

Prince of Songkla University

2019

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ABSTRACT

Effect of different solvents along with ultrasonic assisted extraction (UAE) process on the extraction yield of lipids from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) was investigated. Among all solvents used, the mixture of hexane:isopropanol (1:1) provided the highest yield (3.91 g/100g sample) and carotenoid content (1.97 mg/g lipid). When ultrasonication with different amplitudes (50-90%) was used for 25 min, the highest yield was obtained at 80% amplitude ($p<0.05$). With UAE at the same ultrasonication time, continuous mode rendered the higher yield than pulse mode ($p<0.05$). Lipid extracted with hexane:isopropanol mixture with UAE process had the yield of 8.39 g/100g sample. Phospholipids were major constituents of lipids extracted by solvent extraction. However, more free fatty acids, mono and diglycerides were found in lipids extracted by UAE process, indicating increased hydrolysis. UAE process resulted in higher oxidation of lipids as evidenced by increased peroxide values (PVs) and thiobarbituric acid reactive substances (TBARS). Those alterations were more pronounced in lipids extracted using UAE with continuous mode than pulsed mode as confirmed by Fourier transform infrared (FTIR) spectra.

Impacts of different pre-treatments of cephalothorax before UAE on yield and characteristics of lipids were studied. Autolysis at 50 °C for 3 h in the absence and presence of 0.1% tannic acid (TA), a lipase inhibitor, was implemented. Pre-heating of cephalothorax containing 0.1% TA at 95 °C for different times (15-45 min) was also carried out. When lipids were extracted at amplitude of 80% for 25 min in continuous mode, samples with pre-heating rendered the highest lipid yield (13.3g-14.1g/100g

sample). Pre-heating along with TA addition resulted in suppression of lipid oxidation. Free fatty acid (FFA) content was also found to be lower, whereas the control (without pre-treatment) had higher FFA. FTIR spectra confirmed lower oxidation in lipid from pre-heated samples added with TA. Lipid contained astaxanthin, astaxanthin monoester, astaxanthin diester, canthaxanthin and β-carotene.

After pulsed electric field (PEF) pre-treatment at different electric field strengths (4, 8, 12 and 16 $kVcm^{-1}$) and pulse numbers (120, 160, 200 and 240) was applied on cephalothorax, PEF treated samples were subsequently subjected to lipid extraction UAE (amplitude of 80%) for 25 min in continuous mode. PEF pre-treated samples subjected to UAE rendered the highest lipid yield (10.44 g/100g sample). PEF pre-treatment resulted in reduced lipid oxidation. Lipid from PEF pre-treated samples extracted using UAE had higher content of polyunsaturated fatty acids (PUFAs) as well as carotenoids, including astaxanthin, etc.

Impacts of different pre-treatment conditions and atmosphere on yield and oxidative stability of lipids using UAE were studied. Cephalothorax was subjected to vacuum-microwave (VM) heating (at 95 °C) prior to UAE using a mixture of isopropanol/n-hexane (1:1) as solvent. Nitrogen gas was flushed at two flow rates; low (2.15 L/min) and high (4.35 L/min) into the system during ultrasonication. Vacuum-microwave heating resulted in the increase of lipid yield and the highest yield was observed in the samples extracted by a combination of VM and UAE. Addition of TA at 0.1% in combination with VM, followed by nitrogen flushing resulted in the increased oxidative stability of lipids. Furthermore, astaxanthin content in the lipid was found to be increased by aforementioned treatments.

Shrimp oil extracted from cephalothorax added with 0.1% TA and preheated at 95 °C for 5 min using an innovative UAE under nitrogen atmosphere, was encapsulated in nanoliposomes, prepared using ultrasonication (US) and microfluidization (MF). Nanoliposomes prepared by US and MF were characterized based on particle size, structure and stability. The particle size of US nanoliposomes ranged between 40 and 284 nm, while MF nanoliposomes ranged from 214 to 928 nm. US nanoliposomes exhibited better centrifugal stability than MF counterparts ($p<0.05$). Nanoencapsulation

efficiency (NEE) of US nanoliposomes was higher (93.64%) than that of MF (75.18) and remained constant over the storage of 8 weeks at 30 °C. Nanoliposomes showed higher oxidative stability during the storage than unencapsulated oil ($p<0.05$) with higher retention of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), particularly in US nanoliposomes. Overall, encapsulation of shrimp oil in nanoliposomes could prevent oxidation of oil during storage and mask the undesirable fishy odor.

Shrimp oil nanoliposomes (SONL) prepared using ethanol injection process followed by ultrasonication, were fortified into skim milk at various levels (2 - 10%, v/v), followed by pasteurization at 63 °C for 30 min. Skim milk showed lowered whiteness but increased redness and yellowness as added SONL levels increased ($p<0.05$). Viscosity of fortified samples was also augmented with increasing levels of SONL ($p<0.05$). Fortified milk skim samples had no perceivable fishy odor and were organoleptically acceptable. When skim milk fortified with 10% SONL was stored up to 15 days at 4 °C, microbial load was less than 2.54 log CFU/ml. pH and acidity values were also within the acceptable limits. Shrimp oil in SONL did not undergo oxidation during the extended storage. Fatty acid profile of shrimp oil revealed no loss of polyunsaturated fatty acids taken place during storage of fortified milk. Therefore, nanoliposomes could be an effective carrier for shrimp oil to be fortified in skim milk.

Impact of β-glucan on masking bitterness of skim milk fortified 10 % SONL was examined. β-glucan was added at various levels (0.05–0.2%). With the addition of SONL, fortified skim milk appeared more reddish in color due to the presence of astaxanthin. Addition of β-glucan resulted in the increase in viscosity of the fortified milk by forming network of junction zones. During the storage of skim milk fortified with SONL and 0.1% β-glucan at 4 °C for 15 days, no major quality changes took place. Simulated *in vitro* digestion studies revealed that 45.41% EPA and 48.86% DHA from shrimp oil were bioaccessible for absorption in the gut after digestion.

Shrimp oil was encapsulated into nanoliposomes and dried using freeze drying and spray drying methods. Carboxy methyl cellulose (CMC) and fumed silica (SiO_2), at different proportions were used as wall material and anti-caking agents to improve

the flowability and reconstitution properties of the dried powders. Spray-dried powder was spherical with average particle size of 5.33±2.55 μm, while the freeze-dried powder was irregular in shape with average size of 243.6±256.7 μm. Spray dried powder had better flowability, compared to freeze-dried, which was more porous with much lower bulk density. Encapsulation efficiency and solubility of spray-dried SONL powder was higher than freeze-dried powder ($p<0.05$). However, the wettability of freeze-dried powder was higher with shorter reconstitution time. Freeze-dried powder exhibited lower oxidation of total encapsulated oil and better retention of n-3 fatty acids ($p<0.05$).

Therefore, UAE could be effectively used to increase the extraction yield of lipids and carotenoids from Pacific white shrimp cephalothorax with appropriate treatments. Moreover, the extracted lipids could be encapsulated in nanoliposomes to prevent oxidation and mask the fishy odor of shrimp oil fortified in various foods. Dry nanoliposomes could also be produced with free-flowing behavior.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Shrimp is widely consumed all over the world and Thailand has been a major exporter worldwide. Global shrimp production now stands at approximately 6 million metric ton (MMT) and is expected to increase at a compound annual growth rate (CAPR) of 4.8% between 2016 and 2019 (FAO, 2016). *Litopenaeus vannamei* alone accounts for approximately 76% or roughly 5 MMT of global aquaculture production. Whole shrimp generally gets bifurcated into edible and non-edible portions, in which the latter include exoskeleton and cephalothorax (40-50 g/100g of its total weight). For shrimp processing industry, shell and cephalothorax are discarded and considered as leftover (Sachindra *et al.,* 2005). Most of this by-product ends up as animal feed or protein supplemented diet for aquaculture (Nwanna *et al.*, 2004). Over the past few years, lipids and carotenoids have received increasing interest, due to the presence of some valuable and essential components, especially n-3 fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Amiguet *et al.*, 2012). These fatty acids are known to have excellent health benefits (Harper and Jacobson, 2001). Astaxanthin is the major pigment in crustacean and contributes to reddish-orange color (Higuera-Ciapara *et al.*, 2006). Astaxanthin is a very potent antioxidant and shows ten-fold higher antioxidant property than other carotenoids such as lutein, zeaxanthin, canthaxanthin and carotene (Naguib, 2000) and 100 fold greater than α-tocopherol (Miki, 1991).

Ultrasonic assisted extraction (UAE) has been widely used in food industry to overcome the drawbacks associated with conventional extraction e.g., low extraction efficiency, use of large amount of solvents and subsequent concentration, etc. (Chandrapala *et al.*, 2013). Ultrasound waves are generated in liquid medium and these waves alternately compress and stretch the molecular structure of the medium. During each "stretching" phase (rarefaction), the negative pressure is strong enough to overcome intermolecular binding forces. A fluid medium can be literally torn apart,

thus producing tiny cavities (microbubbles). This phenomenon is termed as acoustic cavitation (Povey and Mason, 1998). In succeeding cycles, these cavities can collapse violently, releasing the large amounts of energy in the immediate vicinity of the microbubbles. Via cavitation effect, huge energy released is strong enough to disrupt cell membranes and causes the release of target compounds in localized areas (Mason, 1999). Ultrasonication under the appropriate conditions could be a powerful method for lipid extraction from cephalothorax owing to its cavitation effect. Extracted lipid could serve as an excellent source of polyunsaturated fatty acids as well as carotenoids.

Pulsed electric field (PEF) is a non-thermal technology, in which a material is placed between two electrodes. High-voltage pulses, for very short periods of time usually between microseconds to milliseconds, are passed through it. There is a negligible increase in temperature of treated sample and cost of operation is low (Töepfl *et al.*, 2006). High voltage pulses with sufficient strength cause electroporation of the cell membrane in plant and animal tissues. Electroporation results in the increased permeability of cell membranes, made up of charged ions and proteins. PEF has been introduced for pretreatment of maize, olives, soybeans and rapeseed before oil extraction (Guderjan *et al.*, 2005; Guderjan *et al.*, 2007). Additionally, PEF is believed to show an inactivation effect on enzymes. Ho *et al.* (1997) reported that the activities of α-amylase, glucose oxidase, and lipase were decreased by 70% - 85% after PEF treatment.

Tannic acid is an antioxidant as well as lipase inhibitor (Horigome *et al.*, 1988; Longstaff and McNab, 1991). Tannins extracted from plants and berries were found to inhibit digestive enzymes (McDougall *et al.,* 2009). Apart from using enzyme inhibitor, heating effectively inactivate the enzyme via thermal denaturation (Klibanov, 1983). Also heat could disrupt the lipid tissues, releasing more lipid for further extraction. Autolysis of hepatopancreas at 60 °C was found to increase the yield and carotenoid from the Pacific white shrimp cephalothorax (Senphan and Benjakul, 2012). Additionally high temperature is shown to have an impact on extraction of lipid by increasing the solubility of solvents (Dubrow *et al.*, 1973). Heat

also causes the proteins in the cell membrane to denature, favoring permeability of solvent (Quinn, 1988). The use of antioxidant and enzyme inhibitors may reduce the deterioration of lipids caused by oxidation and hydrolysis.

The process of entrapping an active substance within the wall material is referred to as encapsulation (Jafari *et al.*, 2008; Mahdavi *et al.*, 2014). Encapsulation has been used widely in food industry for incorporation of various ingredients including enzymes, volatile additives, polyphenols, colorants, etc., inside capsules to preserve them against nutritional losses (Zuidam and Shimoni, 2010). The more recent encapsulation technology is the nano-liposome technology (Munin and Edwards-Lévy, 2011). Liposomes are spherical vesicles consisting of a phospholipid bilayer and an aqueous core with sizes ranging from nanometer to micrometers. The phospholipid bilayer in liposomes forms a protective covering around encapsulated oils, preventing the contact of oils from prooxidants or air. As a result, the oxidation of core is retarded. Nanoliposomes offer better advantages than other encapsulation techniques such as better oxidative stability by avoiding high temperature during drying (Jafari *et al.*, 2008), controlled and targeted release of core material, and increased bioavailability of bioactive components (Chaudhry *et al*., 2008). Encapsulation of unsaturated fatty acids into liposome capsules creates a barrier to prevent the oxidation of these compounds (Hadian, 2016). In dairy industry, liposomes have been used for fortification of vitamins to increase their nutritional value as well as enhance the bioaccessibility (Mozafari *et al.*, 2008). Nanoliposomes can be used to incorporate shrimp oil to enhance its oxidative stability and the application of liposome can be achieved easily due to its hydrophobic nature.

Although nanoliposomes are thermodynamically stable over a considerable period of time, their shelf life of the nanoliposomes in the aqueous system is questionable. The phospholipids in the liposomes are oxidized or hydrolyzed over time. Hydrolysis can be minimized by using pure solvents and removing water as much as possible (Gibbs *et al.*, 1999). Therefore a more stable system can be achieved by removing the water from the nanoliposomal dispersions by drying process. A few studies have shown that liposomes retain their stability and narrow size distribution in

the powder form (Chun *et al.*, 2017). Many drying methods including spray-drying, freeze-drying, or spray freeze-drying have been employed to prepare liposomal dry powder formulations (Courrier *et al.*, 2002). Ideal powder characteristics such as good flow properties, lower reconstitution time and efficient delivery of target compounds are dependent on several factors including drying methods, interparticle friction, cohesive forces, particle size, etc. (Khoe *et al.*, 1991). Most food powders are susceptible to moisture adsorption from the ambient atmosphere, which significantly impacts the flow behavior of powder (Fitzpatrick, 2005). The process of powder particles sticking together either due to strong interparticle cohesion or formation of solid bridges between powder particles is referred to 'caking' (Fitzpatrick, 2005).

Caking is an undesirable powder attribute and is majorly ascribed to the van der Waals forces between the powder particles. Anticaking agents or flow additives are used in food powders to conquer the problems related to agglomeration. Anticaking agents disrupt the cohesive interactions between powder particles by placing themselves between powder particles and reducing the friction (Fitzpatrick, 2005). Fumed silica (SiO_2) is one of anti-caking agents widely used in food and pharmaceutical industries. Fumed silica is hydrophilic and has a large number of hydroxyl groups present on the surface, which can readily interact through hydrogen bonds forming a three dimensional network (Ahmad *et al.*, 2006). Carboxy methyl cellulose (CMC) is a water-soluble cellulose of significant technical importance. It is used as a food thickener and has been widely used in the encapsulation of drugs (Milani and Maleki, 2012). One of the major reason of using CMC as an encapsulating agent (wall material) is the high water solubility and tendency to form a fine and dense network upon dehydration (Benchabane and Bekkour, 2008)

Thus, the extraction of lipids from shrimp cephalothorax with the aid of ultrasound, while maintaining the quality of lipid via preventing hydrolysis or oxidation using several approaches, could be a means to increase the yield of lipid with prime quality. Along with encapsulation in the form of nanoliposome, especially dried form, lipid ions could be stabilized and employed as supplement with ease.

1.2 Review of literature

1.2.1 Pacific white shrimp (*Litopenaeus vannamei*)

1.2.1.1 Anatomy and chemical composition

Pacific white shrimp, (formerly *Penaeus vannamei*), is a decapod crustacean, endemic to the coasts of Peru from south in the eastern Pacific coast of Central and South America to Mexico in the north. In the 1970s, shrimps were introduced widely around the world, however it has become mainly cultivated species in Asia since 2000. It belongs to the family Penaeidae and follows a body format similar to that of most Malacostracans. The body of shrimp is laterally compressed, comprising of a well-developed abdomen adapted for swimming. Each segment is enclosed by a dorsal tergum and ventral sternum (Dall *et al.*, 1990). The laterally-compressed shrimp body is grossly divided into an anterior cephalothorax and a posterior abdomen. The cephalothorax includes the head as well as eight thoracic somites. This section includes the animal's sensory and masticatory apparatus and houses most of its organs. The abdomen is made up of six segments and contains most of the musculature. The cephalothorax includes the prosencephalon and the gnath-thorax. The prosencephalon includes the eyes, antennules, antennae and labrum. The gnath-thorax contains the masticatory apparatus and the thorax proper. The digestive system consists of a mouth, foregut, midgut, hindgut, and hepatopancreas. The shrimp's mouth is located on the ventral surface of the head region anterior to the first maxilliped. Food entering the mouth has undergone initial mastication by the appendages adapted for this purpose. The foregut includes the esophagus and stomach. The posterior chamber is divided into dorsal and ventral sub-chambers (Sis *et al.*, 1991). Shrimp have a translucent body comprising of a bluish-green hue attributed to the pigmented chromatophores (molecules evolved to collect/reflect light) (Bauer, 1981).

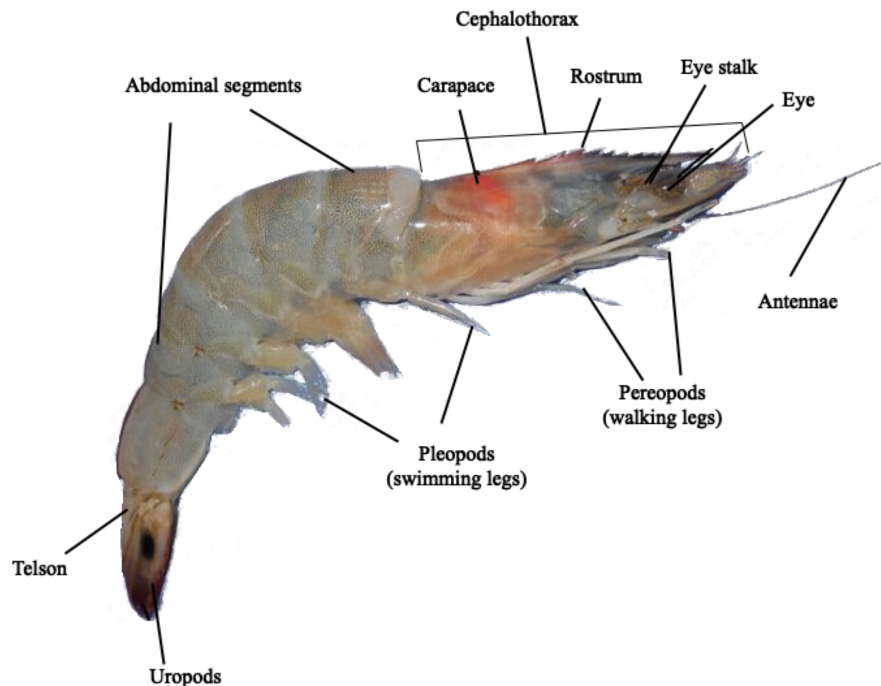


Figure 1. Lateral view of external morphology of *Litopenaeus vannamei*
Source: Hickman *et al.*, (2006)

The hepatopancreas, the shrimp's primary digestive gland, occupies a major part of the posterior cephalothorax and surrounds the posterior stomach and anterior midgut. Principally, hepatopancreas is essential nutrient storage organ for protein (Marsden *et al.*, 2007), lipids (Senphan and Benjakul, 2012), carbohydrates (Zhou *et al.*, 2011), vitamins (Nguyen *et al.*, 2012), carotenoids (Senphan and Benjakul, 2012), essential fatty acids and amino acids (Emerenciano *et al.,* 2013; Zhou *et al.*, 2011).

Farming of crustaceans, particularly shrimps is done widely around the world with Asia holding the share of 90% (FAO, 2014). In 2012, the worldwide estimation of crustacean aquaculture was generally steady at US$19.5 billion, overwhelmed by two species of shrimps (approximately 70% *Litopenaeus vannamei* and 22% *Penaeus monodon*) (FAO, 2014). In Thailand, 90% of shrimp production was farmed, as reported by The Food and Agricultural Organization of the United Nations (FAO, 2010). The processed shrimp/prawn exports from Thailand in 2017, were valued at 1.8 billion U.S.$, which amounts for roughly one-third of the total

seafood exports (Table 1) (GAIN, 2018). The major importers of Thai shrimps are South Korea, Japan and the USA.

Table 1. Thailand’s Seafood exports from 2014-2017

Commodity Exported	**Exports (Million U.S. $)**			
	2014	**2015**	**2016**	**2017**
Fish, fresh/chilled, excluding fish fillets and other fish meat	51.8	45.1	42.8	33.4
Fish, frozen, excluding fish fillets and other fish meat	173.8	127.4	121.6	156.6
Fish fillets and other fish meat, fresh/chilled/frozen	317.6	289.1	281.7	249.1
Fish, dried, salted smoked	110.2	95.1	104.9	90.3
Molluscs, live/fresh/chilled/frozen/dried	448.1	364.1	352.8	373.8
Shrimps and prawns, frozen/not frozen/prepared or preserved	1974.3	1644.2	1952.8	1873.8
Tuna, Skipjack and Boito, prepared/preserved	2354.8	1966.2	1978.8	2061.7
Sardines, Sardinella and Brisling or Sprats, prepared/preserved	168.8	154.5	116.9	108
Salmon, prepared/preserved	122.3	132.8	100.2	113.8
Cuttle fish, Squid, Octopus, live/frozen/chilled	349.8	289.8	283.5	345
Other prepared/preserved fish/seafood	578.8	533.8	514.6	725.9
Total Export Million U.S. $	**6300.5**	**5352.3**	**5567.1**	**5786.4**

Source: Global Trade Atlas (GAIN, 2018)

1.2.1.2 Status

Cultured shrimp is a product that forms a major portion of Thailand's fishery exports. In 2013, the production of Thai cultured shrimps suffered a major setback due to a disease called Early mortality syndrome (EMS), and the shrimp supply was declined by 50 percent from 540,000 metric tons (MT) in 2012 (Figure 2). The shrimp industry, although made progress in diagnosing the disease and farm management practices were improved, never recovered to the previous production levels. In the year 2018, shrimp production is estimated at 350,000 MT (FAO, 2016).

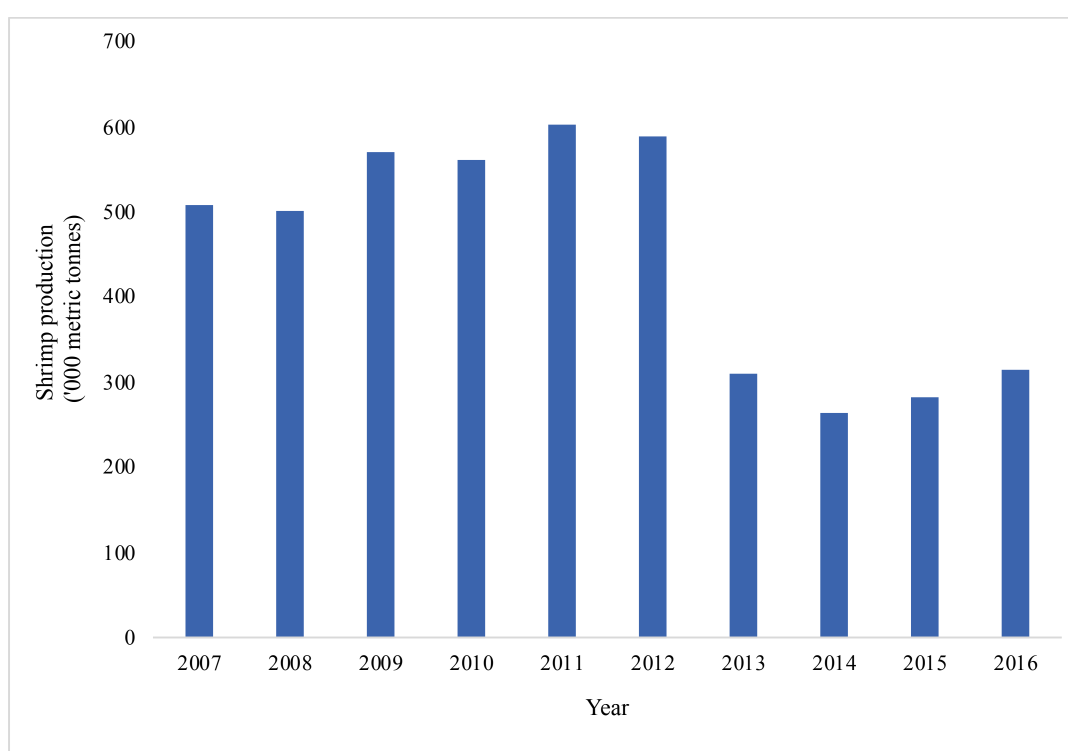


Figure 2. Production of Pacific white shrimp (*Litopenaeus vannamei*) in Thailand
Source: (FAO, 2016)

1.2.2 Compositions of shrimp processing by-products

By-products from shrimp processing include cephalothorax, shell, etc. (Sachindra *et al.,* 2005). Shrimp processing by-products contains valuable components such as chitin, chitosan, lipid, proteins, minerals and carotenoids. Chitosan has been used extensively in food and allied industries due to its bioactivity

(Hirano, 1996). Lipids and carotenoprotein from shrimp processing by-products have been widely used as functional ingredients in food and aquafeed (Armenta and Guerrero-Legarreta, 2009; Auerswald and Gäde, 2008). To better exploit such low market valued resources, there is a need for developing better techniques for utilization of these by-products for value-addition.

1.2.2.1 Proteins and amino acids

Proteins and amino acids are the major constituents of crustacean processing byproducts. Pacific white shrimp cephalothorax constituted 60.6% (dry basis) of protein and essential amino acid content was found to be 340.26 mg/g protein (Cao *et al.*, 2009). Protein hydrolysates from shrimp cephalothorax were found to contain low molecular weight peptides (1–1.5 kDa) which were associated with the regulation of cholecystokinin (CCK), a biomarker associated with satiety (Cudennec *et al.*, 2008). CKK is an important hormone that is responsible for regulation of appetite and gastric emptying. These peptides from shrimp cephalothorax played an important role in stimulating the release of CKK in the intestinal endocrine cells. Such peptides are fortified into functional foods to prevent or treat appetite-related disorders. Moreover, a histone protein has been isolated from hemocytes of Pacific white shrimp having antimicrobial activity (Patat *et al.*, 2004). Amino acids derived from protein hydrolysates through lactic acid fermentation of shrimp cephalothorax consisted of glutamic acid, aspartic acid, glycine, arginine, alanine, serine, histidine, proline, threonine, tyrosine, methionine, valine, leucine, isoleucine and phenylalanine (Bueno-Solano *et al.*, 2009). Free amino acid content from protein hydrolysates from the fermented shrimp cephalothorax was found to be 2,061.79 mg/l (Mao *et al.*, 2013). 50.32% of total amino acids were found to be essential amino acids. Phenylalanine, methionine, and lysine and were present in higher quantities in the fermentation supernatant. Recently, Latorres *et al.* (2018) have reported that protein hydrolysates extracted from the cephalothorax of Pacific white shrimp were the essential sources of bioactive compounds which could be used in the fortification of foods as well as natural antioxidants in the foods containing lipids.

1.2.2.2 Chitin and chitosan

Chitin is the most abundant renewable resource present in the nature only after cellulose (Rinaudo, 2006). Chitin forms the basic structural exoskeleton of crustaceans and amounts for approximately 15-20% of total dry weight (Coward-Kelly *et al.,* 2006). Structurally, chitin is a polymer of 2-acetoamido-2-deoxy-β-D-glucopyranose units linked in linear form. Chitin is naturally found in the cell walls of fungi and yeast or in the exoskeleton of arthropods as the ordered crystalline microfibrils. Commercially, crab and shrimp shells are the main sources of chitin (Paulino *et al.*, 2006). *Bacillus cereus* SV1 was used for extraction of chitin from the crude protease of shrimp shell (Manni *et al.*, 2010). Chitin (89.5%) obtained by the aid of *Bacillus cereus* from crude protease was converted to chitosan by N-deacetylation. Chitosan extracted from shrimp wastes has proven medicinal benefits. It has been used as neuroprotective (Pangestuti and Kim, 2010) and in the prevention of tumor (Quan *et al.*, 2009). Chitosan also possesses antifungal and antibacterial properties (Fernandes *et al.*, 2008) and has been used as an anti-inflammatory agent (Yang *et al.*, 2010). The growth of most gram-positive and gram-negative bacteria was inhibited by the chitosan solution at 50 mg/ml. Chitosan exhibited a bacteriostatic effect on Gram-negative bacteria, *Vibrio cholerae, Escherichia coli* ATCC 25922, *Bacteroides fragilis,* and *Shigella dysenteriae,* except *Salmonella typhimurium* (Benhabiles *et al.*, 2012). Chitosan prepared from shrimp waste was found to have antioxidative properties by inhibiting lipid peroxidation in tuna dark muscles (Si-Trung and Bao, 2015).

1.2.2.3 Carotenoid and carotenoproteins

Carotenoids are fat-soluble pigments found in algae, fungi, plants yeast, photosynthetic bacteria and crustaceans (Sachindra *et al.*, 2005). These pigments were mainly synthesized in several plants and transferred to animals in the form of diet (Liang *et al.*, 2009). Carotenoid can be classified as carotenes (e.g. β-carotene, and lycopene) and xanthophylls (e.g. zeaxanthin and canthaxanthin) (Bernal *et al.*, 2011). These carotenoids have gained the commercial importance due to their natural origin, zero toxicity and amphiphilic soluble compounds (Babu *et al.*, 2008).

These compounds also have immunomodulatory and high antioxidant activities, which can be implemented for the treatments of diseases, such as cardiovascular diseases and several types of cancer (Bernal *et al.*, 2011). Carotenoids are present in the form associated with proteins. Carotenoproteins are more stable than free carotenoids (Ghidalia, 1985). Astaxanthin is also found in carotenoproteins of crustaceans (Chakrabarti, 2002). Black tiger shrimp shells contained 88 % of astaxanthin in the form of carotenoprotein with 20% of other lipid components (Klomklao *et al.*, 2009). Pattanaik *et al.* (2020) extracted carotenoproteins from shrimp shell wastes of *Penaeus monodon, Parapenaeopsis stylifera, Metapenaeus affinis* and *Nematopalemon tenuipes*. Carotenoproteins from *P. stylifera* shell had higher antioxidant activities with increasing quantity of carotenoids.

Astaxanthin is a xanthophyll and its esters are the dominant pigment found in most crustacean species (Sachindra *et al*., 2005; Shahidi and Synowiecki, 1991). *Penaeus monodon* were composed of astaxanthin (free, monoester and diester) as the dominant pigment (86-98 %) followed by β-carotene (3.6%) and zeaxanthin (1.5%) (Okada *et al.*, 1994). *A.alcocki* and *Solonocera indica*, deep sea species, contained 63.5-92.2% astaxanthin and its ester forms (Sachindra *et al.*, 2006). Howell and Matthews (1991) compared the carotenoid contents between wild *Penaeus monodon* and cultured blue counterpart and found that the wild species had higher content of astaxanthin diesters, monoesters and free forms. These variations in amount and types of astaxanthin might be governed by habitat and diet of shrimp (Miniadis-meimaroglou and Sinanoglou, 2012). Oil from shrimp or shrimp processing byproducts is rich in astaxanthin in several forms. For esterified astaxanthin, oleic acid was the major fatty acid, which was esterified with astaxanthin to form astaxanthin monoester in *P.borealis* (Renstrøm and Liaaen-Jensen, 1981). Yang et al. (2015) reported that the carotenoid in *L. vannamei* were composed of 59% astaxanthin monoester, followed by free astaxanthin (33 %) and astaxanthin diester (8 %). Nevertheless Gómez-Estaca *et al.* (2017) found that carotenoids from *L. vannamei* processing waste had higher proportion of astaxanthin diester (43%), with lower contents of astaxanthin monoesters (41%) and free astaxanthin (16%).

Fat soluble vitamins are the part of necessary diet for humans that can be obtained through crustaceans (Merdzhanova *et al.*, 2014; Stancheva and Dobreva, 2013). These fat soluble vitamins including vitamin A (Retinol), D and E (α-Tocopherol) have the vital role in maintaining human health. Vitamin A helps in improving the vision and immune system development. Vitamin E aids in the development of muscular, reproductive and also functions as an antioxidant. Merdzhanova *et al.* (2014) documented the composition of fat soluble vitamins in *Farfantepenaeus aztecus* shrimp meat, consisting of 537.0 µg/100g of vitamin A, 12.99 µg/100g of vitamin D and 7730.9 µg/100g of vitamin E. For oil extracted from cephalothorax of *L. vannamei,* vitamin A content was from 0.9 to 1.6 mg/100 g and vitamin E constituted from 26.2 to 49.0 mg/100 g (López-Cervantes *et al.*, 2006). Since hepatopancreas was termed as lipid storage organ, the level of vitamins was found to be higher than other parts of organs (Wei *et al.*, 2014).

1.2.2.4 Lipids and fatty acids

One of the major source of energy in aquatic organisms, particularly the invertebrates, is the lipid. Lipid is responsible for carrying out essential life processes of growth, reproduction and molting (Zhang *et al.,* 2013). The compositions of lipid from shrimp is dependent on may factors such as growth, age, sex and season (Mili *et al.*, 2011). Oil from krill contained polar non-phospholipids (64–77%), phospholipids (20–33%), and minor triglycerides (1–3%) (Gigliotti *et al.,* 2011). Lipids from krill and shrimp are a rich source of long chain n-3 polyunsaturated fatty acids (PUFAs), mainly EPA (C20:5) and DHA (C22:6) (Sánchez-Camargo *et al.*, 2011). Aquatic foods are one of the promising sources of long chain polyunsaturated fatty acids (PUFA), selectively eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with high impact on health promotion and medical applications (Navaneethan *et al.*, 2014). Higher PUFA content was followed by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) contents in shrimp oil (Sriket *et al.*, 2007). Moreover the total fatty acid profile varied with species, season, trawling location and gender. Fatty acid profiles of shrimp are fluctuated but major fatty acids contain palmitic acid, oleic acid, EPA and DHA as predominant fatty acids. Total SFA,

MUFA and PUFA contents were altered, based upon the survival condition of shrimps (Miniadis-meimaroglou and Sinanoglou, 2012). Higher PUFA contents (44.3, and 43.57%) were also reported for *P. monodon* meat and *L. vannamei* waste (Sriket *et al.,* 2007; Gómez-Estaca *et al.*, 2015). However some shrimp meat (*X. kroyeri*) contained high amount of SFA (55%) (Lira *et al.*, 2014). Fatty acids composition of the shrimps can vary depending on species, size, age, diet, gender, season and geographical location (Miniadis-Meimaroglou and Sinanoglou, 2012). Takeungwongtrakul *et al.,* (2012) reported that during refrigerated storage, a decrease in the triglyceride content and unsaturated fatty acid content, especially EPA and DHA, of shrimp oil and an increase in the free fatty acid of lipid, extracted from Pacific white shrimp cephalothorax and hepatopancreas was observed.

Diet rich in n-3 fatty acids plays a vital role in prevention of cardiovascular diseases. EPA and DHA consumption possibly reduces resting heart rate, improves cardiac diastolic filling and regulates blood pressure (BP) (Ander *et al.*, 2003). Prospective cohort study of congestive heart failure in 2,735 adult patients revealed that combined supplementation of EPA and DHA resulted in the lower incidence of congestive heart failure (CHF) in elder adults (Mozaffarian *et al.*, 2011). In a similar experiment, myocardial infarct survival patients were supplemented with diet consisting of n-3 fatty acids at 2 g/day, while other groups had diet added with mustard seed oil (ALA) or a placebo. The n-3 fatty acids receiving group showed lowered nonfatal myocardial infarction (Singh *et al.*, 1997). Yuan *et al.,* (2001) documented that fish and shell fish consumption in Chinese men reduced the risk of fatal myocardial infarction by 59%. Secondary cardiovascular complications were greatly reduced by adopting a ‘Mediterranean-style’ diet rich in n-3 fatty acids in the Lyon Heart Study (Lorgeril and Salen, 2001). Analysis of the Finnish, Dutch and Italian cohorts in seven countries indicated an inverse relationship between fatty fish consumption and 20-year coronary artery disease **(**CAD) mortality (Oomen *et al.*, 2000). PUFA has the synergistic effect in treatment of cancer chemoprevention by the inhibition of the eicosanoids, which are derived from arachidonic acid (Nie *et al.*, 2002). The eicosanoids formation process is as follows: initially arachidonic acid is transported by the enzyme phospholipase A2 (PLA2) from the cellular membrane

glycerolipid pools, which is further oxidized by three different enzymes namely: (1) the cyclooxygenases (COX-1 and COX-2), (2) lipoxygenase and (3) P450 epoxygenase (EOX). The enhanced expression of those three enzymes has been known for the indicator of cancer development and it also enhances tumor growth and inhibits apoptosis (Bunn and Keith, 2002). EPA and DHA supplementation in cancer patients was studied in many trials (López-Saiz *et al.*, 2013). Inhibitory activity of PUFA in tumor formation may be due to the suppression of cell proliferation and alteration of prostaglandin synthesis (Wang *et al.*, 2014). PUFA supplementation also reduced cachexia in patients with pancreatic cancer (Brown *et al.*, 2003).

1.2.3 Extraction of lipid

Lipids are complex molecules comprising of mixture of polar components and nonpolar components such as glycerides and cholesterol. Therefore extraction of lipids requires use of variety of organic solvents both polar and nonpolar in nature. Principally, solvents used individually, or in combination with appropriate polarity must dissolve the lipid from the cell components for effective extraction (Berry, 2004). Environmental pollution from shrimp processing by products can be minimized by recovering from lipids from them (Handayani *et al.*, 2008). Lipids occur in various tissues from both plant and animal sources. Some lipids are present in complex forms in the membranes and in association with proteins and polysaccharides, and it is difficult to extract such lipids (Xiao, 2010). Lipids and other cellular components are linked together by various bonds such as Van der Waal's forces, ionic bonds, hydrophobic, and hydrogen bonds (Xiao, 2010). Solubility of lipids is governed by the polarity of the solvents and the strengths of interactions between the polar or nonpolar part of the lipid molecules and solvent. The presence of hydrophilic and hydrophobic regions in the lipids determines the type of solvents to be used for extraction. For example, lipids containing low polarity functional groups, such as cholesterol esters or triacylglycerols are soluble in hydrocarbon solvents e.g. cyclohexane or hexane, or in slightly polar solvents, such as ethers or chloroform (Xiao, 2010). Such lipid are insoluble in polar solvents such as methanol, ethanol etc. Chain length of fatty acids determines the solubility of lipids in polar solvents and is

inversely proportional to the chain-length. Degree of saturation also governs the solubility, e.g. unsaturated lipids are highly soluble in most solvents compared to saturated ones. In case of complex lipids containing both polar and nonpolar moieties, the mixture of polar and nonpolar solvents is effective for extraction process (Xiao, 2010). Therefore, effective extraction of lipids from tissues will require the proper knowledge of polarity of solvents and functional groups present in the lipid molecule (Xiao, 2010).

1.2.3.1 Conventional methods

Most commonly and widely used method for extraction of lipids from shrimp processing by-products is the conventional method using non-polar solvents (Meyers and Bligh, 1981; Sachindra *et al.*, 2006; Saito and Regier, 1971). The most common method employed for lipid extraction is the Bligh and Dyer method (Xiao, 2010). This method is modified and economical adaptation of Folch procedure (Folch *et al.*, 1957) which was aimed at reducing the solvent volumes. In this method, the lipid is extracted using a mixture of polar solvent (methanol and chloroform) and water in the ratio of 2:1:0.8, v:v:v, which results in one-phase system. Post extraction, the phases are separated into chloroform and methanol/water phases by the addition of more chloroform and water, in which the lipids are localized in chloroform phase (Xiao, 2010). The advantage of Bligh and Dyer method is that it allows the extraction of all lipids including membrane lipids which contains phospholipids, polar lipids, and the complex lipids bound to other components (Sánchez-Camargo *et al.*, 2011). Lipids from waste of Brazilian redspotted shrimp extracted using Bligh and Dyer method had a yield 4.9% (dry weight basis), whereas the lipids extracted using hexane was found to be 3.3% (dry weight basis) (Sánchez-Camargo *et al.*, 2011). The possible reason for the low extraction yield by hexane could be that hexane is nonpolar in nature which cannot dissolve the phospholipids present in the majority in shrimp oil (Takeungwongtrakul *et al.*, 2012).

Types of solvent play an important role in extraction process. Solvent toxicity, polarity of solvents, handling hazard, ease of solvent removal etc. have been used as criteria for selection of solvent used (Takeungwongtrakul *et al.*, 2015). Some

organic solvents which have been permitted for use in food industries include acetone, ethanol, ethyl acetate, isopropanol, hexane and methyl ethyl ketone. The permitted residual level depends on the type of foods, in which the solvents are used. For example, solvents with high toxicity such as dichloromethane, dimethyl sulfoxide and chloroform are not permitted for use (FDA, 2010). The extraction yield of lipids and carotenoids from shrimp processing by-products varied with the type of solvent used. Owing to the higher solubility of carotenoids in n-hexane and n-heptane and their low boiling points, they are used industrially for the extraction of carotenoids from crustacean processing wastes (Sánchez-Camargo *et al.*, 2011). Shrimp oil majorly comprises phospholipids, which are polar in nature. Therefore, a polar solvent is required for effective extraction. On the other hand, carotenoids are soluble in non-polar solvents and require non-polar solvents for extraction (Sachindra *et al.*, 2006). A mixture of polar and non-polar solvents was used for effective extraction of lipid from shrimp processing by-products.

The extraction yield of lipids from hepatopancreas of Pacific white shrimp using acetone, hexane, isopropanol, a mixture of hexane:acetone, hexane:isopropanol and acetone:isopropanol in different ratios ranged from 7.46 ± 4.53 to 18.22 ± 0.50% wet basis and carotenoid content of extracted oil ranged from 1.60 ± 0.1 to 1.84 ± 0.08 mg/g lipids (Takeungwongtrakul *et al.*, 2015). In addition, the mixture of acetone improved the lipid and carotenoid yields from shrimp head and carapace (Nègre-Sadargues *et al.*, 2000; Sachindra *et al.*, 2006). Findings from the study by Sánchez-Camargo *et al.* (2011) showed highest astaxanthin extraction yield (53 ± 2 mg/kg residue) when isopropanol and hexane (40:60, v/v) mixture was used. Other important aspects concerning the use of solvents for extraction are the environmental issue and proper disposal. Use of inappropriate solvent results in consumption of large volumes of solvent and lower yield is gained. When the solvent is not recycled, it poses potential environmental pollution risks. Toxicity and inflammable nature of solvents together with the difficulty of removal of trace solvents from the final product has stimulated for development of alternative extraction processes, which are green and environment friendly (Delgado-Vargas *et al.*, 2000).

1.2.3.2 Novel methods

1.2.3.2.1 Supercritical fluid extraction

A potential alternative method to conventional solvent extraction process is the supercritical fluid extraction (SFE), that involves the use of supercritical fluids, mostly carbon dioxide under supercritical conditions, to extract bioactive compounds from solid or liquid materials (Junior *et al.*, 2010). SFE is a quick and effective extraction process, which eliminates the use of harmful organic solvents. Another major advantage is the ease of solvent separation after extraction, because CO_2 becomes gas at room temperature (Del Valle and Aguilera, 1999). Supercritical CO_2 (SC-CO_2) is ideal for use in food industry since it is an inert, low-cost, nonflammable, non-toxic solvent and generally recognized as safe (GRAS) (Reverchon and De Marco, 2006; Sahena *et al.*, 2009). CO_2 under supercritical conditions (31.1 °C and 73.8 MPa) has low viscosity and higher diffusivity, and has been used for extraction of non-polar compounds (Mao *et al.*, 2017). SC-CO_2 is particularly used to extract heat labile compounds such as carotenoids and lipids, since SFE does not require high temperatures for processing (Charest *et al.*, 2001; López *et al.*, 2004). The SFE principally consists of a temperature controlled high pressure liquid extraction vessel, a pump for delivering liquid CO_2 at a required rate, a back pressure regulator to regulate the pressure in the vessel and in-line filters to prevent any entrainment of compounds (Markom *et al.*, 2001).

Many studies have been successfully employed SFE for the extraction of lipids and carotenoids from animal and plant sources (Filho *et al.*, 2008; Mendes *et al.*, 1995; Silva *et al.*, 2008). SFE technology has been used to extract oils and lipids from flaxseed (Khattab and Zeitoun, 2013), watermelon seed (Rai *et al.*, 2015), sunflower (Rai *et al.*, 2016), and carotenoids such as lycopene from tomato seeds (Eller *et al.*, 2010) and lutein from spinach (Jaime *et al.*, 2015). To extract oil from SPBP, SFE has been proven as an effective extraction method. Sánchez-Camargo *et al.* (2011) reported that SFE showed a profound impact on the extraction yield of astaxanthin from Brazilian redspotted shrimp (*Farfantepenaeus paulensis*) waste. To increase the efficiency of SFE on extraction yield of lipids and carotenoids from

SPBP, the addition of ethanol as co-solvent to the SC-CO_2 increased the extraction yield of astaxanthin from Brazilian redspotted shrimp waste by 57.9% (Sánchez-Camargo *et al.*, 2012). Since astaxanthin is amphiphilic in nature than oil, while pure SC-CO_2 is non-polar. Therefore extraction of astaxanthin is modified by adding a food-grade polar solvent (Sánchez-Camargo *et al.*, 2012). Moreover, SFE is a preferable method for extraction of astaxanthin owing to the sensitivity of astaxanthin to light and oxygen, which can cause its degradation. Therefore, lipid extraction was enhanced by the incorporation of a polar co-solvent, since shrimp oil is rich in phospholipids (Sahena *et al.*, 2009; Tanaka *et al.*, 2004). SFE also provides a favorable environment for extraction of oils rich in heat sensitive n-3 PUFAs due to modest temperature and oxygen exclusion (Sahena *et al.*, 2009). SC-CO_2 extraction of shrimp processing by-products of Northern shrimp (*Pandalus borealis* Kreyer) resulted in the higher retention of n-3 PUFAs, especially EPA (7.8 ± 0.06%) and DHA (8.0 ± 0.07%) (Amiguet *et al.*, 2012). Krill oil extracted using supercritical CO_2 had higher astaxanthin content and high oxidative stability, compared to oil extracted by hexane (Ali-Nehari *et al.*, 2012). Astaxanthin extracted from *Penaeus monodon* waste using SFE and ethanol as co-solvent had higher yield and better recovery of carotenoids (97.1%) (Radzali *et al.*, 2014).

1.2.3.2.2 Microwave assisted extraction

Another novel method that has gained increasing interest over the past few years is the microwave assisted extraction. The basic principle governing this process is the increased pressure developed inside the cells which causes the rupture of cell walls and releases the target compounds. Microwave also results in the augmented porosity of the cell matrix and allows for increased solvent penetration for effective extraction. Microwave heating takes place by the localized vibrations of water molecules by the high frequency microwaves which allows for the matrix to be heated internally and externally without a thermal gradient being created. Therefore, the microwave energy heats the solvents and solids in contact causing rapid extraction (Oroian and Escriche, 2015; Rosello-Soto *et al.*, 2015; Roselló-Soto *et al.*, 2015; Strati and Oreopoulou, 2014). Microwave extraction has been proven to be an

effective method due to the advantages such as low energy requirement, shorter extraction times, low degradation of target compounds and high efficiency. Some of the factors that affect the microwave extraction include frequency, power and time of microwave applied, uneven tissue matrix, moisture content, temperature and pressure of extraction, type of solvents, and solid-liquid ratio (Rosello-Soto *et al*., 2015; Roselló-Soto *et al.*, 2015; Strati and Oreopoulou, 2014). Microwave assisted extraction of lipids from chicken and pork meat resulted in reduction of extraction time by 75%. Other advantages of lipid microwave assisted extraction were the reduction of manual manipulation, leading to less labor costs and reduced consumption of solvents (Medina *et al*., 2015). Recently, de Moura *et al.* (2018) reported that lipids extracted using microwave assisted process from wet microalgae paste resulted in significantly higher yield and in addition to that, the microwave had no degrading effect on the extracted fatty acids.

1.2.3.2.3 Ultrasonic assisted extraction

In order to conquer the drawbacks associated with conventional extraction, particularly a huge amount of solvents used and lower extraction yield, ultrasonic assisted extraction (UAE) process has been employed to extract lipids and bioactive compounds from plant and animal sources (Mason *et al.*, 2014). UAE is a physical extraction process in which ultrasound waves are used to rupture the cells of the material containing the target compounds for faster and more efficient extraction (Mason *et al.*, 1996). UAE has been implemented in food industry and has gained popularity due to many advantages e.g. augmented extraction yield, lower solvent consumption, faster extraction, better reproducibility, ease to scale-up, and higher purity of final products (Vinatoru *et al.*, 1999). Ultrasound waves are longitudinal waves having frequency higher than 20 kHz, which can carry high energy while traveling through a medium (Mason *et al.*, 2005). During the propagating through a medium, ultrasound waves compress and stretch alternately, building a localized negative pressure. While the stretching phase or "rarefaction" occurs, the pressure is sufficient to overcome intermolecular binding forces, resulting in the creation of tiny cavities or bubbles in the medium. This phenomenon of creating microbubbles by

ultrasound waves is termed as "cavitation" (Povey and Mason, 1998). With each successive cycle, these microbubbles grow bigger and finally collapse violently, thus releasing huge amount of energy. When the bubbles collapse, the localized pressure can reach up to 400 MPa and is able to disrupt cell membranes in the immediate vicinity and causes the liberation of target compounds (Flint and Suslick, 1991). A schematic representation of cavitation phenomenon has been shown in Figure 3.

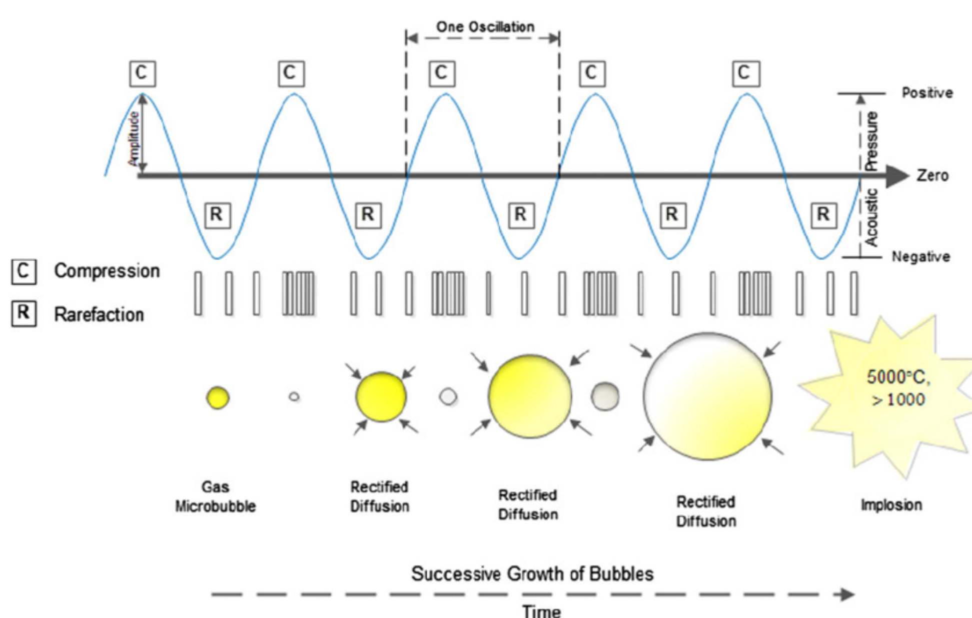


Figure 3. Schematic depiction of growth and collapse of bubble in acoustic cavitation process

Source: Abbas *et al.* (2013)

Ultrasound waves are known to enhance mass transfer in many unit operations related to food industry, particularly in mixing, homogenization and extraction (Fairbanks, 1984; Mason *et al.*, 1996). In case of extraction process involving the solid/liquid extraction by the use of solvents, ultrasound waves enhance the mass transfer by virtue of cavitation phenomena. The implosion of bubbles resulting in the generation of high localized temperature and pressure causes the rupture of cell membranes, releasing the cell components and ultimately the dissolution of target molecules in the solvent (Knorr *et al.*, 2002). Numerous studies have demonstrated that the ultrasound-assisted extraction (UAE) can enhance the

extraction efficiency of target compounds by the cavitation and mechanical effects of ultrasound waves (Chemat *et al.*, 2004; Li *et al.*, 2004; Rostagno *et al.*, 2003). Sivakumar *et al*. (2007) showed the increase in yield of tannin from myrobalan nuts. Lou *et al*. (2010) reported that reported the increase in extraction yield of peanut oil with increasing ultrasonic frequencies. Yao (2012) documented that use of ultrasound at ultrasonic power of 400W for 37.78 min at solid-liquid ratio of 1:5.94 g/ml, resulted in 40.41% of oil extracted from camellia. The extracted oil had superior quality with enhanced oxidative stability and light color, while the fatty acid composition was slightly changed. Zhang (2010) reported that the ultrasound treatment at 120W for 48 min and solid-liquid ratio of 1:7 g/ml enhanced the extraction yield of walnut oil to 94.95%, but increased acid value and peroxide value. Bimakr *et al.* (2012) reported that UAE was quick procedure, resulting in higher extraction yield than conventional extraction methods. In general, ultrasound power causes extra vibration in the sample molecules, improves cavitation and contact surface between sample matrix and liquid solvent phase, thereby enhancing the recovery in the short extraction time (Pan *et al.*, 2012).

The extraction yield of lipids from the lipid-containing solid residue (LSR) obtained from the protein hydrolysis of cephalothorax from Pacific white shrimp was increased from 7.2 ± 0.3 to 12 ± 0.5% dry basis and carotenoid content increased from 5.7 ± 0.2 to 8.6 ± 0.4 mg/g lipid when UAE was used at 80% amplitude for 10 min with 30 s on-and-off pulse mode (Sinthusamran *et al.*, 2018). The extraction yield of pomegranate seed oil (PSO) was found to be higher (25.11% w/w) when UAE was applied compared to the extraction yield by using supercritical fluid extraction (SFE; 15.72%) and soxhlet extraction process (SE; 20.50%). There was a significant difference in the fatty acid compositions among the PSO extracted by SFE, soxhlet extraction, and UAE, in which punicic acid (>65%) was the most dominant when UAE was implemented (Tian *et al.*, 2013). UAE method gave the higher extraction yield of phenolic compounds from olives, requiring less solvent, shorter time and lower temperature than maceration extraction method. Furthermore, a total of 14 phenolic compounds were found in both extracts and UAE offered higher yield than that of ME (Deng *et al.*, 2017). Overall it was found that ultrasonic assisted extraction

of lipids and bioactive compounds resulted in higher extraction yield, shorter processing times and less solvent consumption, compared to the conventional extraction processes. Although UAE has been proven to enhance the extraction yield of lipids and carotenoids, it was shown to induce deteriorative processes including oxidation and hydrolysis of the extracted lipid (Zhang *et al*., 2017). UAE augmented lipid oxidation as shown by the increased peroxide value (PV), TBARS and ρ-anisidine values of extracted lipids (Chemat *et al*., 2004; Li, Pordesimo, and Weiss, 2004; Zhang *et al.*, 2017). Moreover, the localized high temperature and pressure promote the formation of singlet oxygen (Davidson *et al.*, 1987). Furthermore, the sonolysis of water by virtue of ultrasound waves is found to produce numerous free radicals (Riesz and Kondo, 1992). Those radicals, in addition to the trace metal ions such as Cu^{2+} and Fe^{2+} present in the system, trigger the lipid oxidation process (Suslick *et al.*, 1986). Oxidation induced by lipoxygenase toward unsaturated fatty acids could also be one of the possible mechanisms (Gibian and Galaway, 1976).

1.2.4 Lipid hydrolysis and oxidation

Lipid hydrolysis is a critical process that takes place in lipids in crustaceans, in which the breakdown of glycerol–fatty acid esters occurs resulting in the release of free fatty acids (Van Weel, 1970). This process is catalyzed by specific group of enzymes called as lipases and phospholipases (Pacheco-Aguilar *et al.*, 2000). The major source of these enzymes in shrimps is the hepatopancreas, which causes the hydrolysis of the ester bond of triglycerides (Phillips *et al.*, 1984). Shrimp cephalothorax and hepatopancreas have lipase and phospholipase in active form, which facilitate the hydrolysis of the lipids causing the release of free fatty acids (Takeungwongtrakul *et al.,* 2012). Lipase activity is affected by many factors such as temperature, surfactants, pH, chemical additives, solvents and even the shear force on the lipids in aqueous media (Khor *et al.*, 1986; Kosugi *et al.*, 1988; Tsai and Chang, 1993). Hydrolysis of lipids by phospholipase was taken place in silver carp muscle and heating of fish muscle caused the inactivation of phospholipases (Kaneniwa *et al.*, 2000). Lipid from Pacific white shrimp was prone to hydrolysis catalyzed by lipase or phospholipase resulting in the increase in free fatty acid content (Sriket *et al.*, 2007).

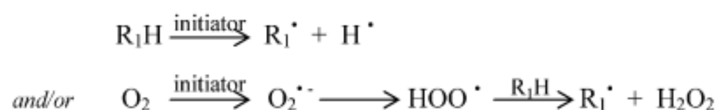
The release of free fatty acids results in more serious problem of lipid oxidation, as these free fatty acids, particularly the unsaturated ones, are more susceptible to oxidation (Sherwin, 1978).

One of the major cause for the deterioration of foods containing lipids, which affects the overall quality of the foods, is the lipid oxidation process. Oxidation of foods rich in PUFAs is a serious problem that affects the shelf-life, overall consumer acceptability, nutritional value, functionality and safety. The oxidation of PUFAs negatively affects the nutritional value of foods (Aladedunye and Przybylski, 2009). Various factors affecting the lipid oxidation in foods include composition of lipids, oxygen levels, presence of prooxidants and antioxidants, environmental factors such as light, temperature and processing conditions (Sikorski and Kolakowska, 2010). Presence of hemeproteins and active metals (Cu, Fe) together with exposure to high levels of oxygen particularly at high temperatures promote accelerated lipid oxidation (Shahidi and Zhong, 2010). Lipid oxidation products, particularly the aldehydes, are known to be carcinogenic and have been shown to induce mutational changes and ageing in humans (Kampa *et al.*, 2007). Nair *et al.* (1986) showed that the toxicity of these aldehydes including malondialdehyde (MDA) and 4-hydroxy-2-nonenal is attributed to their protein cross-linking property which covalently binds the nucleic acids. Foods containing PUFAs are more prone to oxidation. Lipid oxidation results in formation of aromatic hydrocarbons, especially aldehydes and ketones, which cause objectionable off-flavors and odors in foods (Let *et al.*, 2007). PUFAs have low stability towards oxidation, therefore antioxidants are needed to prevent oxidation and stabilize such foods (Sørensen *et al.*, 2010).

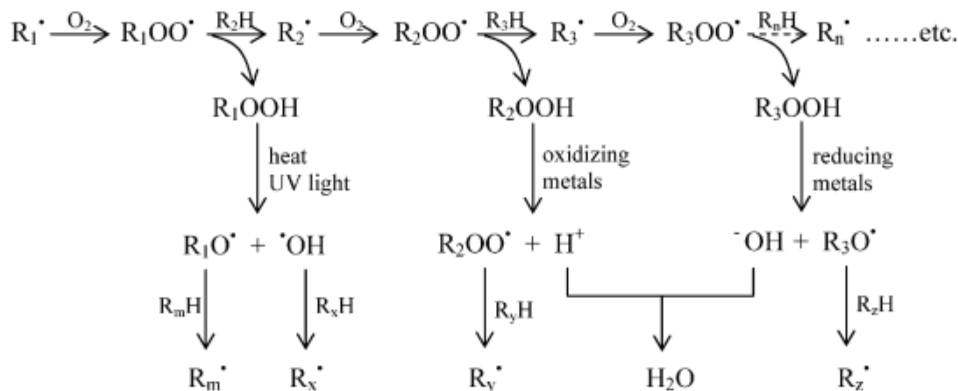
Lipid oxidation process involves the reaction of singlet oxygen with the unsaturated fatty acids and the reaction takes place in three phases: initiation, propagation and termination phase (Figure 4). The free radicals present in the system react with the lipids, creating more unstable products which further react to form other reactive chemical products. Thus, the lipid oxidation continues as a chain reaction, which allows for further oxidation of lipid molecules. This chain reaction is termed as “autoxidation” (Kolanowski *et al.*, 2007). Autoxidation is the reaction of

singlet oxygen or free radicals with lipid molecules taking place under mild conditions. The property of oxygen to behave as electron donor results in the loss of electrons by sequential overlapping reactions (Frankel *et al.*, 2002). The primary products of lipid oxidation are hydroperoxides, which are highly unstable in nature and decompose readily to secondary oxidation products by a series of polymerization reactions. During the decomposition of hydroperoxides, there is a complex mixture of intermediate and secondary oxidation products in the system which includes aldehydes and ketones (Nawar, 1996). Many volatile and non-volatile compounds with different polarity and molecular weights are formed (Dobarganes and Marquez-Ruiz, 2003).

Initiation:



Propagation:



Termination:

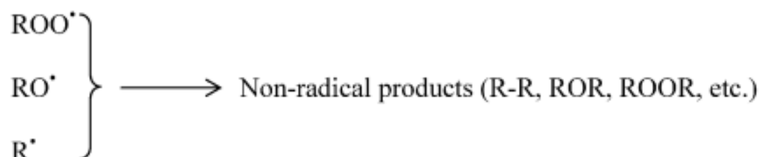


Figure 4. Lipid autoxidation pathways

Source: Shahidi and Zhong (2010)

1.2.4.1 Lipid oxidation in different food systems

Lipid oxidation is a complex process and in one food system it can be more complex than other since it involves the process of electron transfer. Lipid oxidation in emulsions can be a more complex process than in bulk systems (Jacobsen *et al.*, 2008). Even though the basic mechanism governing lipid oxidation can be similar in different food systems, the intrinsic factors like pH, temperature or presence of prooxidants can affect the over lipid oxidation process significantly (Jacobsen *et al.*, 2008). Moreover, the composition of food systems, and moisture content in particular can significantly affect the rate of oxidation reaction. Degree of saturation of lipids is another important factor governing the lipid oxidation process.

1.2.4.2 Impact of lipid composition on oxidation

Composition of foods, particularly the ones containing unsaturated fatty acids are susceptible to oxidation depending on their degree of unsaturation which is represented by the methyl bridge index (MBI, the mean number of methylene positions) (DeMan, 1999). Higher the unsaturation, more intense is the oxidation. Oxidation is more augmented in free fatty acids (FFAs) in bulk food systems than their esterified counterparts. In case of emulsions, linolenic acid showed higher susceptibility to oxidation, followed by linoleic and oleic acids, which clearly indicated that degree of unsaturation directly affected the oxidative capacity (Yi *et al.*, 2013). During the storage, concentration of FFAs in crude soybean oil was increased (Alencar *et al*., 2010). Numerous factors affecting the FFA content in oils include moisture content, initial FFA content, storage temperature, etc. Triacyl glycerides (TAG) containing FFAs can further catalyze the hydrolysis, resulting in the increased FFA content in oil (Chen *et al.*, 2011). FFAs were found to reduce the surface tension of bulk soybean oil and augmented the rate of oxygen diffusion from headspace, resulting in increased lipid oxidation (Mistry and Min, 1987). Furthermore, the FFAs have a capability of concentrating at the surface of the emulsion droplet, thereby attracting the prooxidants such as transition metals, which are known to augment lipid oxidation process (Waraho *et al.*, 2009). In another study by Waraho *et al.* (2011), it was shown that FFAs were able to augment lipid oxidation by evoking the transition

metals and co-oxidizing the TAGs. Higher polarity of FFAs due to the presence of unesterified carboxylic group compared to TAG, makes them more surface active. This ability of FFAs makes them concentrate and diffuse more rapidly at the interface of o/w emulsions (Waraho *et al.,* 2009). Therefore, when pH values of FFAs are above their pKa values (4.8-5.0 for medium and long-chain fatty acids), it can cause the emulsion droplet to be more negatively charged (Lieckfeldt *et al.*, 1995). When oleic acid (0-5.0% of lipid) were added to the emulsions, the hydroperoxides and hexanal concentration were increased (Waraho *et al.*, 2009). As the concentration of oleic acid in emulsion was increased, there was concomitant increase in negative charge of the droplets.

1.2.4.3 Effect of environmental factors on oxidation

Another major factor affecting the lipid oxidation is the exposure of lipids to the environmental factors during storage or processing. During the extraction process of oil and lipids from their plant or animal sources, many unit operations are carried out which may expose the oils to high temperature, oxygen, light, contact with metallic surfaces, moisture etc. (Shahidi and Zhong, 2010). Environmental factors such as heat and light (UV) increase the rate of lipid oxidation (Kolanowski *et al.*, 2007). Transition metal ions (Cu and Fe) present in the food systems, are known to catalyze the lipid oxidation process by acting as prooxidants. Although copper is present in lower quantities in foods, it has not received much interest compared to iron, but it was reported to be much more effective in accelerating the hydroperoxides (Yoshida and Niki, 1992). Oxidation state of these metal ions also plays a major role in oxidation. It was shown that cuprous and ferrous ion were more effective in promoting oxidation that cupric and ferric ion (Wang and Wang, 2008). These transition metal ions are more potent in their activity in the aqueous phase compared to the emulsions (Sun *et al.*, 2011). The mode of action of these transition metals is that they can directly breakdown unsaturated lipids into alkyl radicals, although the rate of reaction is very slow. In emulsion systems, lipid oxidation is catalyzed by the transition metals by decomposition of hydroperoxides (ROOH) into highly reactive and highly unstable peroxyl (ROO) and alkoxyl radicals (RO).

1.2.4.4 The use of antioxidants

One of the most widely used method for preventing or controlling the lipid oxidation process involves the use of antioxidants. It is the most economical, effective and convenient method used by many food manufacturers. Antioxidants are particularly used in highly sensitive and oxidation prone foods to prevent the body from damage caused by deteriorative oxidation products. Antioxidants are the substances which delay or prevent the oxidation, while being present in low concentrations than the substrate being oxidized (Shahidi and Zhong, 2010). Antioxidants are known to extend shelf-life of food products. Antioxidants are used in a wide variety of foods including high and low fat foods, seafoods, cereals, meat products etc. Free radical scavenging antioxidants are the most widely used group of antioxidants. These antioxidants are also referred to as chain-breaking antioxidants, which are known to neutralize metal chelators and free radicals formed during lipid oxidation especially in the initiation and propagation phases (Shahidi and Zhong, 2011). The process of lipid peroxidation results in the change in nutritive and sensory properties of foods (Rajapakse *et al.*, 2005). Hydroperoxide decomposition by any prooxidant such as metals, heat or light, which produces peroxyl and alkoxyl radicals, is the major step in the propagation of lipid peroxidation reaction (Headlam and Davies, 2003; Niki, 1987). The use of antioxidants can cause the chain reaction to break by sacrificing themselves forming unreactive and more stable species. This causes the lipid peroxidation to cease and terminate. Lipid oxidation has been proven to induce diseases specific to ageing (Bing *et al.*, 2008).

1.2.4.4.1 Classification of food antioxidants

The mode of action of antioxidants is that it either protects the target lipids from free radicals which initiate the oxidation or by impeding the propagation of lipid oxidation (Sun *et al.*, 2011). Broadly, antioxidants are classified based on their mode of action into primary antioxidants which are also known as chain breaking antioxidants and secondary antioxidants known as preventive antioxidants (Sun *et al.*, 2011).

1) Primary antioxidants

Also known as chain breaking antioxidants, primary antioxidants which includes a majority of phenolic compounds work by neutralizing the free radicals present in the system by donating an electron. Therefore, they accept free radicals so that the initiation or propagation step of oxidation is inhibited. They convert the highly reactive and unstable peroxy radicals into more stable and unreactive species (Gil, 2011). The effectiveness of primary antioxidants is higher when added in the initiation phase of the oxidation or when the oxidation has not entered into more complex phases. Phenolic compounds including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) are some of the common synthetic antioxidants used in foods. However, at high concentrations, some phenolic antioxidants become prooxidants by losing their activity (Sun *et al.*, 2011). Some of the natural antioxidants that have been exploited over the years to be added in foods include some flavonoids, carotenoids, and tocopherols (Sun *et al.*, 2011). Among the natural antioxidants, carotenoids are more commonly used, due to their singlet oxygen quenching property and neutralizing the peroxy or alkyl-radical intermediates (Matsushita *et al.*, 2000).

2) Secondary antioxidants

There are several mechanisms have been shown to prevent oxidation by the use of secondary antioxidants. They suppress oxidation by inhibiting prooxidants such as metal ions, prooxidative enzymes, singlet oxygen and other oxidants (Leopoldini *et al.*, 2006; Min and Boff, 2002; Sud'Ina *et al.*, 1993). Redox reactions suppress oxidation by using reducing agents. Some of the possible mechanisms by which secondary antioxidants work include the conversion of free radicals to unreactive and more stable products, which in turn slows the rate of reaction. Prevention of oxidation by deactivation of singlet oxygen, metal chelation, ROS detoxification by enzymes, prooxidant enzyme inactivation, UV filtration, antioxidant cofactors of enzymes etc. Hydroperoxides are converted into stable and unreactive products by the use of thioethers in a non-radical pathway (Pokorný,

2007). Secondary antioxidants have been shown to work synergistically with primary antioxidants and enhancing their activity. They can restore the hydrogen to primary antioxidants, act as oxygen scavengers and reducing agents, thus decomposing the hydroperoxides to stable and unreactive species (Sun *et al.*, 2011). Ascorbic acid, citric acid, ascorbyl palmitate, tartaric acid, and lecithin are some of the commonly used secondary antioxidants (Sun *et al.*, 2011).

1.2.4.5 Tannic acid

Tannic acid is a naturally occurring antioxidant comprising of a glucose molecule as a central backbone with hydroxyl groups and one or more galloyl residues, resulting in a molecule of tannic with hydrophilic shell and hydrophobic core (Figure 5) (Gülçin *et al.*, 2010). The antioxidant nature of tannic acid is the resultant of the polyphenolic nature attributed to its structure (Isenburg *et al.* , 2006)

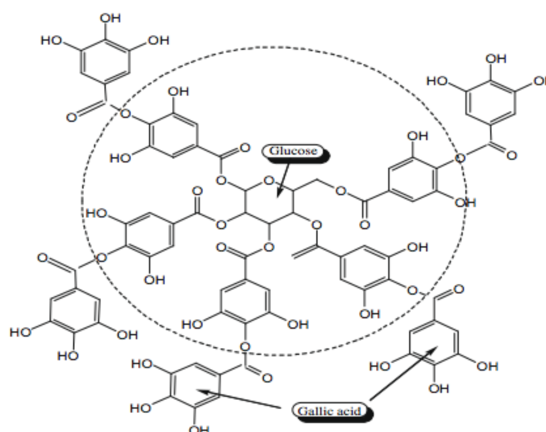


Figure 5. Tannic acid
Source: Gülçin *et al.* (2010)

Tannic acid, also referred as tannins are water-soluble polyphenols which are derived from fruits and tree barks of many plants (Lopes *et al.*, 1999). Numerous studies have demonstrated the free radical scavenging activity of tannic acid. Studies conducted by Maqsood and Benjakul (2010a; 2010b), it was reported that lipid oxidation in fish o/w emulsion, fish mince and fish slices was effectively inhibited by using tannic acid as an antioxidant. Lipid peroxidation of linoleic acid emulsion was suppressed by using tannic acid at a concentration of 15 µg/mL by 97.7% (Gülçin *et*

al., 2010). Food and Drug Administration (FDA) have listed tannic acid as Generally Recognized As Safe (GRAS) compound to be used in foods at a level of 10–400 ppm (Chung *et al.*, 1998). Apart from the antioxidative activity of tannins, it is also an enzyme inhibitor. Tannic acid is reported to inhibit lipase (Horigome *et al.*, 1988; Longstaff ad McNab, 1991). Digestive enzymes were found to be inhibited by the use of tannins extracted berries and plant sources (McDougall *et al.*, 2009). Lipase from porcine pancreas was inhibited by the use of tannic acid (Tamir and Alumot, 1969). In an *in vitro* study on the inhibitory activity of tannins extracted from berries, it was found that tannic acid was potent towards inhibition of porcine pancreatic lipase (McDougall *et al.*, 2009). As a consequence, tannic acid could help retard the hydrolysis of lipids mediated by lipases.

1.2.5 Pulsed electric field

1.2.5.1 Principle

Pulsed electric field (PEF), a non-thermal technology, has been used extensively in food industry for extraction of active compounds. In PEF extraction, a material is placed between two electrodes and high-voltage pulses are passed through it for a very short time usually between microseconds to milliseconds (Redondo *et al.*, 2018). Generally, an ionic gradient or transmembrane potential (TMP) exists across the cell membrane owing to the presence of charged ions and proteins in the cell. When high-voltage pulses for short durations are passed across the cell, polarity is induced in the cells, producing a dipole moment parallel to the applied electric field (Zbinden *et al.*, 2013), which is very strong and causes irreversible pore formation in the cell membrane, or electroporation of the cell membranes (Teissie *et al.*, 2005). Permeability of cell membranes is increased by virtue of electroporation, which leads to the leakage of cell constituents and permeation of solvents into the cell (Antonietta and Matteo, 2016; Vorobiev and Lebovka, 2010). PEF causes negligible rise in the temperature of the samples and cost of operation is also low (Toepfl *et al*., 2006). PEF unit has three major components, a high voltage pulse generator, which is equipped with high capacity capacitors to generate high voltage pulses, a treatment chamber

which consists of the sample to be treated and two electrodes and an oscilloscope to monitor the wave form of applied pulses.

1.2.5.2 The electroporation phenomenon

The process in which cell membranes develop pores by the application of high intensity electric field of sufficient strength is referred to as electroporation or electropermeabilization. When high electric field strength is applied across the cell membrane, an induced potential is created. The induced transmembrane potential (defined as the difference between intra and extra cellular potentials) is unevenly distributed over the surface of cell membrane. The potential difference is high on the membrane sites close to the electrodes and the low as the distance between the membrane sites and the electrodes increases. When the electrocompressive force exceeds the membrane elastic strength, the membrane breakdown or pore formation occurs. The pore formation in the membranes can be either reversible or irreversible depending on the intensity of the applied electric field intensity and the time of operation. With higher field strengths, membranes are broken down over a large part and if the size and number of pores become higher relative to the membrane surface, an irreversible breakdown occurs (Zimmermann *et al.*, 1976). Three possible outcomes during electroporation have been reported. In the first case, when the applied electric field along with the exposure time is insufficient, it will cause to effect on cell membranes. Therefore, no electroporation takes place. Second case is if the applied electric field (E) exceeds the reversible threshold (Ec), which is the minimum electric field strength to cause permeation, for a sufficient time, reversible electroporation takes place (Čorović *et al.*, 2012; Phoon *et al.*, 2008; Rols and Teissie, 1990). In this case, the cell membranes remain permeabilized for a short duration and eventually regain its original form by restoring the cell membrane integrity. The third case of electroporation is the permanent permeation of cell membrane which occurs when the applied electric field is too high causing the cell to lose the homeostasis, eventually killing it by the complete breakdown of plasma membrane (Al-Sakere *et al.*, 2007; Davalos *et al.*, 2005) (Figure 6).

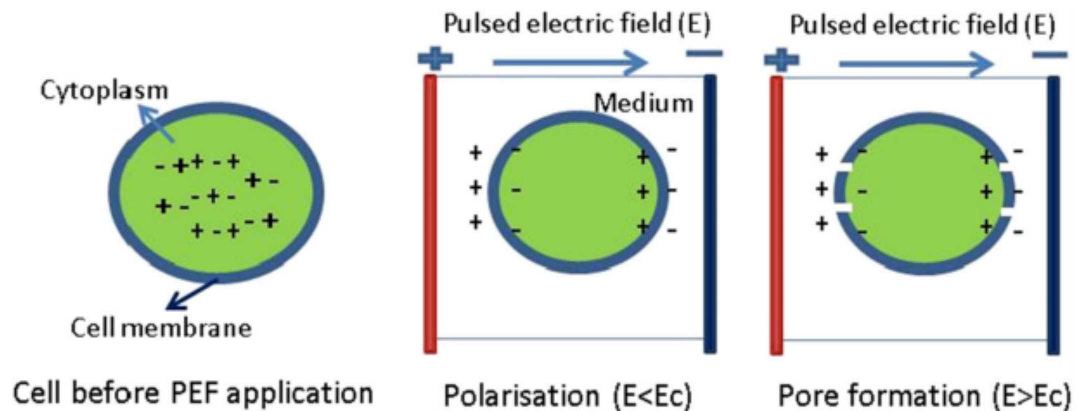


Figure 6. Schematic representation of electroporation mechanism in biological cell membrane exposed to an electric field E.

Source: Kumari *et al.* (2018)

1.2.5.3 Application of PEF

PEF technology can replace conventional extraction processes in improving extraction yields of bioactive compounds, either used individually or in combination with other extraction techniques as intensification pre-treatment (Donsì *et al.*, 2010). The use of PEF in food processing has demonstrated promising results in the extraction process of bioactive compounds from plant and animal sources, particularly microalgae, in the extraction of juices and exudates (Donsì *et al.*, 2010; Fincan *et al.*, 2004; Puértolas, *et al.*, 2012; Vanthoor-Koopmans *et al.*, 2013; Vorobiev and Lebovka, 2009), dehydration of tissues (Ade-Omowaye *et al.*, 2001; Jaeger *et al.*, 2012; Lebovka *et al.*, 2007; Shynkaryk *et al.*, 2008) microbial inactivation by cold sterilization and preservation (Bermúdez-Aguirre *et al.*, 2012; Jayaram, 2000; Marsellés-Fontanet *et al.*, 2012; Mosqueda-Melgar *et al.*, 2012; Qin *et al.*, 1996; Węsierska and Trziszka, 2007; Yeom *et al.*, 2002). PEF has been used for intensification process prior to extraction of oil from maize, olives, soybeans and rapeseed. There was an increase in the extraction yield of oil from maize by 30% and yield of oil from rapeseed was increased by 55%, when PEF was applied (Guderjan *et al.*, 2005; Guderjan *et al.*, 2007). PEF pre-treatment signified oil extraction yield and augmented the amount of bioactive compounds from sunflower seeds extracted using hexane (Shorstkii *et al.*, 2015). PEF pre-treatment of sesame seeds before solvent

extraction resulted in the increase of extraction yield of sesame oil by 4.9% (Sarkis *et al.*, 2015). Application of PEF had a positive effect on the concentrations of polyphenols, tocopherols, phytosterols, and antioxidants in oils (Donsì *et al.*, 2010; Puértolas *et al.*, 2012). When PEF was applied, the oil extraction yield was significantly increased along with the concentration of bioactive compounds. PEF is also known for inactivating enzymes in food systems. The activities of lipase, α-amylase, and glucose oxidase were reduced by 70% - 85% after application of PEF treatment (Ho *et al.*, 1997). The activity of heat-stable papain was reduced by the application of PEF at 50 $kVcm^{-1}$ (Yeom *et al.*, 1999). 97% reduction in the activity of polyphenol oxidase (PPO) from apple extract was observed when PEF was applied for 6000 μs at 24.6 kV/cm and 72% reduction PPO was found when PEF was applied for 6000 μs at 22.3 kV/cm (Giner *et al.*, 2001).

1.2.6 Nanoliposomes

1.2.6.1 Structure

Liposomes are spherical vesicles consisting of a phospholipid bilayer and an aqueous core with size ranging from micrometer to nanometers. Nanometric version of liposomes are referred as nanoliposomes. Nanoliposomal encapsulation is one of the promising encapsulating method for bioactive compounds, widely used in food and nutraceutical industry (Munin and Edwards-Lévy, 2011). Nanoliposomes are widely used encapsulation systems that are capable of controlled release of target molecules (Mozafari and Mortazavi, 2005). Depending upon the size, liposomes can be single-layered (unilamellar) or multiple layered (multilamellar). Encapsulation in liposomes has been widely used to incorporate water-soluble or lipid-soluble components for targeted delivery (Dias *et al.*, 2017). In biological systems, liposomes resemble the cell membranes and are considered as ideal cell models and biomembranes. Liposomes have been used to study the evolution, functioning and emergence of primitive cell membranes (Nomura *et al.*, 2001; Pozzi *et al.*, 1996). Moreover, liposomes are extensively used in agricultural, food, pharmaceutical, and cosmetic industries as carriers for targeted delivery and protection of nutraceuticals, drugs, genetic materials, and pesticides (Mozafari, 2010). The phospholipid bilayer of

liposomes are like concentric layers which contains active compounds inside (Figure 7). The layers can be single or multiple, depending on the method of preparation, and can house lipophilic, hydrophilic and amphiphilic compounds in their lipid or aqueous phases.

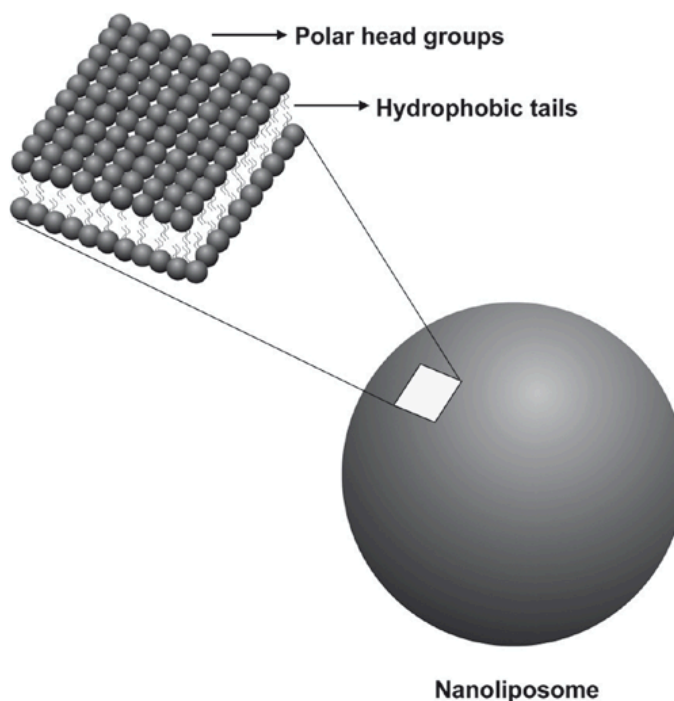


Figure 7. Enlarged section of a nanoliposome bilayer revealing its hydrophilic head groups and hydrophobic parts.
Source: Mozafari (2010)

1.2.6.2 Physicochemical properties

Generally, liposomes and nanoliposomes exhibit same structural, thermodynamic and chemical properties. However, nanoliposomes have larger surface area compared to liposomes which increases the solubility, improves the targeted release of bioactive compounds and thereby enhancing the bioavailability (Mozafari, 2006). Principally, both liposomes and nanoliposomes contain phospholipids as chemical ingredients. Upon ingestion, lipids are acted upon by gastrointestinal lipases to convert them to their constituent molecules, viz fatty acids

and head groups. Triacyl glycerol (TAG) is composed of a glycerol backbone and three fatty acids, whereas monoacyl glycerol (MAG) and diacyl glycerol (DAG) consist of one and two fatty acids, respectively. Phospholipids are polar amphiphilic molecules consisting of a negatively charged polar head, which is hydrophilic and a lipophilic tail (Bowtle, 2000). Nanoliposomes can also contain sterols in their structure, which can greatly affect the properties of the nanoliposomes. Cholesterol is mainly used in the preparation of nanoliposomes (Mozafari, 2010). Naturally, cholesterol does not occur in the liposomal bilayer. However, it is added to modify the properties of bilayer and provide fluidity to the bilayer by preventing the crystallization of acyl chain and providing steric hinderance to the movement. Cholesterol can be added to phospholipid in high molar ratios of up to 1:1 or 2:1 e.g. phosphatidylcholine (PC) (New, 1990). Numerous factors govern the physicochemical properties of nanoliposomes such as temperature, ionic strength and pH (Mozafari, 2010). Nanoliposomes offer low permeability of active material under normal conditions. However, the permeability of the bilayer can be altered by elevated temperature. Nanoliposomes can undergo phase transition at lower temperatures compared to their melting points. Temperature at which the crystallinity of lipid bilayer is lost and there is rise in the fluidity is refered to as phase transition temperature (Tc) (Szoka and Papahadjopoulos, 1980).

1.2.6.3 Methods of preparation

1) Sonication technique

Sonication is a simple method for reducing the size of liposomes and preparation of nanoliposomes (Jesorka and Orwar, 2008; Woodbury *et al.*, 2006). The sonication method involves subjecting hydrated liposomal vesicles to ultrasonication using a probe type ultrasound unit under controlled temperature. The ultrasound waves causes the reduction in the size of liposome vesicles by the cavitation phenomenon which creates a localized pressure by the rupture of microbubbles (Richardson *et al.*, 2007). Size of nanoliposomes was dependent on the sonication power, with higher sonication resulting in smaller nanoliposomes (Richardson *et al.*, 2007). Yamaguchi *et al.* (2009) reported that reduction in the size of liposomes was

the function of ultrasound frequency. At lower frequency, ultrasound waved had higher amplitude and consequently resulted in smaller liposomes.

2) Extrusion Method

Extrusion method of nanoliposome preparation involves the use of filters of smaller pore sizes and forcing the liposomal dispersion through it. Multilamellar vesicular liposomes are modified by this process to form unilamellar vesicles (Berger *et al.*, 2001; MacDonald *et al.*, 1991; Hope *et al.*, 1985). Extrusion of liposomal vesicles through track-etched polycarbonate filters by applying pressure reduces the size of vesicles depending on the pore size of filters, number of extrusion cycles and pressure applied (Frisken *et al.*, 2000; Hunter and Frisken, 1998; Patty and Frisken, 2003).

3) Microfluidization

This method involves the preparation of nanoliposomes without the use of solvents which can be toxic in nature (Jafari *et al.*, 2006; Thompson *et al.,* 2007; Vemuri *et al.*, 1990). A schematic representation of microfluidization technique is shown in Figure 8. Similar apparatus has been used for many years in pharmaceutical industries to prepare liposomal dispersions (Vemuri *et al.*, 1990) and emulsions (Silvestri *et al.*, 1992). Microfluidizers have been used in dairy industries as homogenizers to reduce the size of milk fat globules and prepare flavor emulsions (Jafari *et al.*, 2006). The basic principle governing the microfluidization method is that the pressure is divided through small orifices into two parts, each directed towards each other inside a chamber (Geciova *et al.,* 2002; Jafari *et al.*, 2006). Within the microfluidizer chamber, shear and impact forces, along with cavitation, causes the size-reduction of liposomal vesicles. The pressure in the microfluidizer can go up to 10,000 psi to guide the flow of dispersions through orifices into the impingement area (Kim and Baianu, 1991; Sorgi and Huang, 1996). Microfluidization is advantageous in terms of handling large volumes of liposomes, controlling the average size of vesicles, high efficiencies (>75%), avoiding the use of solvents, and the continuity and reproducibility of operation.

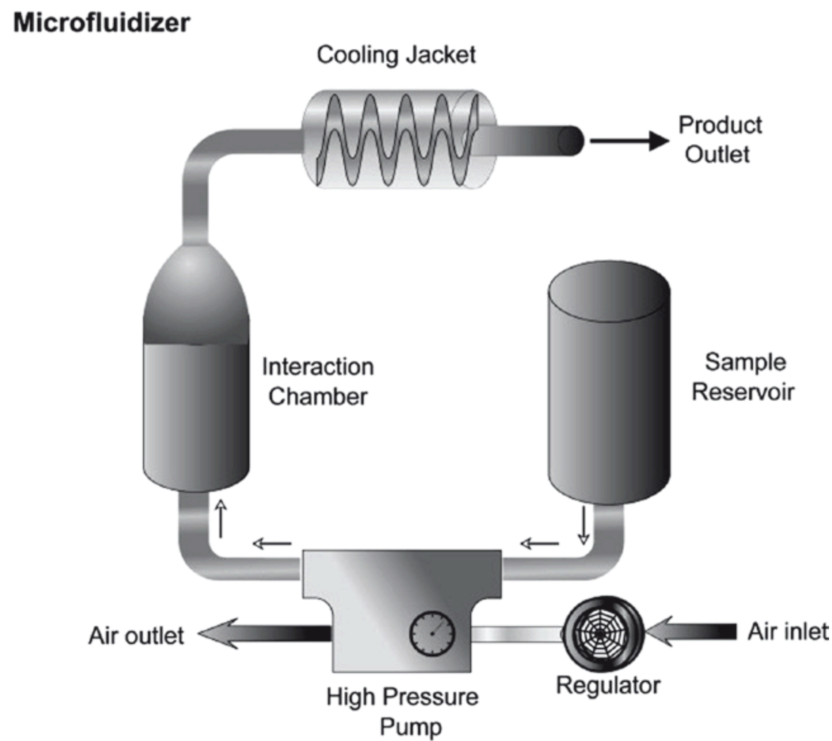


Figure 8. Schematic of a microfluidizer technique
Source: Mozafari (2010)

1.2.7 Nanoencapsulation

The process of packing miniature substances inside nanosized bioactive packing materials is referred as nanoencapsulation (Lopez-Rubio *et al.*, 2006). The major advantage of nanoencapsulation is the controlled release of the their contents (Jimenez *et al.*, 2004). Numerous studies have reported the use of nanoencapsulation in food and pharmaceutical industries for delivery of bioactive compounds (Farokhzad and Langer, 2009; Liu *et al.*, 2008; Müller *et al.*, 2007; Sagalowicz, *et al.*, 2006; Shah *et al.*, 2007; Shimoni, 2009). Nanocarriers have the ability to protect the bioactive component from the detrimental effects of processing conditions and environmental factors such as pH, oxidation and enzymatic degradation (Fang and Bhandari, 2010; Ghosh *et al.*, 2009; Zimet and Livney, 2009). Another major advantage of nanocarriers compared to the micro-size carriers is the increased surface area, improved bioavailability, enhanced solubility, and controlled release of target encapsulated ingredient (Mozafari, 2006; Weiss *et al.*, 2009).

Generally, nanocarriers are identified to have a size of 100 nm or less. Several natural and synthetic polymers have been used as w all materials for the preparation of nanoparticles.

1.2.7.1 Nanoencapsulation in foods

There is a great potential in the nanoencapsulation to conquer the challenges faced by the food industry regarding the efficient delivery of functional bioactive compounds, flavors and other valuable food components. The use of lipid-based liposome system has proven to be effective in catering the requirements by improving the bioavailability, preventing interventions with other foods, enhanced *in vitro* and *in vivo* stability (Mozafari *et al.*, 2008). Liposomes have been used to fortify a wide range of compounds including n-3 fatty acids, vitamin-D, fish oil etc. (Ghorbanzade *et al.*, 2017; Rasti *et al.*, 2017; Walia *et al.*, 2017). Liposomal encapsulation provides better oxidative stability, higher encapsulation efficiency and targeted delivery of encapsulated materials. The phospholipid bilayer protects the active material from the contact with prooxidants and eliminates the exposure to O_2, thus reducing the oxidation. The presence of n-3 fatty acids, particularly the PUFAs in fish oils makes them susceptible to oxidation, resulting in the development of fishy odor. Fortification of fish oil therefore becomes a challenging task. However, recent studies have shown that nano-encapsulated fish oil resulted in enhanced oxidative stability compared to unencapsulated fish oil (Ojagh and Hasani, 2018). Fish oil was released in a controlled manner during *in vitro* digestion of fish oil liposomes (Wang *et al.*, 2015). This could ensure the bioavailability of core oil.

Nano-liposome encapsulation of fish oil in yogurt resulted in a significant reduction in acidity, syneresis and peroxide value. Nano-encapsulation of fish oil and subsequent fortification in yogurt resulted in increased DHA and EPA contents and better sensory characteristics compared to the yogurt fortified with free fish oil (Ghorbanzade *et al.*, 2017). Bread incorporated with omega-3 polyunsaturated fatty acids (PUFAs) nanoliposomes showed better sensory characteristics, compared to that containing microencapsulated ones. Microencapsulated samples had a fishy flavor, while nanoencapsulated bread was similar in flavor to unencapsulated bread.

Moreover, high recovery of omega-3 PUFAs and lower peroxide and anisidine values were observed in nanoliposomal omega-3 PUFAs enriched samples in comparison with other samples (Rasti *et al.*, 2017). Nanoencapsulated fish oil fortified in fruit juice showed better bioaccessibility of DHA and EPA after *in vitro* digestion (Ilyasoglu and El, 2014). Bread fortified with nano-liposomal fish oil had enhanced loaf volume compared to control samples. No negative effect on textural quality and sensory attributes of bread encapsulated with nano-liposomal fish oil was observed (Ojagh and Hasani, 2018). So, using nanoliposomes as carriers of marine oils like fish or shrimp oil can be an effective technique for enhancing the nutritional quality and sensory properties of foods.

1.2.7.2 Bitterness of liposomes

The use of soybean lecithin used as wall material of liposomes has been exploited for a long time due to excellent physicochemical properties. Phospholipase treated modified soybean lecithin and especially lysolecithin A_2, has opened new windows for applicability in food systems due to their increased polarity (Schneider, 1997; Van Nieuwenhuyzen and Szuhaj, 1998). However, the use of these hydrolyzed lecithin in the food systems is limited due to the presence of off-flavor imparted by these phospholipids in the final products. Studies have shown that the unpleasant off-flavors imparted by these phospholipids were described mainly as roasty, strawy, hay-like or nutty (Pardun, 1989; Stephan and Steinhart, 1999), and the overall taste profile of the foods was described as bitter (Pardun, 1982). In contrast to the off-odor of soybean lecithin, there is very little literature describing the bitter compounds and their standardized concentrations in commercial crude and modified soybean lecithin. This limits the possibility of evaluating the impact of addition of lecithin in foods contributing to the bitter taste or changes in the bitterness of lecithin which can result from different modification processes including deoiling and hydrolysis. The possibility of evaluating the bitterness imparted by lecithin is also limited by the use of rational experiments. Numerous studies have demonstrated that oxidation products formed as result of peroxidase and/or lipoxygenase activity (Kalbrener *et al.*, 1974; Rackis *et al.*, 1972), or phospholipid molecules (Sessa *et al.*,

1976) are the major reasons for the bitterness of foods. Wieser *et al.* (1984) reported that the bitterness in fatty alcohols and fatty acids was directly related to the degree of unsaturation and chain length of the fatty acid moiety. In another study, it was found that the major class of substances imparting bitterness to the hydrolyzed lecithin consisted of emulsified FFAs (Stephan and Steinhart, 1999). Soy lecithin was documented to impart bitterness to UHT milk (Stephan and Steinhart, 2000). Bitterness of lecithin could possibly be due to the exposure of its hydrophobic parts to the taste buds, particularly those which were not localized as bilayer but were present as free form in aqueous phase.

1.2.8 β-glucan

1.2.6.1 Source and structure

β-glucan is a non-starch polysaccharide consisting of repeating β-D-glucopyranose units arranged either in linear chains or branched structures, depending upon the source (Lam and Cheung, 2013). β-Glucan extracted from baker's yeast was found to consist of β-(1→3) and (1→6) linkages (Figure 9a). Whereas, β-glucans extracted from other sources, such as cereals contain glucose residues with β-(1→3) and β-(1→4) linkages (Figure 9b) (Zhu *et al.,* 2016). β-glucan is quite diversified and main sources include cereals, mushrooms, yeast, and some bacteria. Among cereals, oat and barley are reported as richest sources of β-glucan ranging between 4 and 7% (Lazaridou and Biliaderis, 2007; Bhatty, 1992). There is a difference in the chain conformation, molecular structure, biological activities, and number of linkage in the β-glucan derived from different sources (Descroix *et al.,* 2006). Generally cereal β-glucans are linear polysaccharides consisting of (1→3) (1→4) glycosidic bonds, in which (1→4)-β-linkages occur mostly in groups of two or three and are interrupted by a single (1→3)-linkage (Wood *et al.,* 1994a). β-glucans structure constitutes predominantly of β-(1→3)-linked cellotriosyl and cellotetraosyl units (~ 90%) and lesser amount of long cellulosic oligosaccharides in (~5 to 10%), with a degree of polymerization (DP) between 5 and 20 (Ebringerova *et al.,* 2005; Woodward *et al.,* 1983). The β-(1→4)-linkage is capable of interchain aggregation,

giving close packing to crystalline structures, whereas the β-(1→3) linkages interrupt the β-(1→4)

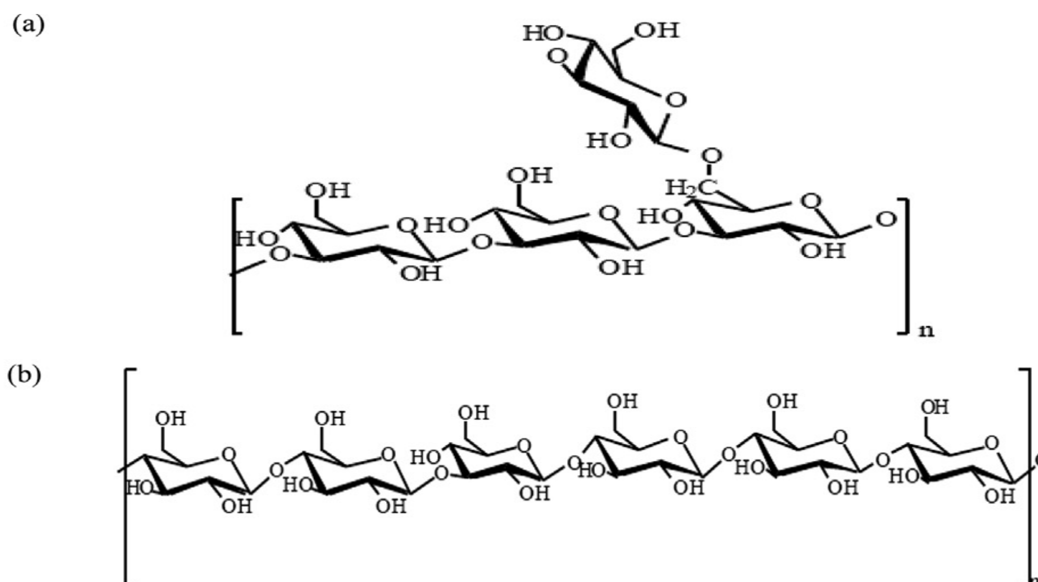


Figure 9. The structure of β-glucans. (a) (1→3) β-glucans with ramifications β (1→6); (b) (1→3) β-glucans with ramifications β (1→4)

Source: Zhu et al. (2016)

linkage sequence and gives rise to kinks in the chain, making it soluble (Woodward *et al.,* 1983; Liu *et al.,* 2015; Shah *et al.,* 2017). Aman and Graham (1987) reported that 20% and 46% of β-glucan was insoluble in oat and barley, respectively. β-glucan is hydrolyzed by lichenase, which specifically cleaves the (1→4)-linkage next to a (1→3)-linkage at its reducing end, yielding oligosaccharides with different degree of polymerization (Lazaridou and Biliaderis, 2007). Based on cereal β-glucan hydrolysis, DP3 (3-O-β-cellobiosyl-D-glucose) and DP4 (3-O-β-cellotriosyl-D-glucose) are the major products, which constitute about 90% of the total β-glucan content. Also a very little cellodextrin-like oligosaccharides which contains more than three consecutive (1→4)-linked glucose residues terminated by a (1→3)-linkage at the reducing end are released. These constitute only 5–10% of the total β-glucan content with degrees of polymerization in the range of 5–20 (Wood *et al.,* 1994a; Wood *et al.,* 1994b; Izydorczyk *et al.,* 1998). The relative amount of DP3 in the β-glucan was found in the range of 67 –72% in wheat, 52 – 69% in barley, and 53 –

61% in oat, whereas DP4 constituted in the range of 25– 33% in barley, 34 – 41% in oat, and 21–24% in wheat. An indicator for structural difference among β-glucans is the ratio of cellotriose to cellotetraose units i.e, DP3:DP4 (Wood *et al.,* 1991; Izydorczyk *et al.,* 1998). DP3/DP4 is considered as a fingerprint of the structure of cereal β-glucans. Wood (2011) reported values of DP3 to DP4 ratio in the range of 2.1–2.4 for oat β-glucan, 3.0–3.8 for wheat β-glucan, and 2.8 – 3.4 for barley β-glucans.

1.2.6.2 Application of β-glucan in food products

Apart from its biological activities including anti-inflammatory, anti-cancer and immune-modulating properties, β-glucan possesses physical properties such as gelation, viscosity and water solubility. Thus, it has been increasingly used in food and other industries (Zhu *et al.,* 2016). β-Glucan had significant effect on physical and sensory properties of sausage. By the combination of β-glucan and resistant starch, production of prebiotic sausage was possible (Sarteshnizia *et al.,* 2015). Gluten free bread based on hydroxypropylmethylcellulose, yeast β-glucan, whey protein isolate and rice starch were developed by Kittisuban *et al.* (2014). Bread made using rice starch and fortified with yeast β-glucan was sensorially acceptable. Sharafbafi *et al.* (2014) studied the effect of fortification of high molecular weight oat β-glucan into milk which can result in the reduced cholesterol and calories in the dairy products. β-glucan at low level (less than 0.2%) did not significantly have any effect on the fortified food products in terms of nutrition significant in food products and caused no phase separation. Upon mixing, different microstructures were obtained at higher concentrations. As a result, there was a change in the bulk rheological properties when β- glucan was added to the mixes. Brennan *et al.* (2013) used fractions of β-glucan from mushroom and barley at higher concentrations to produce extrusion products such as ready to eat snacks. There was a significant reduction in the *in vitro* glycemic response by up to 25% upon addition of β-glucan from mushroom and barley, indicating that β-glucan can considerably reduce the energy content of snacks and help in the modulation of glycemic response.

β-glucan is also known to modulate the rheological properties of foods such as gelation, emulsification, thickening, and stabilization. These properties make β-glucan suitable for use in culinary products such as sauces and soups, and in beverages to control viscosity and several other foods (Dawkins and Nnanna, 1995; Burkus and Temelli, 2000). β-glucan derived from barley has become a preferable stabilizer for imparting desirable viscosity and mouth feel to many beverages. Additionally, β-glucan can be an excellent source of dietary fiber. The use of β-glucan as a thickener in beverages has a potential to replace the currently used traditional thickeners such as alginates, gum Arabic, xanthan gum, carboxymethyl-cellulose and pectin (Giese, 1992). β-glucan has been incorporated in a wide variety of food products and beverages and is not just confined to cereal or dairy based foods (Lyly *et al.*, 2003; Temelli *et al.*, 2004; Konuklar *et al.*, 2004). Some studies have demonstrated the use of β-glucan in manufacturing yogurts and low-fat ice creams (Brennan *et al.*, 2002). β-glucan can also be added in combination with other dietary fibers and fortified into dairy products such as low fat cheese to enhance the rheological properties (Tudorica *et al.*, 2004). The health promoting property of β-glucan derived from barley allows it be incorporated into a variety of foods as an enrichment diet in combination of whey proteins and prevent many diseases (Temelli *et al.*, 2004). With the addition of optimum amounts of β-glucan, good quality soups with better rheological characteristics can be prepared (Lyly *et al.*, 2004; 2007). A soluble β-glucan extracted from rice, known as ricetrim, is also used as thickener and fat replacer to provide better texture to foods. Ricetrim has been used in preparation of pumpkin pudding, cookies, dip for pot crust, layer cake, saute chicken curry, and taro custard (Inglett *et al.*, 2004).

1.2.9 Dehydration of nanoliposomes

1.2.9.1 Freeze-drying

One of the widely used methods for drying is the freeze-drying also referred to as cryodesiccation or lyophilization. Freeze-drying is mostly employed for drying heat-labile products such as flavors, aromas etc. The principle behind freeze-drying is the direct sublimation of frozen product by reducing the pressure and adding

adequate heat to the system (Oetjen and Haseley, 2004). Freeze-drying is also used for encapsulation, which works by homogenizing the active material and the wall materials, followed by freezing and subsequent lyophilization. Freeze-drying is a rather simple method to encapsulate volatile aromas and water-soluble flavors. However the freezing time is longer (generally 20 h) (Desai and Park, 2005). Dehydration of pomace which contained maltodextrin DE20 and anthocyanin using freeze-drying method showed better shelf-life stability and stable water activity when stored at 50 °C for up to 2 months (Delgado-Vargas *et al.*, 2000). Cloudberry extract rich in polyphenols encapsulated using freeze drying having maltodextrins DE18.5 and DE5-8 as wall materials resulted in better protection of polyphenols and showed better antioxidant activity of polyphenols compared to unencapsulated extracts during storage (Laine *et al.*, 2008). Encapsulation using freeze-drying, particularly the volatiles, is dependent on the many factors such as chemical nature of the system, wall materials etc. (Kopelman *et al.*, 1977). Despite long freezing time and dehydration periods, freeze-drying is the most successful process for drying heat-sensitive substances (Fang and Bhandari, 2010; Munin and Edwards-Levy, 2011; Özkan and Bilek, 2014). Freeze-drying has also been employed in the encapsulation of delicate biomaterials using amorphous microstructures made from carbohydrate matrices (Heldman and Hartel 1997; Roos 1995). The use of β-cyclodextrins individually and in combination with soy proteins and polysaccharides such as pectin as wall materials were used for encapsulation using freeze-drying method (Kalogeropoulos *et al.*, 2009; Nori *et al.*, 2011). Food additives including cloudberry polyphenols (Laine *et al.*, 2008), red wine polyphenols (Sanchez *et al.*, 2013), and phenolic compounds extracted from spent coffee grounds (Ballesteros *et al.*, 2017) have been encapsulated using freeze-drying.

1.2.9.2 Spray-drying

Another method used most commonly for dehydrating milk and other liquid foods is the spray-drying. Spray-drying, often referred to as drying by atomization, works on the principle of removing moisture from liquid foods (either is dispersion, solutions or pastes) by disintegrating it into tiny droplets and subsequent

drying at high temperature using hot air. An atomizing system is used which pumps the liquid feed and sprays it into the drying chamber where hot air is used to dry the liquid droplets having smaller diameter (Al-Asheh *et al.*, 2003). The size of powder droplets produced by spray-drying process varies from very fine (between 10-50 μm) to large size (2-3 mm), depending the liquid feed, pump pressure, temperature of hot air used etc. Spray-drying is used extensively in encapsulation of compounds and food additives and is often referred to as microencapsulation. Three basic steps are involved in the spray-drying microencapsulation (Dziezak, 1988). a) To prepare the emulsion or dispersion of the products to be dried; b) to homogenize the dispersion completely with any flocculation; and c) to atomize the liquid feed into the drying chamber using a pump and an atomizer. In the first stage, the stable and fine solution is prepared by mixing the core compounds with the wall materials. The retention of spray-dried microencapsulated core material depends on many factors including properties and composition of the emulsion/dispersion, drying conditions, ratio of wall materials etc. (Jafari *et al.*, 2008). The most important step in spray-drying ins the emulsification process and play a key role in determining the stability and droplet size of the microcapsules and overall efficiency of the process (Barbosa *et al.*, 2005).

Then the solution is homogenized using a high speed homogenizer to ensure no flocculated matter is present. Finally, the homogenized solution is atomized using a atomizer and pumping into the drying chamber, resulting in the formation of microcapsules. As the moisture removal takes place in the drying chamber, the atomized particles assume a spherical shape with the core material encased in the wall material (Gharsallaoui *et al.*, 2007). Depending on the properties of the core materials, the dispersion can be heated to ensure proper homogenization (Gharsallaoui *et al.*, 2007). To prevent the inclusion of air in the dried particles, smaller oil droplets of low viscosity should be used (Drusch, 2007). Shelf-life of encapsulated products particularly the lipids and oils can be enhanced by reducing the droplet size of emulsions and reducing the surface oil on dried particles (Jafari *et al.*, 2007). Emulsions are divided on the basis of their size into nanosized (10-100 nm), micro-emulsion (100-1000 nm) and macro (0.5-100 μm) (Windhab *et al.*, 2005).

1.2.10 Use of anticaking agents

Caking is an undesirable phenomenon in which the powdered material forms aggregates or lumps and hampers the flowability of powders. To conquer the problems related to caking of powders, anticaking agents are used to prevent caking. The basic principle behind anticaking is that these agents compete for water with the host powder particles. In addition to that, they also create a barrier for moisture on the surface of powder particles which are hydrophilic by physically coming in between (Aguilera *et al.*, 1995; Lipasek *et al.*, 2012). Anticaking agents have been lauded for their excellent properties to ensure free flowing powders and their effectiveness has been documented in many studies (Lipasek *et al.*, 2012; Nishanthi *et al.*, 2017). Numerous substances have been used as anticaking agents in food industries including calcium phosphate ($Ca_3(PO_4)_2$) and silicon dioxide (SiO_2) (Food-grade), which have been generally recognized as safe (GRAS) (FDA, 2019; Yang *et al.*, 2016; Chang *et al.*, 2018; Lipasek *et al.*, 2011). Several mechanisms have been postulated for the working of anticaking agents. They are known to compete for moisture with the host powder particles, create a protective barrier around the hygroscopic powder particle surface to prevent it from absorbing moisture, they eliminate the friction between the powder particles and prevent the growth of crystals in the formation of solid bridges (Aguilera *et al.*, 1995, Hollenbach *et al.*, 1983, Peleg, 1978, Peleg and Hollenbach, 1984).

Anticaking agents are small sized (1-4mm) inert chemicals added to bulk powders (Bradley, 1999). Anticaking agents work by disrupting the interparticle cohesive forces, such as liquid bridge formation and van der Waals interaction, by placing themselves between powder particles (Flitzpatrick, 2005). Many anticaking agents are porous in nature and preferentially adsorb large amount of moisture on the surface (Peleg, 1984). When the moisture is increased further, there is a large increase in the equilibrium RH, which determines the optimum condition and performance of anticaking agents (Aguilera *et al.*, 1995). For exerting the anticaking property, these agents do not have to essentially cover the entire surface of powder particles (Aguilera *et al.*, 1995). Flow additives have been commonly and widely used in the food industry to ensure a more free-flowing product. Many products in which

anticaking agents are used include spices, salt, powdered drink formulations, chees powders, food flavorants, and gravy. For the use in food industry, not many food additives have been approved so far. Some of the additives used include calcium silicate and silicon dioxide (FDA, 2019). Some dairy-based infant formula powders increased the T_g of amorphous phase by adding high molecular weight carbohydrates (Chuy and Labuza, 1994). With the increase in T_g, caking of the powders is reduced considerably. It was observed that lower degree of hydrolysis in protein hydrolysates diminishes caking effect (Slade *et al.*,1989). Some lipids have been shown to exert an antiplasticization effect in a lipid-compatible polymer plasticized by water, thus increasing T_g (Slade *et al.*, 1989).

1.3 References

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CHAPTER 2

ULTRASOUND WAVES INCREASE THE YIELD AND CAROTENOID CONTENT OF LIPID EXTRACTED FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP (*LITOPENAUS VANNAMEI*)

2.1 Abstract

Lipids from cephalothorax of Pacific white shrimp (*Litopenaus vannamei*) were obtained using solvent extraction and ultrasonic assisted extraction (UAE). Among all solvents used, the mixture of hexane:isopropane (1:1) provided the highest yield (3.91 g/100g sample) and carotenoid content (1.97 mg/g lipid). When ultrasonication with different amplitudes (50-90%) was used for 25 min, the highest yield was obtained at 80% amplitude ($p<0.05$). With the same ultrasonication time, UAE with continous mode rendered the higher yield than pulse mode ($p<0.05$). Lipid extracted with hexane:isopropanol mixture with UAE process had the yield of 8.39 g/100g sample. Phospholipids were major constituents of lipids extracted by solvent extraction. However, more free fatty acids, mono and diglycerides were found in lipids extracted by UAE process, indicating increased hydrolysis. UAE process resulted in higher oxidation of lipids as evidenced by increased peroxide values (PVs) and thiobarbituric acid reactive substances (TBARS). Those alterations were more pronounced in lipids extracted using UAE with continuous mode than pulsed mode as confirmed by fourier transform infrared (FTIR) spectra. Overall, UAE process markedly increased the yield of lipids from shrimp cephalothorax, but oxidation and hydrolysis took place to some degree.

2.2 Introduction

Shrimp is widely consumed all over the world and Thailand has been a major exporter worldwide. Whole shrimp generally gets bifurcated into edible and non-edible portions, in which the latter includes exoskeleton and cephalothorax accounting 40-50% of its total weight. For shrimp processing industry, shell and cephalothorax are discarded and considered as leftover (Sachindra *et al.,* 2005). Most of this by-product ends up as animal feed or protein supplemented diet for aquaculture (Nwanna

et al., 2004). Over the past few years, lipids and carotenoids have received increasing interest, due to the presence of some valuable and essential components, especially n-3 fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Treyvaud, *et al.*, 2012). These fatty acids are known to have excellent health benefits (Harper and Jacobson, 2001). Astaxanthin is the major pigment in crustacean and contributes to reddish-orange colour (Higuera-Ciapara *et al.*, 2006). Astaxanthin is a very potent antioxidant and shows ten-fold higher antioxidant property than other carotenoids such as lutein, zeaxanthin, canthaxanthin and carotene (Naguib, 2000) and 100 fold greater than α-tocopherol (Miki, 1991). Lipid extracted from Pacific white shrimp waste contained trans-astaxanthin, astaxanthin monoesters and diesters (Gómez-Estaca *et al.*, 2017). PUFAs, α-tocopherol and astaxanthin in lipid extracts from Pacific white shrimp cephalothorax were shown to have anti-inflammatory effect, thereby preventing inflammation-related diseases (Gómez-Guillén *et al.*, 2018).

Lipids from shrimp wastes can be varied in terms of yield and compositions. Solvents have been reported to affect the extraction yield (Takeungwongtrakul *et al.*, 2012). To enhance efficiency in extraction, some technology such as supercritical fluid extraction (SFE) was employed to extract the lipid from shrimp. Supercritical fluid extraction using supercritical CO_2 had low effect on extraction yield of lipids but exhibited significant impact on astaxanthin extraction yield (Lopes and Cabral, 2011). Furthermore, the cost of extraction using SFE is much higher than other methods such as solvent extraction or ultrasonic extraction (Rosa and Meireles, 2005). Despite having the lowest extraction yield compared to other processes, solvent extraction also poses serious issues of toxicity and difficulty in removal of trace solvents (Delgado-Vargas and Paredes-Lopez, 2003).

Ultrasonic assisted extraction (UAE) has been widely used in food industry to overcome the drawbacks associated with conventional extraction e.g., low extraction efficiency, use of large amount of solvents and subsequent concentration, etc. (Chandrapala and Oliver, 2013). Ultrasound waves are generated in liquid medium and these waves alternately compress and stretch the molecular structure of the medium. During each "stretching" phase (rarefaction), the negative pressure is strong

enough to overcome intermolecular binding forces. A fluid medium can be literally torn apart, producing tiny cavities (microbubbles). This phenomenon is termed as acoustic cavitation (Povey and Mason, 1998). Via cavitation effect, huge energy released is strong enough to disrupt cell membranes and cause the release of target compounds in localised areas (Mason, 1999). The advantages of ultrasound for extraction include reducing extraction time, improving reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, and giving higher purity to the final product (Vinatoru and Toma, 1999). UAE has been found to increase the extraction yield of Pomegranate seed oil by 25% (Tian *et al*., 2013). UAE rendered flaxseed oil with an increased yield, while solvent consumption was low (Zhang *et al.*, 2008). Ultrasonication under the appropriate conditions could be a powerful method for lipid extraction from cephalothorax owing to its cavitation effect. Extracted lipid could serve as an excellent source of polyunsaturated fatty acids as well as carotenoids.

2.3 Objectives

To determine the impact of different solvents on lipid extraction from cephalothorax of Pacific white shrimp.

To investigate the effect of ultrasonic assisted process on yield, composition and oxidative stability of lipids.

2.4 Materials and methods

2.4.1 Chemicals

ρ-anisidine, ammonium thiocyanate, palmitic acid and cupric acetate were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulphate, isooctane, ethanol and ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were procured from Fluka (Buchs, Switzerland). Methanol, chloroform, n-hexane, petroleum ether and hydrochloric acid were purchased from Lab-Scan (Bangkok, Thailand).

2.4.2 Collection and preparation of shrimp cephalothorax

Cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. The samples (10 kg) were placed in a polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkhla University, Hat Yai, Songkhla, within approximately 1 h. Upon arrival, insulated box containing samples and ice was kept in a walk-in-cold room (4 °C) until the samples were used for lipid extraction.

Prior to lipid extraction, shrimp cephalothorax was ground to obtain a homogenous paste using a blender (National Model MK-K77, Tokyo, Japan) for 10 min. Ground cephalothorax contained 80.98 ± 1.54% moisture, 3.81 ± 0.11% ash, 3.62 ± 0.34% fat and 10.56 ± 0.22% protein as determined by AOAC method (AOAC, 2000).

2.4.3 Effect of different solvents on lipid extraction

Different solvents including n-hexane, isopropanol, acetone, chloroform, the mixture of acetone:isopropanol (1:1, v/v) and hexane:isopropanol (1:1, v/v) were used. Ground cephalothorax (25 g) was homogenised with 125 mL of solvent at a speed of 9500 rpm using an IKA Labortechnik homogeniser (Selangor, Malaysia) at 4°C for 2 min. The homogenate was centrifuged at 3000g for 15 min at 4°C using a Hitachi centrifuge (Hitachi Koki Co., Ltd, Tokyo, Japan). The supernatent was transferred into a flask containing 2-5 g of anhydrous sodium sulphate, followed by filtration using a Whatman no. 4 filter paper (Whatman International Ltd., Maidstone, England). The filtrate was transferred into a round bottom flask and evaporated using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 25°C. The residual solvent was removed by nitrogen flushing. The lipid samples were flushed with nitrogen gas, sealed tightly in a vial and kept at -40°C for further analysis. The solvent rendering the highest yield and carotenoid content was selected for ultrasonic assisted extraction process.

2.4.4 Analyses

2.4.4.1 Determination of extraction yield

Weight of extracted lipid was measured. The yield was calculated as g/100 g starting ground cephalothorax.

2.4.4.2 Measurement of total carotenoid content

Total carotenoid content in the lipid was determined according to the method of Saito and Regier (1971) with slight modifications. Thirty milligram (30 mg) of lipid sample was mixed with 10 mL of petroleum ether. The absorbance of the mixture, appropriately diluted, was measured at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Concentration was calculated using the following equation with a slight modification as follows:

$$C(\mu\text{g/glipid}) = \frac{A_{468} \times volume\ of\ extract \times dilution}{0.2 \times weight\ of\ sample\ used\ in\ grams}$$

where 0.2 is the A_{468} of 1 μg/mL standard astaxanthin.

2.4.5 Effect of Ultrasonic assisted extraction (UAE) on lipid extraction

2.4.5.1 Impact of various amplitudes

Twenty five grams (25 g) of ground cephalothorax paste was homogenised with 125 mL of the mixture of n-hexane and isopropanol (1:1, v/v) using a homogeniser for 2 min at 4°C. The sample was subsequently subjected to ultrasonic extraction using an Ultrasonic Processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA). The maximum power output of the ultrasonic transducer was 750W. The whole irradiated volume was approximately 150 mL. Ultrasonic extraction was carried out at different amplitudes of 50%, 60%, 70%, 80% and 90% using a pulsed mode with an on-time and off-time cycle of 5 seconds. The extraction times were 30, 40, 50 and 60 min. The sample was kept in ice bath and the temperature was kept below 1 °C during the ultra-sonication process. The resonance frequency of the transducer was 20 kHz output. After the ultrasonic treatment, the solution was centrifuged at 3000g for 25 min at 4°C and filtered using Whatman No.

4 filter paper. Finally, the solvent in the filtrate was removed by a rotary evaporator as previously described and the samples were stored in vials. All samples were subjected to determination of yield and carotenoid content.

2.4.5.2 Comparative study on ultrasonication using pulse and continuous mode

Ultrasonication was performed using pulse and continuous mode for different sonication time (15, 20, 25 and 30 min). For pulsed mode, the time of operation was 30, 40, 50 and 60 min. Since the pulse mode was carried out with an on an off pulse of 5 s, the sonication time was halved in each operation. Therefore both the modes of operation had the same sonication time of 15, 20, 25 and 30 min. The temperature during extraction was controlled to be below 4°C using the ice bath. The lipids were recovered and analysed as previously described.

2.4.6 Characterization of lipid extracted using different extraction methods

Lipid extracted using typical solvent method (n-hexane:isopropanol, 1:1), ultrasound with pulse mode (50 min) and continuous mode (25 min) were characterised

2.4.6.1 Determination of lipid compositions

2.4.6.1.1 Lipid classes.

The types of lipids were analysed using a thin layer chromatography/flame ionisation detection analyser (IATROSCAN TLC/FID Analyser, IATRON Laboratories, Inc., Tokyo, Japan). The chromarods S-III (silica gel powder-coated Chromarod S-III, Iatron Laboratories Inc., Tokyo, Japan) were cleaned by soaking in 50% nitric acid solution and washed with tap water, distilled water and acetone, respectively. The rods were dried and checked twice before use. The lipid samples dissolved in chloroform were spotted on the rods. The lipid classes were isolated utilising the mixed solvents: n-hexane: diethyl ether: formic acid (50:20:10, v/v/v) for approximately 15 min, followed by hexane: benzene (1:1, v/v) for approximately 30 min. Then the rods were dried in an oven (105 °C) for 10 min

before being analysed with the (flame ionisation detector) FID detector. The analyses were carried out under the following conditions: flow rate of hydrogen, 150 mL/min; flow rate of air, 700 mL/min; scan speed, 30 s/scan. Peak area was quantitated and expressed as % of total lipid.

2.4.6.1.2 Fatty acid profile.

Fatty acid profile was determined by gas chromatography (GC) according to the method of AOAC (AOAC, 2000). Fatty acid methyl esters (FAMEs) were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with a 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 injection port was set at 250 °C and detector was set at 270 °C. The oven was programmed from 170 to 225 °C at a rate of 1 °C / min (no initial or final hold). The retention times of FAME standards were used to identify the chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

2.4.6.1.3 Fourier transform infrared (FTIR) spectra

Fourier transform infrared (FTIR) analysis of lipid samples was performed in a horizontal attenuated total reflectance trough plate crystal cell (45 ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Before analysis, the crystal cell was cleaned with acetone, wiped off dry with soft tissue and the background scan was run. Lipid sample (200 μL) was applied directly onto the crystal cell and the cell was mounted to the FTIR spectrometer. Spectra of mid-infrared region of the range of 4000–500 cm^{-1} with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were resized against a background spectrum recorded from the clean empty cell at 25 °C. Spectral data analysis was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

2.4.6.2 Measurement of lipid oxidation/hydrolysis

2.4.6.2.1 Peroxide value (PV)

PV was determined using the ferric thiocyanate method Chaijan *et al.* (2006). Lipid sample (50 µL) was 10-fold diluted using 75% ethanol, (v/v). To the prepared sample, a mixture of 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µL of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added. The mixture was mixed well and the absorbance was read at 500 nm using a spectrophotometer. The blank was prepared in the same way, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

2.4.6.2.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined by the method as described by Buege and Aust (1978). Lipid sample (0.5g) was mixed with 2.5 mL of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was then heated in boiling water (95–100°C) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at 3600g at 25°C for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was read at 532 nm using a spectrophotometer. A standard curve was prepared using 1,1,3,3- tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde/ kg lipid.

2.4.6.2.3 *ρ*-Anisidine value (AnV)

ρ-Anisidine value (AnV) of sample was analysed according to the method of AOAC (AOCS, 1990). One hundred mg lipid sample were dissolved in 25 mL of isooctane. Then 2.5 mL of the solution was pipetted out and mixed with 0.5 mL of 0.5% ρ-anisidine in acetic acid for 10 min. The absorbance was read at 350 nm using a spectrophotometer and the ρ-anisidine value was calculated using the following formula:

$$\rho - \text{AnV} = 25 \times \frac{(1.2 \times A2) - A1}{W}$$

where A_1 and A_2 are the absorbance value at 350 nm before and after adding ρ-anisidine, respectively; W = weight of sample (g).

2.4.6.2.4 Free fatty acid (FFA) content

FFA content was determined according to the method of Lowry and Tinsley (1976). To a lipid sample (0.1 g), 5 mL of isooctane were added and swirled vigorously to dissolve the sample. The mixture was added with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, which was prepared by dissolving 5 g of the reagent grade cupric acetate in 100 mL of water, filtering and adjusting the pH to 6.0–6.2 using pyridine. The mixture was shaken vigorously using a Vortex- Genie2 shaker (Bohemia, NY, USA) for 90 s and allowed to stand for 20 s. The absorbance of upper layer was read at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 µmol/ mL. FFA content was expressed as g FFA/100 g lipid.

2.4.7 Statistical analysis

All experiments were performed in triplicate and results were expressed as means ± SD. The data was analysed by analysis of variance (ANOVA) and comparison of means was carried out by the Duncan's multiple range test. For pair comparison, the t-test was used (Steel and Torrie, 1980). Statistical Analysis was carried out using SPSS by IBM (SPSS 22.0 for windows, IBM, New York, USA).

2.5 Results and discussion

2.5.1 Impact of different solvents on lipid extraction from shrimp cephalothorax.

The yields and carotenoid content in lipid extracted from cephalothorax of Pacific white shrimp using different solvents are shown in Figure 10. The yield of lipid ranged from 1.45-3.91 g/100 sample. Some organic solvents have been permitted for use in food industries. Those include acetone, ethyl acetate, hexane, isopropanol, methyl ethyl ketone and ethanol. Nevertheless, solvents such as

dichloromethane, dimethyl sulfoxide and chloroform are not allowed because of their toxicity (USFDA, 2010). Amongst all the single solvents, isopropanol gave the highest yield (3.28 g/100 sample). However the mixture of n-hexane and isopropanol (1:1, v/v) showed the higher yield (3.91 g/100 sample) than single solvents and the mixture of acetone and isopropanol (1:1) ($p<0.05$). This finding was in accordance with the findings of Sánchez-Camargo *et al.* and Takeungwongtrakul *et al.*, who reported the highest extraction yield from red shrimp waste and hepatopancreas of Pacific white shrimp, respectively. It was noted that types of solvents played a significant role in the extraction of lipids from cephalothorax. Appropriate polarity of solvents used was the key factor governing the extraction efficiency. Lipids in shrimp cephalothorax majorly comprised phospholipids (Takeungwongtrakul *et al*., 2012). These phospholipids are polar in nature and hence require a polar solvent for effective extraction. Carotenoids, on the other hand, are soluble in non-polar solvents (Sachindra *et al.*, 2006). Hexane, a non-polar solvent, is an appropriate choice of solvent for carotenoid extraction from the lipids extracted from Brazilian redspotted shrimp waste (Sánchez-Camargo *et al.*, 2011). The use of a mixture of polar and nonpolar solvents for extraction of carotenoids from shrimp by-product generally resulted in the highest yield (Khanafari *et al.*, 2007). A mixture of polar solvent such as acetone with non-polar solvent have been used to improve the extraction yield of carotenoid from shrimp head and carapace (Sachindra *et al.*, 2006). Carotenoid contents in lipids extracted using various solvents were varied Figure 10B. The highest carotenoid content was found in lipids extracted using the mixture of hexane:isopropanol (1:1) ($p<0.05$), followed by that extracted using isopropanol. Nevertheless, carotenoid contents were inversely proportional to extraction yield, when acetone, chloroform and hexane were used as solvents. These solvents are non-polar in nature and might not be appropriate to extract lipids from shrimp cephalothorax, having phospholipids as major components.

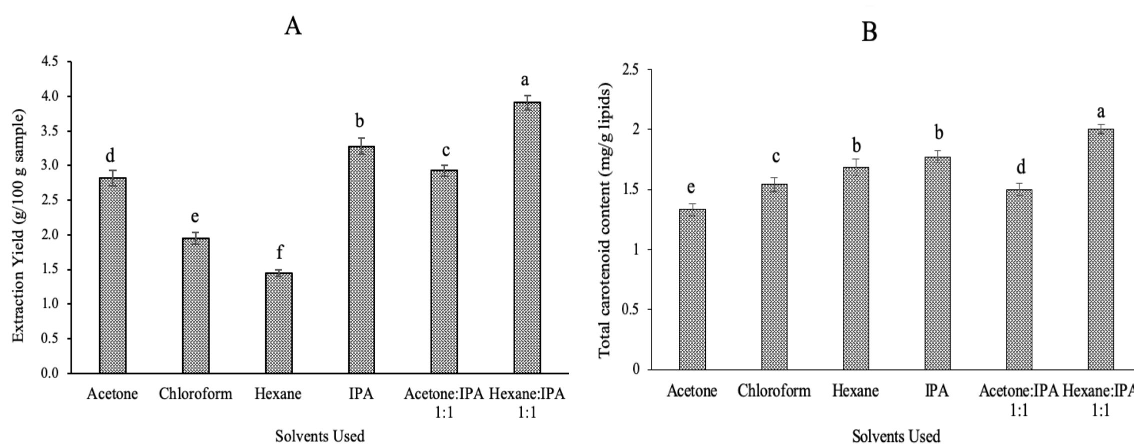

Figure 10. Effect of different solvents on yield (A) and carotenoid content (B) of lipids from cephalothorax of Pacific white shrimp. Ground cephalothorax to solvent ratio of 1:5 was used. Different letters on the bars indicate significant difference ($p<0.05$). Bars represent standard deviation (n=3).

2.5.2 Effect of ultrasonic assisted extraction at different amplitudes on lipid extraction from shrimp cephalothorax.

The effect of ultrasound at different amplitudes using pulse mode (with 5 sec on-off pulse) on extraction yields is shown in Figure 11A. The extraction yields with ultrasonic treatment were higher than that from non-ultrasonic treatment (solvent extraction). Generally the yield was increased when the amplitudes increased up to 80% ($p<0.05$). The increased yield was more likely attributed to the acoustic cavitation phenomenon and mechanical effects. Sonication is widely used for extraction of various organic substances, such as plant extracts and oils. Ultrasonic waves generate microbubbles inside the solution (Vinatoru, 2001). When microbubbles collapse, high shear gradient is generated, thus disrupting cell wall (Jaki *et al.*, 2006). This helps in penetration of solvent into cells and release of target components. It is imperative to note that the recovery of lipids by the ultrasonic assisted extraction and selection of appropriate solvents was much higher than the soxhlet method. It is attributed to the fact that petroleum ether used in the soxhlet method is a non-polar solvent and cannot extract the polar compounds in shrimp oil. Moreover, due to the lack of cavitation of any physical membrane rupturing process, not all the lipids are released during the extraction process. Sivakumar *et al.* (2007)

and Lou *et al.* (2010) showed the increase in yield of tannin from myrobalan nuts and oil from chickpeas when ultrasonic assisted extraction was used. Zhang *et al.* (2017) reported the increase in extraction yield of peanut oil with increasing ultrasonic frequencies. No difference in yield was obtained when amplitude of 80 and 90% were used ($p>0.05$). This could be explained by the relationship between ultrasonic frequency and cavitation threshold (Hauptmann *et al.*, 2013). At a certain optimum energy level, the cavitation was highest and there was no significant effect on the extraction yield when energy beyond that level was employed.

When the continuous mode (without on-off pulse) was used for lipid extraction, the yield was higher than those obtained using pulsed mode (Figure 11B). Similar to the yield obtained from pulse mode, no difference in the yield was found when the amplitudes of 80 and 90% were applied ($p>0.05$). During the offset time of 5 seconds in pulsed mode, the particles underwent agglomeration and precipitation, owing to their mass. As a result, the energy dissipation was not uniform (Doulah, 1977). The cavitation effect of ultrasonics is viewed as a transfer of sonic energy into hydrodynamic energy by the release of elastic waves. When these waves propagate into the liquid system, they tend to disintegrate into eddies, which in turn will break-up to form new set of eddies. This process continues until the eddies are so small that they get damped by the viscosity of medium. At continuous ultrasonication, these elastic waves will form a continuous source of eddies (Doulah, 1977).

Additionally, with continuous mode of ultrasonication, the particles were dispersed evenly in the medium and uniformly with maximum energy dissipation. At the same ultrasonication time for both the modes, higher temperature was obtained in the continuous mode. Collapse of bubbles was greater in continuous mode, leading to the localised generation of heat to higher extent (Suslick, 1989). However, the temperature of system was controlled to be lower than 4 °C throughout extraction in the present study.

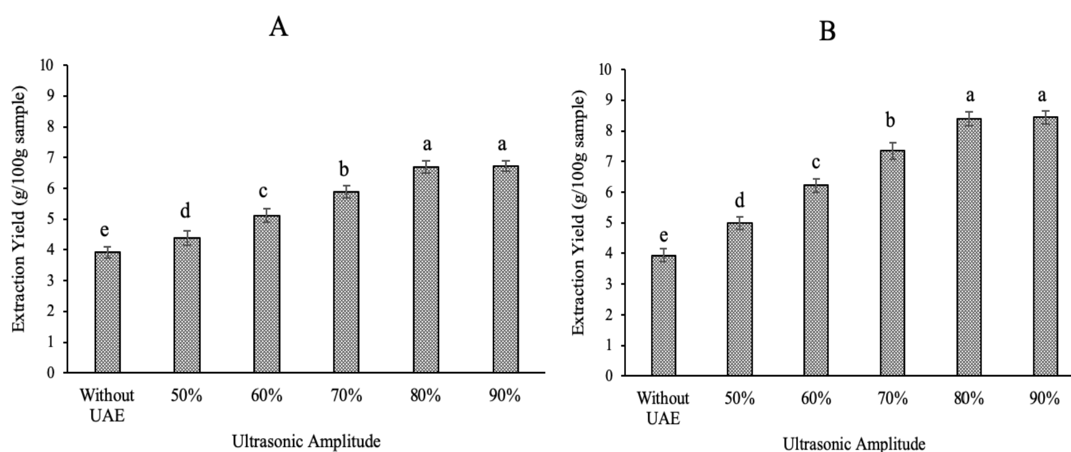

Figure 11. Effect of ultrasonication at different amplitudes on yield of lipids from cephalothorax of Pacific white shrimp using pulsed mode (A) and continuous mode (B). Sonication time was 25 min. Different letters on the bars indicate significant difference ($p<0.05$). Bars represent standard deviation (n=3).

The effects of ultrasonication time on yield of lipids using UAE process with pulsed and continuous mode are shown in Figure 12. Time of operation played a vital role in overall yield of lipid extraction from cephalothorax. It was observed that the yield was generally increased with increasing total operation time up to 25 min and 50 min for continuous mode and pulsed mode, respectively, which were equivalent to ultrasonication time of 25 min. At time higher than 60 min for pulsed mode and 30 min for continuous ultrasonic extraction, slight decrease in yield was noticeable. This can be explained by the phenomenon of re-adsorption of target molecules by higher surface area of particles caused by excessive mechanical shear (Dong *et al.*, 2010). Since there were no differences in yield between lipids extracted for 50 and 60 min (pulse mode) or between 25 and 30 min (continuous mode), ultrasonic assisted extraction with 50 min in pulsed mode and 25 min for continuous mode were chosen as the optimal processes.

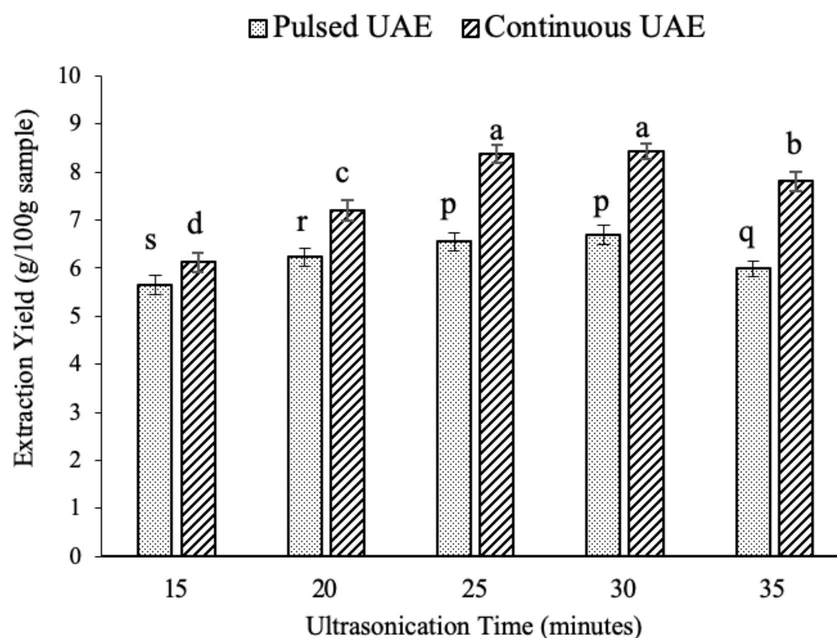

Figure 12. Effect of ultrasonication time on yield of lipids from cephalothorax of Pacific white shrimp using pulsed and continuous ultrasonic process. The ultrasonic amplitude was 80%. Operation time for pulsed mode was two-fold longer than continuous mode. Different letters among the same mode on the bars indicate significant difference ($p<0.05$). Bars represent standard deviation (n=3).

2.5.3 Characteristics of lipids extracted with selected processes

2.5.3.1 Lipid classes

The compositions of lipids extracted from cephalothorax of Pacific white shrimp by solvent extraction and UAE process with pulsed and continuous modes are shown in Table 2. The sample with solvent extraction method contained phospholipid as the dominant constituent, followed by triglyceride. It was noted that mono- and di-glyceride were also found in the lipid extracted with solvent. This might be cause by the rapid hydrolysis of ester bond caused by endogenous lipases, occurring in shrimp cephalothorax during handling or grinding prior to extraction. On the other hand, lipid extracted using UAE process showed high levels of free fatty acids, mono and diglycerides, regardless of modes used. This coincided with the decrease in phospholipids. It was noted that lipid samples extracted using UAE

process with continuous mode showed the lowest phospholipid content among all samples. Lower triglycerides but higher diglycerides were found in lipid extracted with UAE process with continuous mode in comparison with that using pulsed mode ($p<0.05$). The result indicated that the glycerol-fatty acid ester bonds were largely disrupted by the mechanical shear forces and cavitation effect of UAE process, especially with continuous mode. With continuous mode, the ester bonds were more destroyed, thus releasing more free fatty acids. Ultrasonic treatment of sunflower oil under 20 kHz and 150 W for 1 min resulted in the development of fishy and rancid odour due to hydrolysis of triglycerides into free fatty acids (Chemat *et al.*, 2004). Additionally, lipase and phospholipase in shrimp were postulated to play an essential role in the hydrolysis of lipids during extraction (Lopez and Maragoni, 2000). Lipase-catalysed hydrolysis was more profound in ultrasonic assisted extraction methods due to larger interfacial area and smaller droplet size of bubbles obtained by ultrasonication (Huang *et al.*, 2010). Increasing ultrasonic power was found to enhance the degree of lipase-catalyzed hydrolysis of soy oil in solvent-free system (Liu *et al.*, 2008).

Table 2. Composition of lipids extracted from cephalothorax of Pacific white shrimp using various extraction methods.

Extraction Method	Lipid Composition (%)				
	Monoglyceride	Diglyceride	Triglyceride	Phospholipid	Free fatty acid
Solvent	12.85±3.26a	8.69±3.77b	18.71±3.82b	45.98±4.67a	13.81±2.57b
Ultrasonic (pulsed)	16.99±2.91a	13.86±2.09b	23.62±1.13a	9.52±3.15b	34.9±2.03a
Ultrasonic (continuous)	12.04±1.38a	28.13±3.37a	18.68±2.97b	6.25±2.08b	36.13±3.12a

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

2.5.3.2 Fatty acid profiles

The fatty acid profiles of lipids extracted from cephalothorax of Pacific white shrimp by solvent extraction and UAE process are shown in Table 3. The lipids extracted by solvent extraction contained 30.4% saturated fatty acids, 22.25% monounsaturated fatty acid (MUFA) and 37.5% PUFA. Lipids extracted by pulsed UAE process consisted of 30.86% saturated fatty acids, 22.47% MUFA and 35.99% PUFA, whereas lipids extracted by continuous UAE process consisted of 32.32% saturated fatty acids, 22.13% MUFA and 33.32% PUFA. The PUFAs were found to be the major fatty acids in both the samples extracted by two different processes. This result was in agreement with Lin et al. (2003) and Takeungwongtrakul *et al.* (2012), who found that PUFAs were the major fatty acids in Pacific white shrimp. Shrimp cephalothorax contained palmitic acid as the most abundant fatty acid, followed by oleic acid and linoleic acid (Soultani and Strati, 2016). Takeungwongtrakul *et al.* (2012) also reported similar composition of fatty acids in shrimp cephalothorax. The lipids also had higher contents of n-3 fatty acids, docosohexanoic acid (DHA) and eicosopentanoic acid (EPA). The high content of DHA is more likely attributed to the presence of high phospholipids, which contain high amount of PUFAs. The lipids extracted via UAE process had lower contents of DHA and EPA, compared to that extracted by solvent extraction. The decrease in the DHA and EPA can be attributed to their oxidation during ultrasonication process by the incorporation of oxygen. The DHA and EPA of shrimp cephalothorax decreased by 0.49% and 0.54% in pulsed UAE process and by 1.93% and 2.08% in continuous UAE process, respectively. Thiansilakul *et al.* (2010) reported that during extended storage, DHA and EPA contents in the lipid decreased due to the susceptibility to oxidation.

Table 3. Fatty acid profile of lipids extracted from cephalothorax of Pacific white shrimp using various extraction methods.

Fatty acids (g/100 g lipids)	Solvent	Ultrasonic (p)*	Ultrasonic (c)*
C14:0	0.53 ± 0.00a	0.51 ± 0.00b	0.49 ± 0.00c
C15:0	0.30 ± 0.00c	0.32 ± 0.00b	0.38 ± 0.00a
C16:0	16.95 ± 0.15b	17.07 ± 0.00b	18.08 ± 0.19a
C16:1t9	0.34 ± 0.01b	0.30 ± 0.03b	0.43 ± 0.01a
C16:1 n-7	1.27 ± 0.00a	1.25 ± 0.01a	1.16 ± 0.00b
C17:0	1.02 ± 0.00c	1.04 ± 0.00b	1.43 ± 0.01a
C18:0	10.40 ± 0.00b	10.68 ± 0.15a	10.77 ± 0.02a
C18:1 n-9	16.26 ± 0.10a	16.35 ± 0.02a	16.32 ± 0.11a
C18:1 n-7	2.37 ± 0.00a	2.38 ± 0.00a	2.25 ± 0.00b
C18:2 n-6	11.87 ± 0.03a	11.7 ± 0.08b	11.63 ± 0.04b
C18:3 n-3 (ALA)	0.69 ± 0.00a	0.65 ± 0.00b	0.52 ± 0.00c
C20:0	0.34 ± 0.00a	0.34 ± 0.00a	0.31 ± 0.00b
C20:1 n-9	1.33 ± 0.00a	1.34 ± 0.00a	1.29 ± 0.01b
C20:2 n-6	1.68 ± 0.01b	1.68 ± 0.00b	1.79 ± 0.01a
C20:3 n-6	0.26 ± 0.00a	0.25 ± 0.00a	0.22 ± 0.00b
C20:3 n-3	0.17 ± 0.00	ND	ND
C20:4 n-6 (ARA)	5.72 ± 0.40a	5.29 ± 0.40b	5.89 ± 0.40a
C20:5 n-3 (EPA)	9.17 ± 0.04a	8.63 ± 0.12b	7.09 ± 0.08c
C22:0	0.47 ± 0.00b	0.49 ± 0.00a	0.44 ± 0.0c
C22:1 n-9	0.16 ± 0.00	ND	ND
C22:6 n-3 (DHA)	8.11 ± 0.05a	7.62 ± 0.07b	6.18 ± 0.05c
C24:0	0.39 ± 0.00b	0.41 ± 0.00a	0.42 ± 0.00a
C24:1	0.68 ± 0.00a	0.69 ± 0.01a	0.68 ± 0.00a
Unidentified peak	9.86 ± 0.10b	10.70 ± 0.59a	9.53 ± 0.61b
Saturated fatty acid (SFA)	30.4 ± 0.00c	30.86 ± 0.00b	32.32 ± 0.00a
Monounsaturated fatty acid (MUFA)	22.25 ± 0.00b	22.47 ± 0.00a	22.13 ± 0.00c
Polyunsaturated fatty acid (PUFA)	37.5 ± 0.00a	35.99 ± 0.00b	33.32 ± 0.00c

*(p): pulsed mode; (c): continuous mode. Different lowercase letters in the same row indicate significant difference ($p<0.05$).

2.5.3.3 Carotenoid content of shrimp cephalothorax

The carotenoid content of lipids extracted using UAE process was higher than those of samples extracted with only solvent (without ultrasonication) ($p<0.05$). This could be attributed to the fact that during ultrasonication, the cavitation could induce the breakdown of protein matrices, thus releasing more astaxanthin from the complex. The total carotenoid contents of lipids extracted were 3.91 ± 0.11 mg/g lipid for pulsed ultrasonic extraction, 4.64 ± 0.14 mg/g lipid for continuous ultrasonic extraction and 2.01 ± 0.08 mg/g lipid for non-ultrasonic extraction as shown in Figure 13A. Takeungwongtrakul *et al.* (2012) reported that carotenoid content was 3.10 ± 0.02 mg/g in lipid from shrimp cephalothorax of Pacific white shrimp extracted by mixture of n-hexane and isopropanol. Sachindra *et al.* (2005) reported that carotenoid content was varied, depending upon the feed and habitat. Indian shrimps contained carotenoids varying from 35 to 153 µg/g, and the major pigment was astaxanthin and its esters. Astaxanthin is the main pigment found in crustacean and salmonids, and it gives the desirable reddish-orange colour in these organisms (Higuera-Ciapara *et al.*, 2006). Lipids from Pacific white shrimp waste contained all trans-astaxanthin, two cis-astaxanthin isomers, five astaxanthin monoesters and ten astaxanthin diesters (Gómez-Estaca *et al.*, 2017). The higher content of carotenoid was concomitant with the increasing yield. Lipid extracted using ultrasonication with continuous mode showed the higher carotenoid content than that using pulsed mode ($p<0.05$). With continuous mode, the hexane was able to extract carotenoid more efficiently as evidenced by the higher level of carotenoid content. In general, carotenoids which were soluble in lipids could be co-extracted with lipids especially via cavitation. Goula *et al.* (2017) reported extraction yield of 93.8% of the total carotenoids from pomegranate waste using vegetable oils by ultrasonic assisted extraction.

2.5.3.4 Peroxide value (PV)

Figure 13B shows the peroxide value (PV) of the lipids extracted by different methods. PV of lipid sample extracted by UAE was higher than the lipid extracted by using solvent ($p<0.05$). Oxidation leads to formation of hydroperoxides which are the intermediate products and subsequently decomposed to aldehydes, ketones and other oxides. This result was in agreement with the findings of Zhang *et*

al. (2017) who reported the increase in PV of Peanut oil with ultrasonic extraction, compared to leaching method. The increase in PV was due to the formation of hydroperoxides formed by the reaction of singlet oxygen with unsaturated lipids or by the action of lipoxygenase on the oxidation of polyunsaturated fatty acids (Nawar, 1996). The difference in PV of lipids extracted from two different methods was governed by several factors, especially oxygen concentration. When comparing PV between lipids from two UAE processes, the higher value of PV was observed in the sample with continuous mode ($p<0.05$). The ultrasonic mechanism is based on mechanical effects particularly cavitation, which resulted in the increase in the localised temperature and pressure. This could induce lipid oxidation as evidenced by the increased PV. Due to the presence of higher oxygen incorporated during UAE process, the oxidation of unsaturated fatty acids present in the lipids took place to higher extent. It was found that continuous mode resulted in higher PV of lipid extracted than pulsed mode. This might be due to the agitation of lipids, allowing the contact with oxygen more effectively. Moreover, the increase in PV was plausibly attributed to the higher levels of lipoxygenase in ultrasonicated samples due to the disruption of cell matrix to release more enzymes. Lipoxygenase was found to be present in the shrimp head (Shye *et al.*, 1987).

2.5.3.5 TBARS

TBARS value of lipid with UAE process was found to be higher than that extracted using solvent ($p<0.05$). The values of TBARS for lipids from solvent extraction process, UAE with pulsed mode and continuous mode were 11.16, 17.08, 19.87 mg MDA/kg sample respectively (Figure 13C). The result indicated that the lipid oxidation occurred mostly in the samples extracted using UAE process ($p<0.05$). Thiobarbituric acid reactive substances have been used as the indices for relatively polar secondary oxidation products including aldehydes and ketones, etc. (Nawar, 1996). The increase in the TBARS value of lipids indicated the formation of the secondary lipid oxidation products (Chaijan *et al.*, 2006). Since lipids contained high content of PUFA, (Table 3), they were susceptible to oxidation. With the continuous ultrasonication, TBARS value was found to increase. During the ultrasonic extraction, mechanical effect could enhance mass transfer, and oxygen could be incorporated to a

higher degree. The instantaneous high temperature and pressure when the bubbles collapse might induce faster oxidation of lipids (Zhang *et al*., 2017). Hydroperoxides could be decomposed into aldehydes, ketones and others with ease under harsh condition as indicated by the increased TBARS value. The loss of natural antioxidants might occur during ultrasonic process and thus favouring lipid oxidation in UAE samples particularly with continuous mode.

2.5.3.6 *ρ*-Anisidine value (AnV)

AnV of lipid sample with UAE process were higher than that using solvent extraction method ($p<0.05$), (Figure 13D). The results reiterated the generation of secondary oxidation products, mainly non-volatile aldehydes and ketones in the lipid samples. ρ-Anisidine value is another index of lipid oxidation, which determines the amount of non-volatile aldehyde (principally 2-alkenals and 2,4-alkadienals) in lipids (Choe and Min, 2006). Aldehydes present in lipids react with the ρ-anisidine reagent under acidic conditions, producing yellowish products. The aldehydes formed can be further oxidised or participate in dimerisation or condensation reactions (Gulla and Waghray, 2011). The result was in accordance with Zhang *et al.* (2017) who showed the higher AnV of peanut oil extracted via ultrasonic extraction, compared to extraction by leaching. Takeungwongtrakul *et al.* (2012) reported that lipids extracted from shrimp hepatopancreas had the increased AnV with increasing storage time. During the ultrasonic extraction, cavitation and mechanical effect enhanced mass transfer, in which oxygen might be incorporated into lipids to a higher extent. Hence, the quality of the oils continued to deteriorate, particularly lipid extracted using UAE process with continuous mode.

2.5.3.7 Lipid hydrolysis

Free fatty acid content of lipids extracted from cephalothorax of Pacific white shrimp by solvent extraction and UAE process are shown in Figure 13E. FFA content of lipid extracted by UAE process increased drastically ($p<0.05$). The hydrolysis of glycerol–fatty acid esters is one imperative change that takes place in lipids, causing the release of free fatty acids and is normally catalysed by lipases and phospholipases (Pacheco-Aguilar *et al*., 2000). The sharp increase in FFA induced by

ultrasonic extraction was more likely due to mechanical effects. Cleavage of ester bonds due to high shear forces mediated by cavitation led to the release of free fatty acids. Furthermore, the release of lipases by ultrasonication process from cell matrix made hydrolysis more probable. As expected, the highest FFA content was found in lipid extracted using UAE process with continuous mode, followed by pulsed mode. The lowest FFA was found in lipid with solvent extraction method. Free fatty acids liberated were prone to oxidation as shown by increments in TBARS and AnV (Figure 13C and 13D). Therefore, extraction methods had the profound effect on the hydrolysis and oxidation of lipid from Pacific white shrimp cephalothorax.

Since the FFA content of shrimp oil extracted using UAE process was higher than the standard limits of edible oils, it is suggested that some refining process such as neutralization should be carried out to remove the FFA before consumption. The common practice used for neutralization of edible oils involves the use of sodium hydroxide. Alkaline neutralization of crude soybean oil by NaOH resulted in 75% removal of FFAs (Kuleasan and Tekin, 2008).

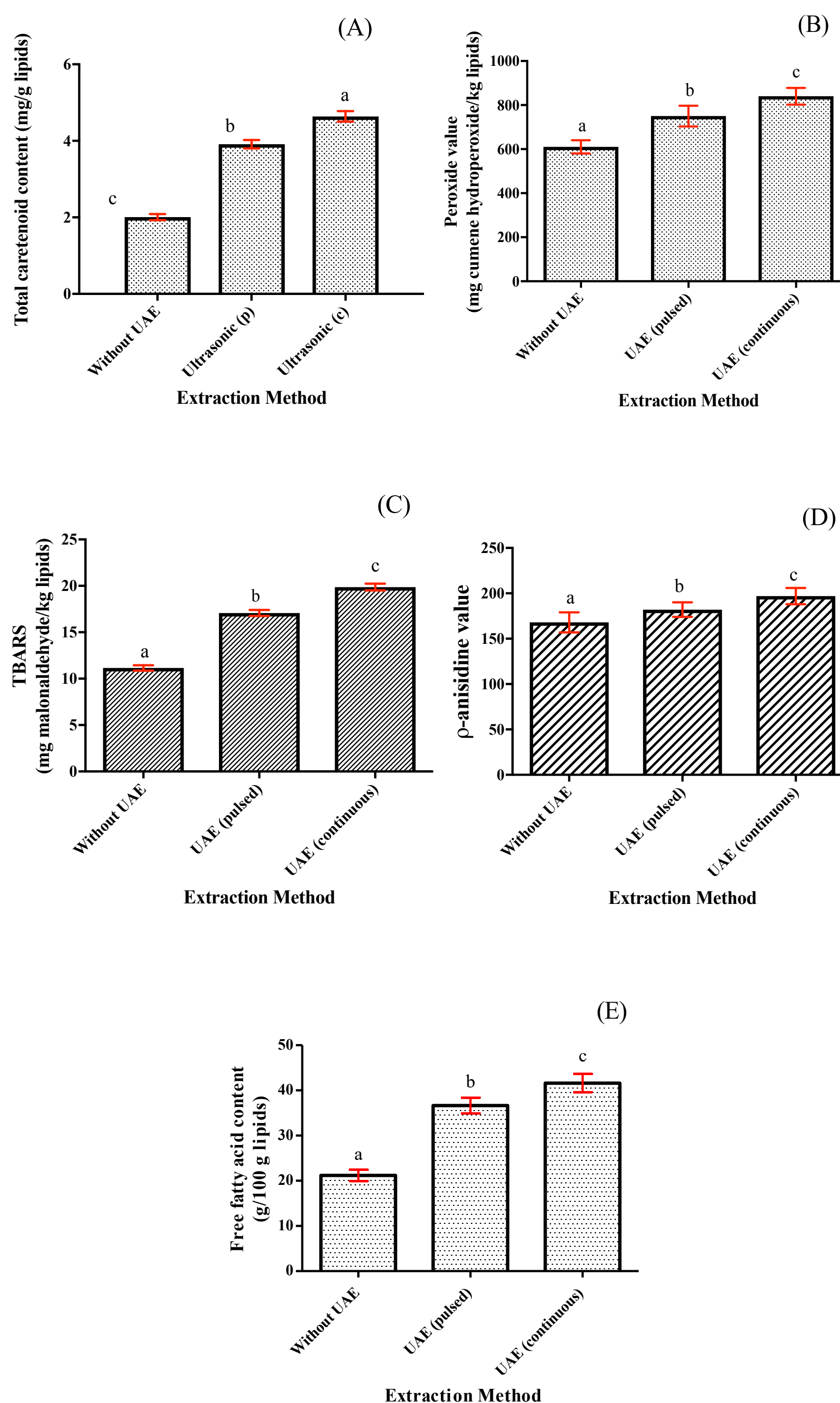

Figure 13. Total carotenoid content (A), Peroxide value (B), TBARS value (C), ρ-anisidine value (D) and free fatty acid content (E) of lipids from cephalothorax of Pacific white shrimp extracted by various extraction methods. Ultrasonic amplitude was 80%. Sonication time was 25 min. Different letters on bars indicate significant difference ($p<0.05$). Bars represent standard deviation (n=3).

2.5.4 FTIR spectra

FTIR spectra of lipids from cephalothorax of Pacific white shrimp are illustrated in Figure 14. A higher amplitude of peak with a wavenumber of 3600–3200 cm^{-1} representing –OH, stretching was observed in the lipids from cephalothorax. The highest amplitude of peaks with wavenumber range of 3400-3300 cm^{-1} representing OH group was found in lipid extracted using UAE with continuous mode, followed by pulse mode. OH group of glycerol backbone could be liberated after the phospholipid or triglyceride were hydrolysed. This was in accordance with the higher free fatty acid contents in both samples, when compared with the lipid extracted using solvent (Table 2). The peak observed at 3400 cm^{-1} was due to their –OO–H stretching vibrations (Van de Voort *et al.*, 1994). Guillén and Cabo (2004) reported that the ratio between the absorbance band at 2854 cm^{-1}, due to the symmetrical stretching vibration of $–CH_2$ groups and the absorbance band between 3600 and 3100 cm^{-1} ($A_{2854}/A_{3600–3100}$), could be used to monitor the oxidation process. When a similar ratio of $A_{2900}/A_{3500–3150}$ was determined, it was found that the value was 98-133 and 87-112 for lipids extracted with UAE using pulsed mode and continuous mode respectively. The ratio of 109-154 was attained in the lipids extracted by solvent extraction. The lower the value of this ratio, the more advanced oxidation in the samples occurred (Guillén and Cabo, 2004). This was concomitant with the higher PV (Figure 13B) and TBARS (Figure 13C) of lipids extracted by UAE process, especially with continuous mode. The increased amplitude at 2900 cm^{-1} indicated the higher amounts of aldehyde formed in lipids (Van de Voort *et al.*, 1994). Such a peak showed the higher amplitude for lipid extracted using UAE, compared with typical method. The peaks at wavenumbers of 1643 and 1641 cm^{-1} represents the C=O carboxylic group of free fatty acids (Guillen and Cabo, 1997). It was noted that the high amplitude was in accordance with higher free fatty acid content in lipid extracted using UAE process (Table 2). The higher amplitude was noticeable in lipid extracted using UAE with continuous mode, compared to pulsed mode. The lipids extracted by UAE with continuous mode had the highest amplitude of 1640 cm^{-1} representing free fatty acids, followed by that extracted by UAE with pulsed mode. The lowest amplitude was found in the sample using solvent extraction. This result confirmed that mechanical

shearing and molecular disruption was more pronounced when lipids were extracted by ultrasonic process, particularly with continuous mode.

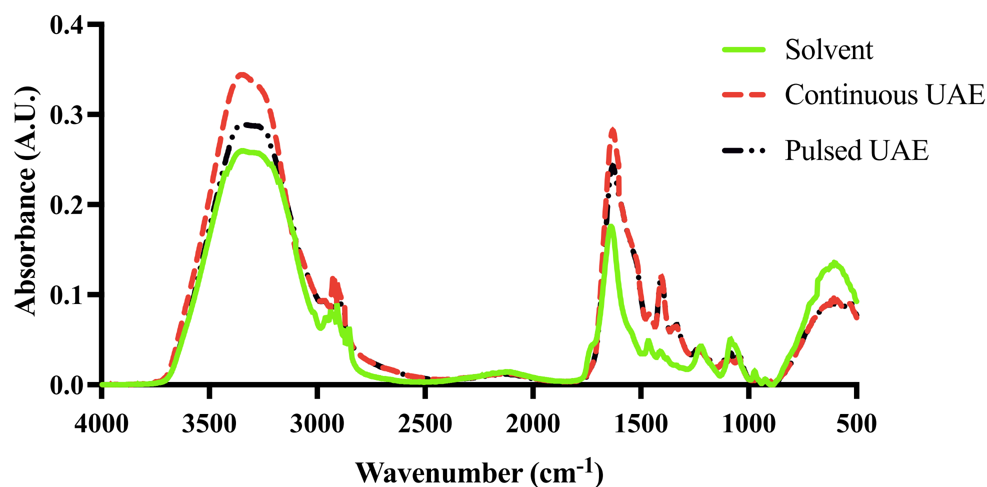

Figure 14. FTIR spectra of lipids extracted from cephalothorax of Pacific white shrimp

2.6 Conclusion

Ultrasonic assisted extraction (UAE process) increased the yield of lipids and carotenoids extracted from shrimp cephalothorax significantly. The highest yield was obtained by continuous mode along with increased carotenoid content. However, lipid was susceptible to oxidation and hydrolysis due to incorporation of oxygen and breakage of bonds by mechanical shear during the ultrasonication process. Hydroperoxides were generated and underwent decomposition to form the secondary oxidation products, especially aldehydes. Free fatty acids were liberated due to the cleavage of glycerol-ester bonds which also resulted in enhanced lipid oxidation overall.

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CHAPTER 3

EFFECT OF PRE-TREATMENTS ON YIELD AND PROPERTIES OF LIPID EXTRACTED FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) BY ULTRASONIC ASSISTED PROCESS

3.1 Abstract

Impacts of different pre-treatments of cephalothorax from Pacific white shrimp before ultrasound assisted extraction (UAE) on yield and characteristics of lipids were studied. Autolysis at 50 °C for 3 h in the absence and presence of 0.1% tannic acid (TA), a lipase inhibitor, was implemented. Pre-heating of cephalothorax containing 0.1% TA at 95 °C for different times (15-45 min) was also carried out. When (UAE) was used to extract the lipids at the ultrasonic amplitude of 80% for 25 min in continuous mode, samples with pre-heating rendered the highest lipid yield (13.3g-14.1g/100g sample). Pre-heating along with TA addition resulted in suppression of lipid oxidation as evidenced by decrease in peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). Free fatty acid (FFA) content was also found to be lower, whereas the control (without pre-treatment) had higher FFA. Fourier transform infrared (FTIR) spectra confirmed lower oxidation in lipid from pre-heated samples added with TA. Lipid contained astaxanthin, astaxanthin monoester, astaxanthin diester, canthaxanthin and β-carotene. Overall, the combination of pre-heating and addition of TA was a promising means to increase the yield and maintain the quality of lipid from cephalothorax using UAE.

3.2 Introduction

Shrimp is one of the major seafoods consumed all over the world. Global shrimp production now stands at approximately 6 million metric ton (MMT) and is expected to increase at a compound annual growth rate (CAGR) of 4.8% between 2016 and 2019. *Litopenaeus vannamei* alone accounts for approximately 76% or roughly 5 MMT of global aquaculture production. Asia alone produces by 71% (Anderson *et al.*, 2016). Thailand holds a share of about 0.5 MMT and has made a

good recovery after a downfall in production in recent years. Shrimp is processed before consumption or exporting to other countries. Inedible part, accounting around 40-50%, is leftover with low market value (Sachindra *et al.*, 2005). These byproducts consisting of cephalothorax, carapace and tail, mainly end up either as animal feed or supplementary diet for aquaculture (Nwanna *et al.*, 2004).

Over the past few years, shrimp-processing by-product has received increasing interest because of the presence of useful bioactive compounds, especially lipid from shrimp cephalothorax. It was rich in n-3 fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Amiguet *et al*., 2012) and carotenoids including β-carotene and astaxanthin. Astaxanthin being a very potent antioxidant reportedly exhibits 10-fold greater antioxidant property than carotenoids such as canthaxanthin, zeaxanthin and lutein (Naguib, 2000) and 100-fold greater than α-tocopherol (Miki, 1991).

Many new techniques have been exploited for extracting the lipid from the shrimp cephalothorax. Those include supercritical fluid extraction (Haloui and Meniai, 2017), microwave assisted extraction (Hu *et al*., 2017) and ultrasonic assisted extraction (UAE) (Gulzar and Benjakul, 2018). UAE has been demonstrated as a promising method in enhancing the yield of lipids and carotenoids. However, UAE had a disadvantage by inducing the deterioration, both oxidation and hydrolysis, of extracted lipid. Lipid oxidation was stimulated by incorporation of oxygen and mechanical effects caused by cavitation phenomenon. UAE was found to facilitate hydrolysis, particularly by exposing more substrates to enzymes (Gulzar and Benjakul, 2018). Flavonoid content (FC) and total phenolic content (TPC) in ethanolic extract from *Ocimum tenuiflorum* leaves were increased ($p<0.05$) with increasing ultrasonic energy density (Upadhyay *et al.*, 2015). UAE also resulted in an increase in the extraction yield of phenolic acids from Satsuma mandarin (*Citrus unshiu Marc*) peels under the controlled temperatures (Ma *et al.*, 2009).

Tannic acid is an antioxidant as well as lipase inhibitor (Horigome *et al.*, 1988; Longstaff and McNab, 1991). Tannic acid at a concentration of 15 μg/mL inhibited lipid peroxidation of linoleic acid emulsion by 97.7% (Gülçin *et al.*, 2010).

Additionally, tannins extracted from plants and berries have been found to inhibit digestive enzymes (McDougall *et al.*, 2009). Apart from using enzyme inhibitor, heating effectively inactivate the enzyme via thermal denaturation (Klibanov, 1983). Also heat could disrupt the lipid tissues, releasing more lipid for further extraction. Autolysis of hepatopancreas at 60 °C was found to increase the yield and carotenoid from the Pacific white shrimp cephalothorax (Senphan and Benjakul, 2012). Additionally high temperature is shown to have an impact on extraction of lipid by increasing the solubility of solvents (Dubrow *et al.*, 1973). Heat also causes the proteins in the cell membrane to denature, favouring permeability of solvent (Quinn, 1988). To maintain the quality of lipid from cephalothorax using UAE, the appropriate pre-treatment should be implemented before extraction. To lower both hydrolysis mediated by enzymes and oxidation of lipid extracted by ultrasound waves, inactivation of enzymes by pre-heating or use of inhibitor along with antioxidant could pave the way for lowering deterioration of lipid.

3.3 Objective

To investigate the effect of autolysis, pre-heating and tannic acid under different conditions on the yield and properties of lipid extracted from Pacific white shrimp cephalothorax using UAE process.

3.4 Materials and methods

3.4.1 Chemicals

ρ-nitrophenyl palmitate, palmitic acid, ρ-anisidine and cupric acetate were procured from Sigma (St. Louis. MO, USA). Isooctane, ethanol, ferrous chloride and trichloroacetic acid were acquired from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were obtained from Fluka (Buchs, Switzerland). Petroleum ether, n-hexane, isopropanol and hydrochloric acid were procured from Lab-Scan (Bangkok, Thailand).

3.4.2 Procurement and preparation of Pacific white shrimp cephalothorax

Ten (10 kg) samples of Pacific white shrimp (*Litopenaeus vannamei*) cephalothorax were procured locally from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. The samples were kept in polyethylene bags and imbedded inside a polystyrene box, which contained ice, twice as much as the sample by weight. Cephalothorax was ground for 10 min with the help of a blender (National Model MK-K77, Tokyo, Japan) to obtain a homogenous paste.

3.4.3 Extraction of crude lipase

Ground cephalothorax was defatted by acetone following the method of Senphan *et al.* (2015). Crude lipase extraction was performed by the method of Bouchaâla *et al*. (2015) with slight modifications. Acetone powder (50 g) was mixed with extraction buffer (25 mM Tris–HCl, pH 8.0 containing 2.5 mM benzamidine and 1 mM $CaCl_2$) at a ratio of 1:50 (w/v) and stirred at 4 °C for 3 h. To remove the tissue debris, the suspension was centrifuged for 10 min at 4 °C at 10,000×g using a refrigerated centrifuge (model CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant was filtered through a Whatman No.1 filter paper. The filtrate was used as crude lipase.

3.4.4 Lipase activity assay

Lipase activity was examined as per the method of Kurtovic *et al.*, (2010) using ρ-nitrophenyl palmitate (ρ-NPP) as substrate. 15mM concentration of ρ-NPP dissolved isopropanol was used as stock solution. 0.25 mM ρ-NPP mixed with 20 mM Tris–HCl buffer (pH adjusted to 8.0) containing 5 mM Na cholate, 20 mM CaCl2 and 0.01% gum arabic was used as substrate working solution. The substrate working solution was pre-incubated for 20 min at 50 °C before the assay. Appropriately diluted enzyme solution (50 μL) was added to 3.0 mL of substrate working solution and the reaction mixture was then incubated for 5 min at 50 °C. Absorbance was measured at 410 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) to quantify the release of ρ-nitrophenol (ρ-NP). The amount of enzyme producing 1 μmol ρ-NP per min under the assay condition was defined as one unit (U)

of activity..

3.4.5 Effect of tannic acid at different levels on lipase inhibition.

Tannic acid was dissolved in deionized water to obtain different concentrations (0.1%, 0.5%, 0.75%, 1% and 1.5%, w/v). Fifty (50 μL) of tannic acid solution was added to 50 μL enzyme solution. The mixtures were left at room temperature for 20 min. Remaining lipase activity was measured in the same fashion as described above. Control was also prepared in the same manner, but deionized water was used instead of tannic acid solution.

Inhibitory activity of lipase was calculated as follows:

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100$$

where C and T are lipase activity of the control and solution containing tannic acid, respectively.

3.4.6 Effect of pre-heating and autolysis in combination with tannic acid on extraction yield and properties of lipid extracted by ultrasound waves

Ground cephalothorax (50 g) was added with 0.1% TA (w/w) and subjected to a pre-heating at a temperature of 95 °C for different times (15, 30 and 45 min). For another portion, ground cephalothorax without and with addition of 0.1% TA was incubated at 50 °C for 3 h for autolysis. After pre-heating or autolysis, all samples were suddenly cooled in ice water before lipid extraction.

To extract lipid, samples (50 g) was homogenized with 250 mL of the mixture of equal proportions of isopropanol and n-hexane at 4 °C for 2 min using IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 9500 rpm. Subsequently, the sample was subjected to ultrasonic assisted extraction (UAE) by an Ultrasonic Processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA). Ultrasonic extraction was performed following the method of Gulzar and Benjakul (2018). The input ultrasonic amplitude was fixed at 80% and the time of operation was 25 min at continuous mode. In order to calculate the actual energy dissipated during the sonication process, the ultrasonic energy density (UED) was

calculated using the following formula:

$$UED = \frac{\left(\frac{dT}{dt}\right) \times m \times C_p}{V}$$

where m and C_p are the mass (kg) and heat activity (J/kg-K) of the solvent, respectively, $\frac{dT}{dt}$ is the change in temperature during exposure to ultrasound energy and V is the volume of irradiated medium.

The UED value corresponding to the applied amplitude of 80% was calculated to be 0.12 W/cm^3. During the ultra-sonication process, the temperature of the sample was kept below 1 °C by placing in ice-bath. After the extraction, centrifugation of the solution was carried out at 4 °C for 25 min at 3000g followed by filtering through Whatman No. 4 filter paper. Rotary evaporator (EYELA N-1000, Tokyo Rikakikai, Co., Ltd.,Tokyo, Japan) was used to remove the solvent in the filtrate at 25 °C. The remainder solvent was removed by nitrogen flushing. Lipid samples were collected in vials, further flushed with nitrogen gas and then sealed tightly before being stored at −40 °C until analysis.

3.4.7 Analyses

3.4.7.1 Determination of extraction yield and total carotenoid content

Extraction yield was calculated by measuring the weight of extracted lipid and expressed as g/100g of starting ground cephalothorax.

Total carotenoid content in the lipid was measured by the method of Saito and Regier (1971) with slight modifications. Lipid sample (30 mg) was mixed with 10 mL of petroleum ether. The solution was appropriately diluted using petroleum ether as diluent. The absorbance was measured at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and the carotenoid concentration was calculated using the following equation:

$$\text{C } (\mu\text{g/g lipid}) = \left(\frac{\text{A468} \times \text{volume of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in grams}}\right)$$

where 0.2 is the A_{468} of 1 μg/mL standard astaxanthin.

3.4.7.2 Measurement of lipid oxidation/hydrolysis

3.4.7.1 Peroxide value (PV)

PV content were determined by the methods as described by Chaijan *et al.* (2006). Lipid sample (50 μL) was 10-fold diluted using 75% ethanol, (v/v). To the prepared sample, a mixture of 2.35 mL of 75% ethanol (v/v), 50 μL of 30% ammonium thiocyanate (w/v) and 50 μL of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added. The mixture was mixed well and the absorbance was read at 500 nm using a spectrophotometer. The blank was prepared in the same way, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

3.4.7.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined by the method described by Buege and Aust (1978). Lipid sample (0.5g) was mixed with 2.5 mL of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was then heated in boiling water (95–100°C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600g at 25°C for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was read at 532 nm using a spectrophotometer. A standard curve was prepared using malonaldehyde (MDA) at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde/ kg lipid.

3.4.7.3 Free fatty acid (FFA) content

FFA content was determined according to the method of Lowry and Tinsley (1976). To a lipid sample (0.1 g), 5 mL of isooctane were added and swirled vigorously to dissolve the sample. The mixture was added with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, shaken vigorously for 90 s and allowed to stand for 20 s. The absorbance of upper layer was read at 715 nm. A standard curve was

prepared using palmitic acid in isooctane at concentrations ranging from 0 to 10 μmol/mL. FFA content was expressed as g FFA/100 g lipid.

3.4.8 Characterization of lipids extracted from the selected pre-treated cephalothorax

3.4.8.1 Fatty acid profile

Fatty acid profile was examined by using fatty acid methyl esters (FAMEs) in the gas chromatography (GC) according to the AOAC method (AOAC, 1990). FAME was prepared by using BF_3/MeOH (14% boron trifluoride). Samples (100 mg) were saponified with 4 mL of 0.5 N methanolic sodium hydroxide solution for 15 min with boiling. The mixture was homogenized with 4 mL of BF_3/MeOH reagent and boiled for 10 min. After cooling, 6 mL saturated NaCl solution was added to the mixture, and 3 mL of n-hexane was added. The upper layer of the mixture was transferred to a 20 mL bottle containing anhydrous $Na_2S_2O_3$. The supernatant was directly analyzed by injecting to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with a 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 injection port was set at 250 °C and detector was set at 270 °C. The oven was programmed from 170 to 225 °C at a rate of 1 °C/min (no initial or final hold). The retention times of FAME standards were used to identify the chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100g lipid.

3.4.8.2 FTIR spectra

FTIR analysis of lipid samples was performed in a horizontal attenuated total reflectance trough plate crystal cell (45 ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany) as tailored by Takeungwongtrakul, Benjakul and H-Kittikun, (2012. Lipid sample (200 μL) was applied directly onto the crystal cell and the cell was mounted to the FTIR spectrometer. Spectra of mid-infrared region of the range of 4000–500 cm^{-1} with the

automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were resized against a background spectrum recorded from the clean empty cell at 25 °C. Spectral data analysis was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

3.4.8.3 Determination of carotenoids

Thin-layer chromatography (TLC) was employed for determination of carotenoids using activated 20 × 20 cm silica gel plates (silica gel G type 60, Merck) following the method of Takeungwongtrakul *et al.* (2015). The sample applied to the plates was placed in the mobile phase (acetone: hexane, 25:75, v/v) for the separation to take place. Identification of carotenoids was done by comparing the retention factor (R_f) of individual band with those of standards.

3.4.9 Statistical analysis

All the experiments were performed in triplicates and the results were expressed as means ± SD. Analysis of variance (ANOVA) was used for data analysis and means were compared by the Duncan's multiple range test. For pair comparison, the t-test was used (Steel and Torrie, 1980). SPSS by IBM (SPSS 22.0 for windows, IBM, Armonk, NY, USA) was used for statistical analysis of all the data recorded.

3.5 Results and discussion

3.5.1 Effect of tannic acid at different levels on inhibition of lipase from Pacific white shrimp cephalothorax

Lipase activity in crude extract from Pacific white shrimp cephalothorax had the specific activity of 0.15±0.02 units/mg protein. Lipases could hydrolyze the ester bond in ρ-nitrophenyl palmitate (ρ-NPP), used as a substrate, to release ρ-nitrophenol and palmitic acid. Inhibitory effects of TA at different concentrations on lipase activity of crude extract from Pacific white shrimp cephalothorax are illustrated in Figure 15. Inhibition was in dose-dependent manner. Nevertheless, there was no difference in the relative inhibition between 1% and 1.5% (w/v) TA ($p>0.05$). Inhibition of lipase might be partly due to the formation of a

substrate-tannin complex, in which lipase could not hydrolyze the substrate (Kumar and Singh, 1984). Furthermore, lipase could be inhibited by TA, known as protein cross-linker, might bind enzyme, thus inducing the conformational change. As a result, lipase activity was lowered. TA was found to inhibit lipase extracted from porcine pancreas (Tamir and Alumot, 1969). Berry extract rich in tannin showed *in vitro* inhibitory activity towards porcine pancreatic lipase (McDougall *et al*., 2009). The results indicated that lipase was strongly inhibited by TA. This could help lower the hydrolysis of lipid from shrimp cephalothorax caused by lipase.

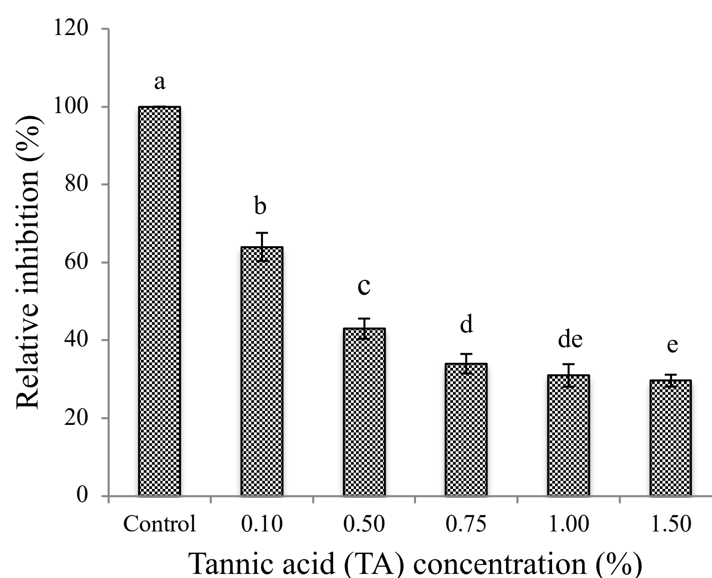

Figure 15. Effect of tannic acid (TA) concentration on inhibition of lipase in crude extract from Pacific white shrimp cephalothorax. Bars represent the standard deviation (n=3). Different lowercase letters on the bars denote significant difference ($p<0.05$).

3.5.2 Effect of pre-treatment and tannic acid incorporation on extraction yield and properties of lipid

3.5.2.1 Yield and carotenoid content

Effect of pre-heating at 95 °C at various times and autolysis in the presence of 0.1% TA on extraction yield of lipid from shrimp cephalothorax extracted by UAE is shown in Figure 16A. Heat treatment at high temperature (95 °C)

increased the extraction yield significantly ($p<0.05$). Samples subjected to pre-heating for 30 min and 45 min showed the highest yield ($p<0.05$). Heating could assist in extraction process by thermally disrupting the tissues and facilitating the solvent penetration into the tissues. Lipid extraction from whole red hake increased with increasing temperature used for heating (Dubrow *et al*., 1973). The sample subjected to autolysis (in the presence of TA) for 3 h at 50 °C also showed an increase in the extraction yield ($p<0.05$), compared to the control (without any heat treatment or autolysis). Sample subjected to autolysis without addition of TA also had a higher yield than control sample but the yield was lower than that obtained from sample autolyzed in the presence of TA. This could be attributed to the hydrolysis of lipoproteins, thereby causing the release of additional free lipids, which could be subsequently extracted. Autolysis of Pacific white shrimp hepatopancreas prior to extraction at for 60 min at 60 °C increased the extraction yield (Senphan and Benjakul, 2012). In the present study, after the autolysis or pre-heating at high temperature in the presence of tannic acid, lipase inhibitor, the sample was extracted with the aid of ultrasound. Cavitation effect more likely enhanced the extraction of lipid associated with tissues. Carotenoid content was also found to be higher in heat-treated samples prior to extraction by UAE. The similar trend was obtained to the extraction yield as shown in Figure 16B. Hydrolysis of carotenoproteins in the autolyzed samples could have resulted in the increase in the carotenoid content of the extracted lipids.

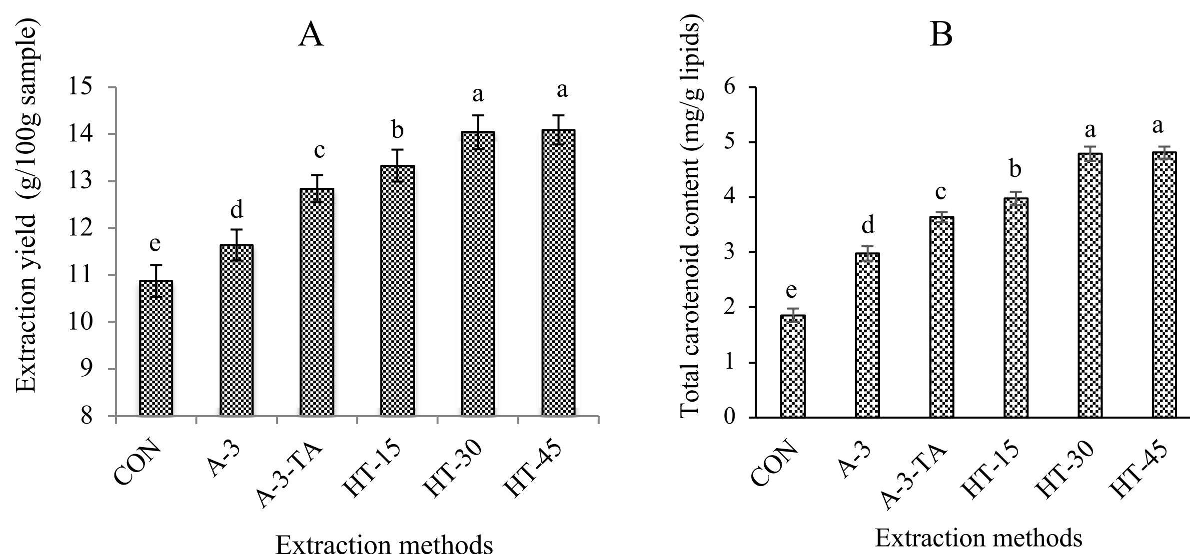

Figure 16. Effect of different pre-treatments on extraction yield of lipids (A) and carotenoid content of lipids (B) from cephalothorax of Pacific white shrimp. CON: Without pre-treatment; A-3: Autolysis at 50 °C for 3 h; A-3-TA: Autolysis at 50 °C for 3 h in the presence of 0.1% tannic acid; HT-15, HT-30, HT-45: Pre-heating at 95 °C for 15, 30 and 45 min, respectively, in the presence of 0.1% tannic acid. All lipids were extracted using UAE (continuous mode, 80% amplitude) for 25 min. Bars represent the standard deviation (n=3). Different lowercase letters on the bars denote significant difference (p<0.05).

3.5.2.2 Lipid oxidation/hydrolysis

3.5.2.2.1 Peroxide value (PV)

PV of lipid samples extracted by UAE from cephalothorax with different pre-treatments is depicted in Figure 17A. Highest PV was found in sample subjected to autolysis for 3 h at 50 °C without adding TA, followed by the control sample (without any pre-treatment). For the sample with autolysis in the presence of TA, lower PV was obtained. TA could act as the potential antioxidant toward lipid oxidation during incubation at 50 °C for 3 h. Furthermore, the samples pre-heated at 95 °C had significantly lower PV (p<0.05) than others, regardless of pre-heating time

used. PV of lipid is determined by many factors including method of extraction, oxygen saturation, fatty acid composition etc. (Gulzar and Benjakul, 2018). In general, lipid extracted with UAE showed higher PV, particularly when higher amplitudes were applied. Due to cavitation effect, higher oxygen was incorporated into the system, along with the increase in localized temperature (Gulzar and Benjakul, 2018). PV was increased as a result of hydroperoxides formed by the reaction of unsaturated lipids and singlet oxygen or the oxidation of polyunsaturated fatty acids by the action of lipoxygenase (Nawar, 1996). Shrimp head was found to be containing lipoxygenase, indigenously (Shye *et al.*, 1987). Lipid oxidation is generally more pronounced during autolysis (50 °C) (Senphan and Benjakul, 2012). This was indicated by the highest PV in the sample autolyzed without the addition of TA. TA with free radical scavenging activity was able to prevent lipid oxidation by scavenging lipid radicals (Maqsood and Benjakul, 2010). The results indicated that addition of TA suppressed the lipid oxidation effectively ($p<0.05$). Pre-heating at high temperature might cause the denaturation of lipoxygenase, resulting in lower PV values ($p<0.05$). However, pre-heating time (15-45 min) had no significant effect on PV of resulting extracted lipid ($p>0.05$).

3.5.2.2.2 TBARS

Figure 17B shows TBARS values of lipids extracted from cephalothorax of Pacific white shrimp with different pre-treatment conditions by UAE. TBARS values for all samples varied between 29 and 74 MDA/kg lipid. Highest TBARS value was found in sample autolyzed for 3 h at 50 °C without the addition of TA. This showed similar trend to PV result (Figure 17A). The control sample (without pre-treatment) had slightly lower PV. TBARS of samples autolyzed for 3 h with TA addition was lower ($p<0.05$) than that without TA and the control. TA was found to retard lipid oxidation in striped catfish (Maqsood and Benjakul, 2010) and TBARS values were increased at lower rate during refrigerated storage. TBARS is generally used as an index for quantifying relatively polar secondary oxidation products including aldehydes and ketones, etc (Nawar, 1996). Chaijan *et al.* (2006) reported that the increase in TBARS value of lipids was caused as a result of

formation of the secondary lipid oxidation products. Lipids extracted with UAE had higher TBARS, primarily due to oxidation of PUFAs, which are more prone to oxidation (Gulzar and Benjakul, 2018). TBARS value of lipids extracted from Pacific white shrimp hepatopancreas increased with increasing temperature (Senphan and Benjakul, 2012). For the samples subjected to pre-heating, TBARS were slightly lower as temperature increased ($p<0.05$). Heat more likely inactivated most enzymes including lipase and phospholipase, in which free fatty acids were less liberated. Also, lipoxygenase might be inactivated, leading to less oxidation. The hydroperoxides are decomposed to produce off-flavor volatile compounds that deteriorate the lipid quality (Choe and Min, 2006). This coincided with the increase in TBARS. The results therefore established the fact that lipid oxidation was markedly lowered when cephalothorax was pre-heated at high temperature in the presence of TA before UAE process. TA most likely functioned as antioxidant and enzyme inhibitor (Figure 15).

3.5.2.2.3 Free fatty acid (FFA) content

FFA contents of lipids extracted from cephalothorax of Pacific white shrimp are shown in Figure 17C. The sample subjected to autolysis at 50 °C for 3 h without TA addition showed the highest FFA content ($p<0.05$). This was primarily due the action of lipase on the glycerol ester bond of lipid and phospho-ester bond of phospholipid. Lipases catalyze the hydrolysis of glycerol–fatty acid esters and phospholipases, causing the release of free fatty acids (Pacheco-Aguilar *et al.*, 2000). During ultrasonication process, hydrolysis was further enhanced by the release of lipases from cell matrix. Sharp increase in FFA content was found in lipids extracted from hepatopancreas of Pacific white shrimp when autolyzed at 60 °C (Senphan and Benjakul, 2012). Free fatty acids liberated were also susceptible to oxidation as shown by the increases in PV and TBARS (Figure 17A and 17B). PV and TBARS assessments include oxidation of all kinds of lipid molecules. Accumulation of FFA has been shown to accelerate lipid oxidation and produce off odour (Mackie, 1993). FFAs, particularly PUFAs which arise from phospholipid hydrolysis, are more sensitive to oxidation (Moerck and Ball, 2018). However, the drastic decrease in FFA was noticeable when TA was added during autolysis of cephalothorax prior to UAE

process. The samples pre-treated at high temperature and added with TA showed the lower FFA content ($p<0.05$). This was plausibly due the inactivation of lipases as demonstrated by the addition TA and heat treatment at high temperature. The increase in FFA content is also governed by the shear and mechanical effects of UAE mediated by cavitation (Gulzar and Benjakul, 2018). In the presence of TA, it could scavenge some radicals generated via cavitation. This could lower the breakdown of ester bond.

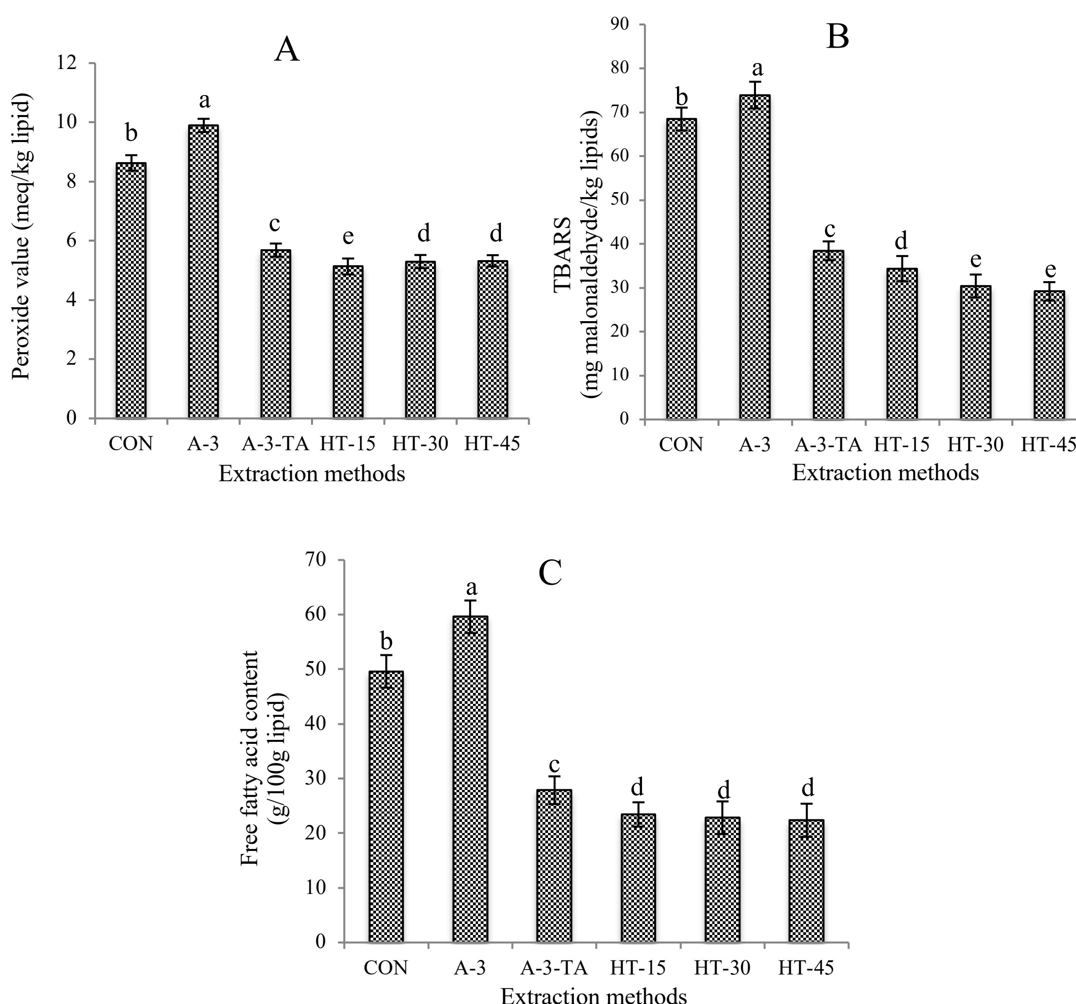

Figure 17. Peroxide value (A), TBARS value (B) and free fatty acid content (C) of lipids from cephalothorax of Pacific white shrimp extracted by UAE as affected by different pre-treatments. Caption see Figure 16.

3.5.3 Characteristics of lipids extracted from cephalothorax with the selected pre-treatment

3.5.3.1 Fatty acid profiles

Table 4 illustrates the fatty acid profiles of lipids extracted from Pacific white shrimp cephalothorax by UAE process (without pre-treatment) (CON) and lipids extracted from pre-treated sample added with 0.1% TA (95 °C for 30 min) followed by UAE, (PH-TA). CON sample contained 28.36 g/100g saturated fatty acids, 20.89 g/100g MUFA and 36.5 g/100g PUFA. PH-TA consisted of 28.22 g/100g saturated fatty acids, 20.63 g/100g MUFA and 37.76 g/100g PUFA. PUFAs were the major fatty acids found in both the samples. Takeungwongtrakul *et al.*, (2012); Gulzar and Benjakul, (2018) also found PUFAs to be the major fatty acids in Pacific white shrimp lipids. Overall, palmitic acid was present as the most abundant fatty acid, followed by oleic acid and stearic acid. When comparing the contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) between both samples, slightly lower contents of both EPA and DHA were found in CON sample, compared to PH-TA counterpart. The decreases in EPA and DHA were plausibly attributed to their oxidation during ultra-sonication process by the incorporation of oxygen. For PH-TA sample, cephalothorax was heated in the presence of TA. Hydrolytic or oxidative enzymes were mostly inactivated. Also, during UAE process, TA might play a role in suppressing the oxidation of lipids. As a consequence, DHA and EPA were more retained after the extraction by UAE process. PH-TA sample had the increase in polyene index (ratio of DHA+EPA/C16:0) by 10.72%, compared to that of CON sample. This reconfirmed the oxidative stability of the former. Due to lipid oxidation, DHA and EPA were decreased, while palmitic acid (C16:0) was unchanged. This in turn decreased the polyene index (Belhaj *et al.*, 2010). EPA and DHA reportedly have been shown to possess excellent health benefits especially anti-inflammatory, anti-thrombosis and anti-arrhythmia properties. They are beneficial for people suffering from cardio vascular diseases (Sahena *et al.*, 2009). Thus, pre-heating and incorporation of 0.1% TA in cephalothorax prior to extraction using UAE process could retard lipid oxidation, especially PUFA

Table 4. Fatty acid profile of lipids extracted from cephalothorax of Pacific white shrimp using different methods.

Fatty acids (g/100 g lipids)	CON*	PH-TA*
C12:0	0.05 ± 0.00a	0.04 ± 0.01a
C14:0	0.51 ± 0.01a	0.50 ± 0.00a
C14:1	0.11 ± 0.01a	0.11 ± 0.00a
C15:0	0.34 ± 0.05a	0.37 ± 0.01a
C15:1	0.25 ± 0.01b	0.51 ± 0.03a
C16:0	15.99 ± 0.08a	15.84 ± 0.02a
C16:1 n-7	1.34 ± 0.01a	1.27 ± 0.00b
C17:0	1.17 ± 0.00a	1.17 ± 0.00a
C18:0	9.29 ± 0.04b	9.08 ± 0.01a
C18:1 n9 trans	0.19 ± 0.01a	0.21 ± 0.00a
C18:1 n11 trans	0.15 ± 0.00a	0.15 ± 0.00a
C18:1 n-9	14.35 ± 0.06b	13.92 ± 0.05a
C18:1 n-7	2.49 ± 0.01b	2.39 ± 0.00a
C18:2 n-6	11.62 ± 0.01b	11.39 ± 0.02a
C18:2t9t12 trans	0.09 ± 0.00a	0.10 ± 0.01a
C18:3 n-3 (ALA)	0.66 ± 0.01a	0.62 ± 0.01a
C18:4 n-3	0.08 ± 0.00a	0.08 ± 0.00a
C20:0	0.39 ± 0.00a	0.40 ± 0.01a
C20:1 n-9	1.20 ± 0.00a	1.20 ± 0.01a
C20:2 n-6	1.58 ± 0.01a	1.59 ± 0.02a
C20:3 n-6	0.16 ± 0.00a	0.16 ± 0.00a
C20:3 n-3	0.23 ± 0.00a	0.22 ± 0.01a
C20:4 n-6 (ARA)	4.91 ± 0.01a	4.91 ± 0.03a
C20:4 n-3	0.14 ± 0.00a	0.12 ± 0.00a
C20:5 n-3 (EPA)	7.09 ± 0.03a	7.92 ± 0.02b
C21:0	0.11 ± 0.00a	0.12 ± 0.01a
C22:0	0.51 ± 0.01a	0.57 ± 0.01a
C22:1 n-9	0.13 ± 0.00a	0.15 ± 0.01a
C22:6 n-3 (DHA)	9.84 ± 0.00a	10.65 ± 0.02b
C23:0	ND	0.13 ± 0.00
C24:1	0.68 ± 0.00a	0.72 ± 0.01b
Unidentified peak	14.24 ± 0.00b	13.38 ± 0.19a
Saturated fatty acid (SFA)	28.36 ± 0.01b	28.22 ± 0.01a
Monounsaturated fatty acid (MUFA)	20.89 ± 0.01b	20.63 ± 0.00a
Polyunsaturated fatty acid (PUFA)	36.5 ± 0.01a	37.76 ± 0.01b

CON represents sample extracted by UAE without pre-treatment; PH-TA represents sample extracted by UAE with prior heating (95 °C for 30 min) in the presence of 0.1% tannic acid.

Different lowercase letters in the same row indicate significant differences ($p<0.05$).

3.5.3.2 FTIR spectra

Figure 18 represents the FTIR spectra of CON and PH-TA samples. Similar spectra were observed in both the samples with the dominant peaks observed at the wavenumber range of 3150–2800 cm^{-1}, representing C–H stretch vibrations and overlapping with the –OH group in carboxylic acid. C–H stretching vibration bands between at 3100 and 2854 cm^{-1} represents the degree of unsaturation of fats and oils (Afran and Newbery, 1991). This was concomitant with the results obtained from fatty acid profile (Table 4), showing that lipids extracted from shrimp cephalothorax had high levels of PUFAs. Stretching peaks found between 3600 and 3100 cm^{-1} represented the symmetrical stretching vibration of $–CH_2$, OH or NH groups. The peak observed at 3400 cm^{-1} was due to their –OO–H stretching vibrations (Van De Voort *et al.*, 1994), reflecting the formation of hydroperoxide. It was found that the PH-TA sample had smaller peaks at 3400 cm^{-1}, compared to the CON counterpart, indicating less presence of hydroperoxides, as confirmed by PV (Figure 17A). The peaks at wavenumbers of 1736-1745 cm^{-1} represent the ester carbonyl group of triglycerides (Takeungwongtrakul *et al.*, 2015). Similar peaks were observed at 1744 cm^{-1}, indicating that triglycerides were present in both the samples. However, the PH-TA sample showed a larger peak. The decrease in the peak of CON sample was plausibly due to the hydrolysis of the ester bond by lipases. The stretching vibration bands near 1237 and 1209 cm^{-1} are directly proportional to the presence of saturated acyl groups in oil associated with the C–O ester groups and with the bending vibration of the CH_2 group. The bands at approximately 1117 and 1099 cm^{-1} are inversely to the proportion of saturated acyl groups associated with the stretching vibration of the C–O ester groups (Guillén and Cabo, 1997). The FTIR result confirmed that lipid oxidation was suppressed to some degree in pre-treated sample added with 0.1% TA (PH-TA sample), while sample without pre-treatment and TA incorporation exhibited higher oxidation and hydrolysis due to the cavitation and mechanical shearing by ultrasonic process.

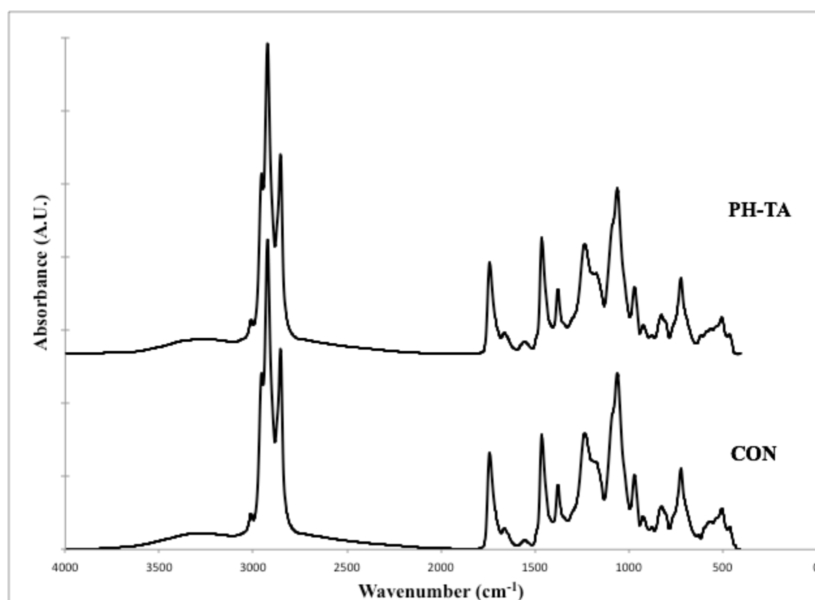

Figure 18. FTIR spectra of lipids extracted from cephalothorax of Pacific white shrimp using the selected extraction methods. CON: Lipids extracted by UAE without pre-treatment. PH-TA: Lipids extracted by UAE with prior heating (95 °C for 30 min) in the presence of 0.1% tannic acid.

3.5.3.3 Carotenoids

Thin-layer chromatograms of CON and PH-TA samples are illustrated in Figure 19. TLC is a fast and simple technique used to separate carotenoids, and is widely used due to its low cost (Sánchez-Camargo *et al.*, 2011). Prominent bands at retention factors (R_f) of 0.33, 0.43, 0.48, 0.69, 0.77, 0.85 and a small band at R_f 0.97 were obtained in the chromatogram. Bands with R_f of 0.33 and 0.97 were identified to be free astaxanthin and β-carotene, respectively. Free astaxanthin from lipid of Brazillian redspotted shrimp corresponded to R_f of 0.33 (Sánchez-Camargo *et al.*, 2011). The bands with R_f of 0.40, 0.50 and 0.75 correspond to canthaxanthin, astaxanthin monoester and astaxanthin diester, respectively (Takeungwongtrakul *et al.*, 2015). There was the marked difference in the band intensity and color between both samples. PH-TA sample showed the bands with higher color intensity, compared to CON sample, indicating higher presence of carotenoids of the former. This was plausibly due to the fact that TA incorporated could act as antioxidant during pre-treatment and UAE extraction. This could lower the oxidation of carotenoids in the

PH-TA sample.

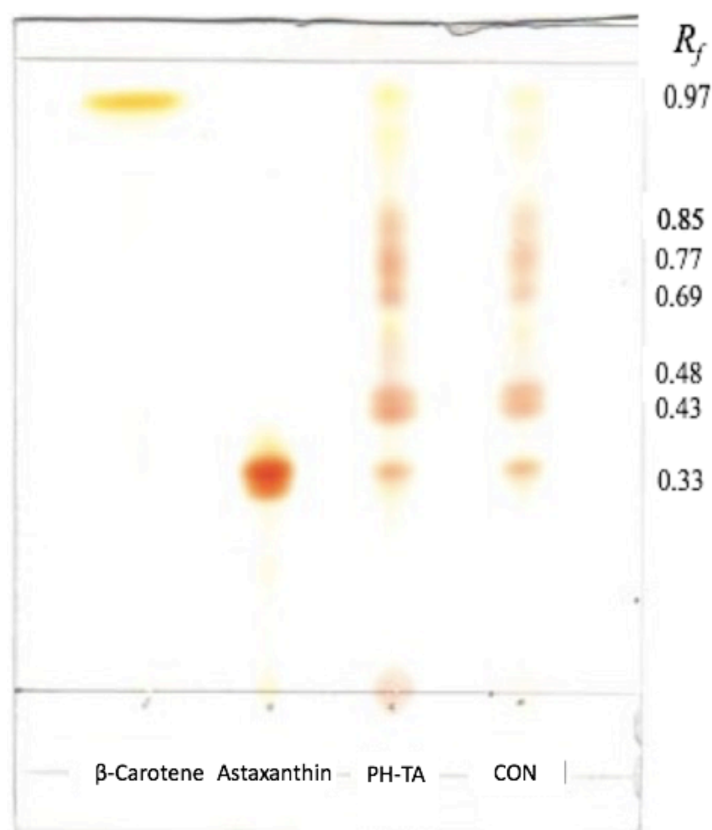

Figure 19. Thin-layer chromatography (TLC) of lipids extracted from cephalothorax of Pacific white shrimp extracted using the selected extraction methods. CON: Lipids extracted by UAE without pre-treatment. PH-TA: Lipids extracted by UAE with prior heating (95 °C for 30 min) in the presence of 0.1% tannic acid.

3.6 Conclusion

Pre-heating at 95 °C of cephalothorax of Pacific white shrimp in the presence of TA (0.1%) proved to be a beneficial pre-treatment for lipid extraction by UAE process. The yield of lipids and carotenoids from Pacific white shrimp cephalothorax were markedly increased. Conversely, prior autolysis was detrimental to the quality of lipid, especially when UAE was implemented. The lipid suffered less oxidation and hydrolysis due to the combined effect of pre-heating and addition of TA, which suppressed the hydrolysis by inhibiting the lipase and lowered the oxidation.

Therefore the combination of pre-heating and TA addition was successful in maintaining the quality of lipid from cephalothorax of Pacific white shrimp extracted by UAE, while providing high yield of lipids.

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CHAPTER 4

IMPACT OF PULSED ELECTRIC FIELD PRE-TREATMENT ON YIELD AND QUALITY OF LIPID EXTRACTED FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) BY ULTRASOUND ASSISTED PROCESS

4.1 Abstract

Pacific white shrimp cephalothorax was subjected to pulsed electric field (PEF) pre-treatment at different electric field strengths (4, 8, 12 and 16 $kVcm^{-1}$) and pulse numbers (120, 160, 200 and 240). PEF treated samples were subsequently subjected to lipid extraction using ultrasonic assisted extraction (UAE) process at ultrasonic amplitude of 80% for 25 min in continuous mode. PEF pre-treated samples subjected to UAE rendered the highest lipid yield (10.44 g/100g sample). PEF pre-treatment resulted in reduced lipid oxidation as affirmed by the decreases in peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). Lipid from PEF pre-treated samples extracted using UAE had higher content of PUFAs as well as carotenoids, including astaxanthin, astaxanthin monoester, astaxanthin diester, canthaxanthin and β-carotene. Overall, PEF pre-treatment helped in enhancement of extraction yield of lipids and carotenoids from shrimp cephalothorax and reduce lipid oxidation to some extent.

4.2 Introduction

Shrimp processing industry in Thailand holds a share of approximately 8% of global shrimp production. In 2017, Thailand exported processed shrimp/prawns having a value of $1.8 billion, which is roughly one-third of the total seafood exports (GAIN, 2018). A huge amount of by-products including cephalothorax, carapace and tail are generated from shrimp processing industry, which mainly end up either as feed for animals or supplementary food for aquaculture (Nwanna *et al.*, 2004). Several studies have demonstrated that lipid from Pacific white shrimp (*Litopenaeus vannamei)* cephalothorax contains useful bioactive compounds such as ω-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and

carotenoids including β-carotene and astaxanthin (Amiguet *et al.*, 2012). Astaxanthin is a carotenoid found in crustaceans and is more potent antioxidant compared to canthaxanthin, zeaxanthin and lutein (Naguib, 2000). It is commercially sold in the form of capsules and is proven to have anti-oxidative and anti-inflammatory effects (Gómez-Estaca *et al.*, 2017).

Lipids and carotenoids from cephalothorax of Pacific white shrimp have been extracted using ultrasonic-assisted extraction (UAE). UAE was found to be a promising method in enhancing the extraction yield (Gulzar and Benjakul, 2018). However, UAE had limitations and disadvantages since it induced both oxidation and hydrolysis of extracted lipid. Therefore non-destructive pre-treatment or process is required before lipid extraction. Pulsed electric field (PEF) is a non-thermal technology, in which a material is placed between two electrodes. High-voltage pulses, for very short periods of time usually between microseconds to milliseconds, are passed through it. There is a negligible increase in temperature of treated sample and cost of operation is low (Toepfl *et al.*, 2006). High voltage pulses with sufficient strength causes electroporation of the cell membrane in plant and animal tissues. Electroporation results in the increased permeability of cell membranes, made up of charged ions and proteins. Due to the ionic gradient caused by these charged ions, a transmembrane potential (TMP) exists across the cell membrane. When an external electric field is applied above TMP, polarity is induced in the cells, creating a charge separation, and produces a dipole moment that is parallel to the external field (Zbinden *et al.*, 2013). Due to the presence of this external field with short duration pulses, pores are formed (Teissie et al., 2005).

PEF has been introduced for pre-treatment of maize, olives, soybeans and rapeseed before oil extraction (Guderjan *et al.*, 2005; Guderjan *et al.*, 2007). PEF pre-treatment improved oil extraction yield significantly and increased the amount of bioactive compounds in the oil. Additionally, PEF is believed to show an inactivation effect on enzymes. Ho *et al.* (1997) reported that the activities of α-amylase, glucose oxidase, and lipase were decreased by 70% - 85% post PEF treatment. An irreversible reduction of heat-stable papain was documented after PEF treatment at 50 $kVcm^{-1}$.

(Yeom *et al.*, 1999). Drastic reduction (97%) in polyphenol oxidase (PPO) was found in the apple extract with PEF treatment at 24.6 $kVcm^{-1}$ for 6000 s and PPO was reduced by 72% in pear extract when PEF at 22.3 $kVcm^{-1}$ for 6000 s was applied (Yeom *et al.*, 1999).

4.3 Objective

To study the effect of PEF pre-treatment on the extraction yield and quality of lipid extracted from Pacific white shrimp cephalothorax using UAE process.

4.4 Materials and methods

4.4.1 Sample procurement and preparation

Cephalothorax of freshly harvested Pacific white shrimp (*Litopenaeus vannamei*) was procured from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. The samples (10 kg) kept in a polyethylene bag were further imbedded in a styrofoam box, which contained ice (20 kg), and transported to the seafood chemistry and biochemistry laboratory, Department of Food Technology, Prince of Songkhla University, Hat Yai, Songkhla, within approximately 1 h.

4.4.2 PEF set-up and treatments

PEF set-up is shown in Figure 20A. The apparatus used for the PEF treatment consisted of a high voltage power supply (PEF LAB-400W, Febix International Inc., Chiang Mai, Thailand), a stainless steel treatment chamber with 1.5 L capacity equipped with a stainless steel plate electrode 20 cm × 10 cm, a plexiglass cabinet with aluminium frame and a digital storage oscilloscope (DSO) (UTD2052CEX, UNI-T®, Dongguan city, Guangdong, China) with a UT-P03 oscilloscope probe (60 MHz, ×10). Firstly, whole cephalothorax (100 g) was placed on the perforated plastic mesh to avoid the contact with the bottom electrode. Thereafter tap water (500 mL) was added and samples were cross-checked for the complete immersion of samples in water. A gap of 1.5 cm was maintained between the electrode and the bottom of the chamber (including the plastic mesh). High voltage pulses were passed through the sample. The circuit layout of the PEF system

is illustrated in Figure 20B. Different treatment voltages were applied by changing the amplitude and number of pulses for each treatment. The voltage (4 - 16 $kVcm^{-1}$) was varied using the voltage regulator and the pulse number (120 - 240) was varied by adjusting the gap between spark caps. The DSO was used to monitor the electric field strength, frequency and pulse width of the applied electric pulses. The temperature was maintained at below 1 °C by placing the chamber in the iced water.

After PEF treatment, water used for submerging cephalothorax was taken and dried in the oven at 105 °C for 24 h (ULM 400, Memmert GmbH, Schwabach, Germany) to estimate the oil lost in the water. The cell disintegration index of sample was calculated by using the following formula

$$Z_c = \frac{\sigma_t - \sigma_i}{\sigma - \sigma_i}$$

where σ_t is the conductivity of the sample after pulsed electric treatment; σ_i is the conductivity of the initial intact sample and σ is the conductivity of the maximally disintegrated sample in water (by treating the samples at 30 $kVcm^{-1}$ for 20 min).

The conductivity was determined using LCR meter (UT612, UNI-T®, Dongguan city, Guangdong, China.

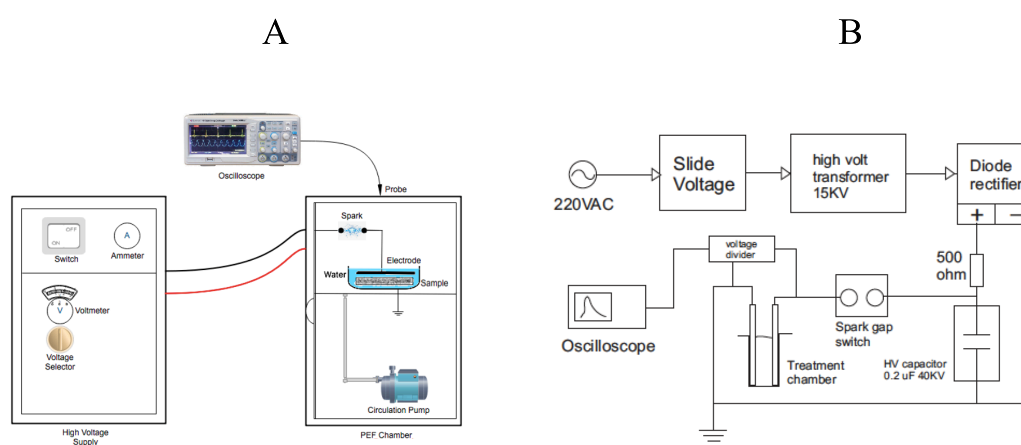

Figure 20. Pulsed electric field (PEF) setup (A) and its circuit layout (B)

4.4.3 Extraction of lipid with PEF pre-treatment using solvent with the aid of ultrasound assisted extraction (UAE)

Different PEF treated cephalothorax samples were ground for 5 min using a blender (National Model MK-K77, Tokyo, Japan) to obtain a homogenous paste. For each sample with different PEF conditions, the sample was divided into 2 portions. The first portion was subjected to extraction using isopropanol and hexane (1:1) for 2 min as described by Gulzar and Benjakul, (2018). Ground cephalothorax (50 g) was homogenized with 250 mL of solvent at a speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) at 4 °C for 2 min. The homogenate was centrifuged at 3000g for 15 min at 4 °C using a Hitachi centrifuge (Hitachi Koki Co., Ltd, Tokyo, Japan). The supernatent was transferred into a flask containing 2–5 g of anhydrous sodium sulphate, followed by filtration using a Whatman no. 4 filter paper (Whatman International Ltd., Maidstone, England). The filtrate was transferred into a round bottom flask and evaporated using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 25 °C. The residual solvent was removed by nitrogen flushing. The lipid obtained from typical solvent extraction process (without PEF pre-treatment) was termed as 'S' and the lipid obtained by solvent extraction with prior PEF pre-treatment was termed as 'PEF-S' For the second portion, the prepared samples (50 g) were homogenized with 250 mL of the mixture of isopropanol and n-hexane (1:1) at 4 °C for 2 min at a speed of 9500 rpm. Thereafter, the sample was subjected to UAE by an Ultrasonic Processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA). The solvent was removed as mentioned above. Lipids obtained from the sample without and with prior PEF pre-treatment, followed by UAE were named 'S-U' and 'PEF-S-U', respectively. The lipid extracted using solvent extraction with prior PEF pre-treatment was referred to as 'PEF-S'. The input ultrasonic amplitude was fixed at 80% and the time of operation was 25 min at continuous mode. In order to calculate the actual energy dissipated during the sonication process, the ultrasonic energy density (UED) was calculated using the formula

$$UED = \frac{\left(\frac{dT}{dt}\right) \times m \times C_p}{V}$$

where m is the mass (kg), C_p is the specific heat (J kg-K^{-1}) of the solvent, $\frac{dT}{dt}$ is the change in temperature during ultrasonication and V is the volume of irradiated medium. The UED value corresponding to the applied amplitude of 80% was calculated to be 0.12 Wcm^{-3}. To avoid the temperature rise during sonication process, the sample was placed in an iced-bath. After the ultra-sonication, the lipids were separated from the solvent by centrifugation and evaporation by the process described by Gulzar and Benjakul, (2018). Lipids were collected and transferred into vials, flushed with nitrogen gas and tightly sealed before being stored at −40 °C until analyses.

4.4.4 Analyses

4.4.4.1 Determination of extraction yield of lipids

Extraction yield was calculated by measuring the weight of extracted lipid and expressed as g/100g of starting ground cephalothorax.

4.4.4.2 Determination of total carotenoid content

Carotenoid contents (µg/g lipid) in the obtained lipids were determined by measuring the absorbance of appropriately diluted sample at 468 nm, according to the method of Saito and Regier (1971)

4.4.4.3 Determination of oxidation and hydrolysis of lipids

Peroxide Value (PV) and thiobarbituric acid reactive substances (TBARS) are used as indicators for lipid oxidation, while free fatty acid (FFA) content is used as an indicator for lipid hydrolysis. PV was determined using the ferric thiocyanate method as described by Chaijan *et al.* (2006). TBARS were examined by the method tailored by Buege and Aust (1978). FFA content was determined following the method of Lowry and Tinsley (1976).

4.4.4.4 Characterization of lipids extracted using the selected methods

Lipid samples including S, S-U and PEF-S or PEF-S-U, in which optimal PEF condition (at 16 $kVcm^{-1}$ and 240 pulse number) was used, were subjected to characterization.

4.4.4.4.1 Fatty acid profile

Fatty acid profile was examined in the form of fatty acid methyl esters (FAMEs) using the gas chromatography (GC). Fatty acids were firstly transmethylated by 2 M methanolic sodium hydroxide, along with 2 M methanolic hydrochloric acid. FAMEs were analyzed by gas chromatography (GC) using Agilent 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. FAME dissolved in hexane was determined using Agilent J&W fused silica capillary column (100 m × 0.25 mm × 0.20 μm) (a split ratio of 1:20). The injection port temperature was kept at 250 °C and detector (flame ionization detector, FID) temperature was 270 °C. The oven temperature ranged from 170 to 225 °C with a flow rate of 1 °C /min. The chromatographic peaks of the samples were identified based on retention times, compared to those of standards. Peak area ratio was used for calculation and the content was expressed as g fatty acid/100 g lipid.

4.4.4.4.2 Fourier transform infrared (FTIR) spectra

Lipid sample (200 μL) was introduced onto the horizontal attenuated total reflectance trough plate crystal cell (45 ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies, Inc., Madison, WI, USA). The cell was mounted to FTIR spectrometer (Bruker Co., Ettlingen, Germany). Spectra of the mid-infrared region of the range of 4000–500 cm^{-1} with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were resized against a background spectrum recorded from the clean empty cell at 25 °C. Spectral data analysis was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

4.4.4.4.3 Analysis of carotenoids

Thin-layer chromatography (TLC) was employed for determination of carotenoids using activated silica gel plates 20 × 20 cm (silica gel G type 60, Merck, Darmstadt, Germany) following the method of Takeungwongtrakul *et al.* (2015). The sample applied to the plates was placed in the mobile phase (acetone: hexane, 25:75, v/v) for the separation to take place. Identification of carotenoids was done by comparing the retention factor (R_f) of individual band with those of standards.

4.4.5 Statistical analysis

All the experiments were performed in triplicates and the results were expressed as means ± SD. Analysis of variance (ANOVA) was used for data analysis and means were compared by the Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was done using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

4.5 Results and discussion

4.5.1 Effect of pulse electric field (PEF) pre-treatment on cell disintegration of shrimp cephalothorax

Cell disintegration index (Z_c) of samples treated with PEF at different electric field strengths and pulse numbers is shown in Figure 21. The Z_c of 0 was obtained for the intact samples (without PEF). Value of 1.0 was reported for completely disintegrated samples (Guderjan *et al.*, 2005). Z_c was increased with increasing electric field strength and number of pulses ($p<0.05$). Highest disintegration was achieved at 12 or 16 $kVcm^{-1}$ when 240 pulses were applied ($p<0.05$). Electrical conductivity used as the disintegration index is directly related to the degree of tissue damage (Lebovka *et al.*, 2002). Z_c increased ($p<0.05$) as PEF up to 12 $kVcm^{-1}$ was employed. No further tissue damage took place when electric field strength was higher than 12 $kVcm^{-1}$ ($p>0.05$), regardless of pulse numbers. The turbidity of water, in which cephalothorax was immersed, was visually increased when electric field strength and number of pulses increased. This was evidenced by the increase in total solids in the submerging water (data not shown).

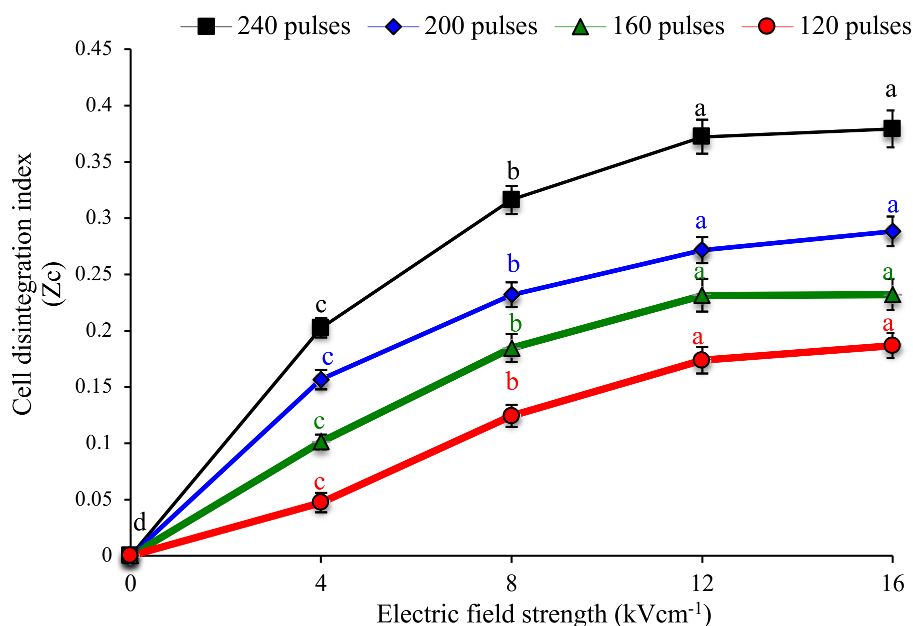

Figure 21. Effect of electric field strength and pulse numbers on cell disintegration index (Z_c) of Pacific white shrimp cephalothorax. Bars represent the standard deviation (n=3).

4.5.2 Effect of PEF pre-treatment on extraction yield of lipids extracted by typical solvent and UAE process

The extraction yields of lipids from samples subjected to PEF under various conditions, followed by solvent extraction are shown in Figure 22A. The lowest yield was obtained for the control (extracted by typical solvent extraction without PEF treatment). Extraction yield of lipids was increased when electric field strength and pulse number were increased ($p<0.05$). Due to high electric field strength, electroporation occurred in the sample to a higher extent. Electroporation resulted in both reversible and irreversible permeabilization of cephalothorax. This could improve the transfer of solvent into the starting material, thereby enhancing extraction efficiency of lipids (Guderjan *et al.*, 2007). The main objective of an oil extraction process is the rupture of cell walls to allow solvents to diffuse into cells, thus enhancing extraction efficiency of oil (Carr, 1995). PEF pre-treatment increased the oil yield from sunflower seeds extracted by hexane (Shorstkii *et al.*, 2015). PEF was

also documented to be an effective pre-treatment for extraction of oil from sesame seeds (Sarkis *et al.*, 2015). PEF, as a pre-treatment, increased the yield and the bioactive compounds content in oil from rapeseed extracted using hexane (Guderjan *et al.*, 2007). Thus PEF pre-treatment in combination with solvent extraction could increase the yield of lipid from shrimp cephalothorax.

There was a drastic increase in the extraction yield of lipids when UAE and UAE with PEF pre-treatment was applied, as compared to typical solvent extraction process. The yield of lipids extracted with PEF pre-treatment was higher than UAE alone (at 0 $kVcm^{-1}$) (Figure 22B), particularly with increasing electric field strengths and pulses. Generally, UAE is a more severe extraction process than PEF in terms of damaging impact on the cells. Nevertheless UAE lowered the quality of extracted lipid due to cavitation effects and oxygen incorporation (Gulzar and Benjakul, 2018). Overall, with increasing electric field strength and pulse numbers, yields of lipids extracted by the aid of UAE were increased ($p<0.05$). Thus PEF could be used as an effective pre-treatment for lipid extraction from shrimp cephalothorax since it could induce the generation of pores in the matrix. As a consequence, solvent could be penetrated thoroughly, especially when UAE was applied. Turbulence caused by cavitation in UAE process could enhance the penetration of solvent into the pores previously generated by PEF. Both PEF and UAE had therefore the combined effect or synergism on lipid extraction from shrimp cephalothorax. Based on yield of lipid, pre-treatment using PEF of 240 pulse number was selected for further study.

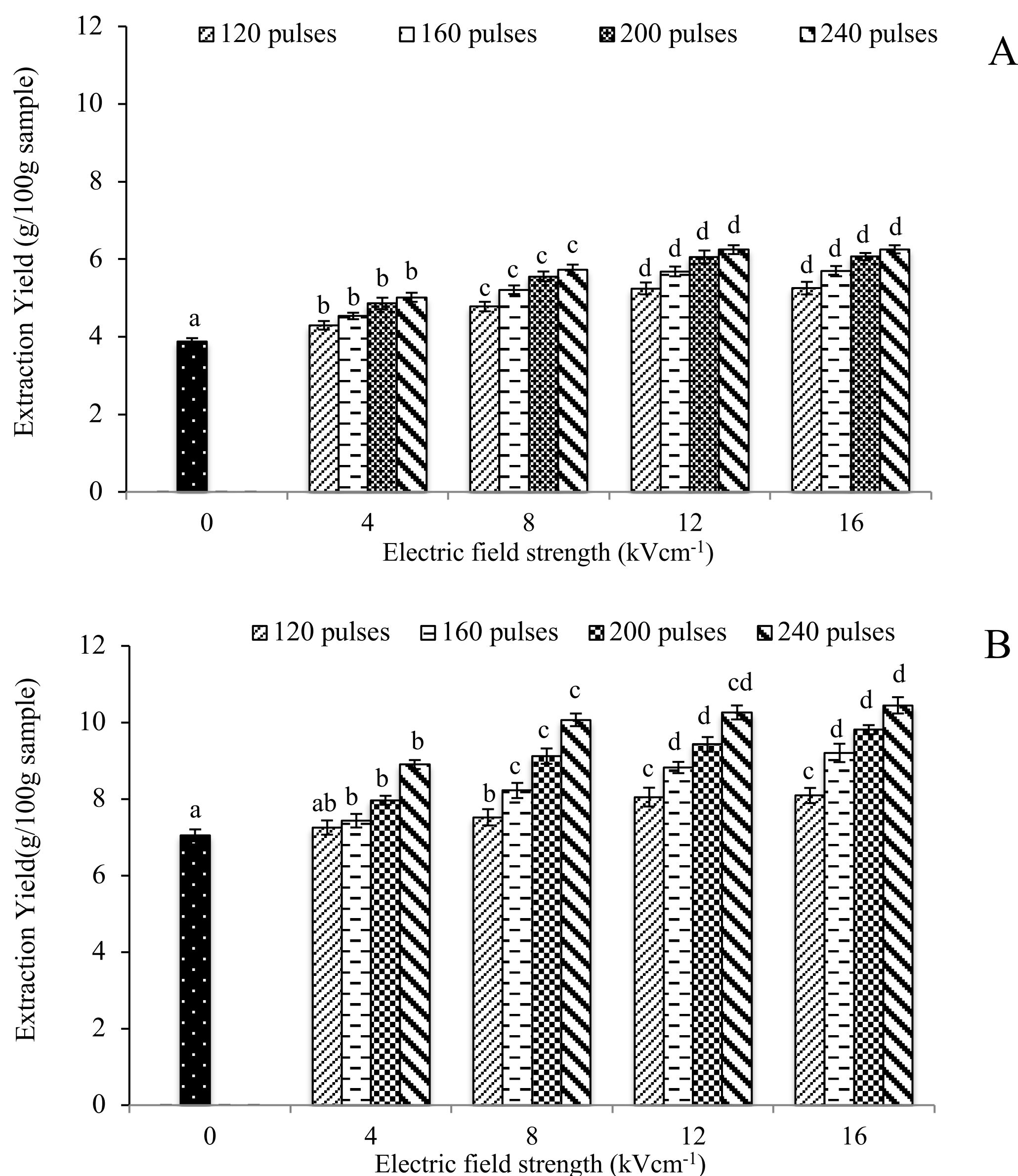

Figure 22. Effect of electric field strength and pulse numbers on yield of lipids extracted from Pacific white shrimp cephalothorax by solvent extraction with prior PEF pre-treatment at different electric field strengths and pulse numbers (A) or by solvent extraction process with prior PEF pre-treatment, followed by UAE (B) processes. Bars represent the standard deviation (n=3). Different letters on the bars within the same electric field strength including the control denote significant difference ($p<0.05$). Values at 0 $kVcm^{-1}$ in (A) and (B) represent the samples extracted by typical solvent extraction and UAE process, respectively.

4.5.3 Impact of PEF pre-treatment on oxidation and hydrolysis of lipids extracted by typical solvent and UAE processes

4.5.3.1 Peroxide value (PV)

Effect of PEF treatments at 240 pulse number with different electric field strengths on PV of lipid samples using two different extraction methods, typical solvent and UAE methods, is shown in Figure 23A. PV was decreased with increasing electric field up to 12 $kVcm^{-1}$ for both PEF-S and PEF-S-U samples. At the same electric field strength, samples from PEF-S-U process exhibited the higher PV than those from PEF-S process ($p<0.05$). UAE treatment generally increased the oxidation of lipids by enhancement of oxygen incorporation (Gulzar and Benjakul, 2018). However, PEF treatment, particularly at higher electric fields helped lower the oxidation ($p<0.05$). This was possibly due to the inactivation of enzymes present in the cephalothorax by PEF treatment. PEF is known to inactivate or reduce the activity of enzymes. PEF treatment retarded the activity of peroxidase and polyphenol oxidase *in vitro,* especially with increasing treatment time and electric field (Zhong *et al.*, 2007). Inactivation of enzymes by PEF is influenced by several factors such as electric field strength, electrical conductivity and pH of the media (Yang *et al.*, 2004). PV is an index of hydroperoxides generated during oxidation of unsaturated fatty acids by the action of lipoxygenase or antioxidation (Nawar, 1996). Shrimp cephalothorax was found to contain lipoxygenase (Shye *et al.*, 1987). PV of lipid is determined by many factors including method of extraction, oxygen saturation, fatty acid composition etc. (Gulzar and Benjakul, 2018). The PV of peanut oil treated with PEF was found to decrease with increasing PEF strength, indicating that the rate of fatty acid oxidation was decreased as PEF strength increased (Zeng *et al.*, 2010). It can be inferred that PEF pre-treatment was advantageous for lowering the oxidation of PUFAs in the lipids extracted by both extraction processes.

4.5.3.2 TBARS

Figure 23B shows TBARS values of lipids with different PEF pre-treatments using solvent or UAE process. TBARS values showed similar trend to PV results (Figure 23A). Lowest TBARS value was found in lipid from cephalothorax

pre-treated with PEF at 12 $kVcm^{-1}$ ($p<0.05$), irrespective of extraction methods. However, no difference in TBARS between samples treated with PEF at 12 and 16 $kVcm^{-1}$ ($p>0.05$). TBARS decreased as electric field strength was increased, regardless of extraction methods used. TBARS values were higher in PEF-S-U samples than PEF-S ($p<0.05$) when the same PEF strength was used ($p<0.05$). The hydroperoxides are decomposed and off-odor volatile compounds are produced, thus deteriorating the quality of lipids (Choe and Min, 2006). Lipids from cephalothorax extracted with UAE had high TBARS (Gulzar and Benjakul, 2018). Carbonyl group value (CGV) of peanut oil was reported to decrease with increasing electric field strength applied (Zeng *et al.*, 2010). The results suggested that the PEF pre-treatment reduced the oxidation of lipids in an electric field strength dependent manner, more likely by inactivation of oxidative enzymes.

4.5.3.3 Free fatty acid (FFA) content

FFA contents of lipids as influenced by PEF pre-treatment and extraction methods are shown in Figure 23C. PEF treatment at electric field strength of 4 $kVcm^{-1}$ showed no effect on FFA content on both PEF-S and PEF-S-U samples. There was a slight decrease of FFA content as the electric field strength was higher than 4 kVcm-1 ($p<0.05$). Nevertheless, electric field strength in the range of 8 – 16 $kVcm^{-1}$ had no different impact on FFA content in the obtained lipids ($p>0.05$). With the same PEF condition, PEF-S-U samples had higher FFA content than PEF-S counterpart ($p<0.05$). Drastic increase in FFA content of lipids was mediated by the shear and mechanical effects of UAE via cavitation effect (Gulzar and Benjakul, 2018). Increase in FFA content is also triggered by action of lipase or phospholipase, which hydrolyzes ester bond of lipid and phospho-ester bond of phospholipid and releases free fatty acids (Pacheco-Aguilar *et al.*, 2000). Hydrolysis was further enhanced by the release of lipases during ultrasonication process from cell matrix. With PEF treatment, especially at high electric field strength, hydrolytic enzymes were partially inactivated. Acidity of peanut oil treated with PEF was found to be lower than untreated samples after 100 days of storage (Zeng *et al.*, 2010). Nevertheless, a slight increase in the acidity value (as oleic acid) of olive paste was

reported after PEF treatment of olives at electric field intensity of 7 $kVcm^{-1}$ (Puértolas and Martínez de Marañón, 2015). PEF pre-treatment was found to have an effect on lowering FFA content of lipids extracted from Pacific white shrimp cephalothorax to some degree, irrespective of extraction processes.

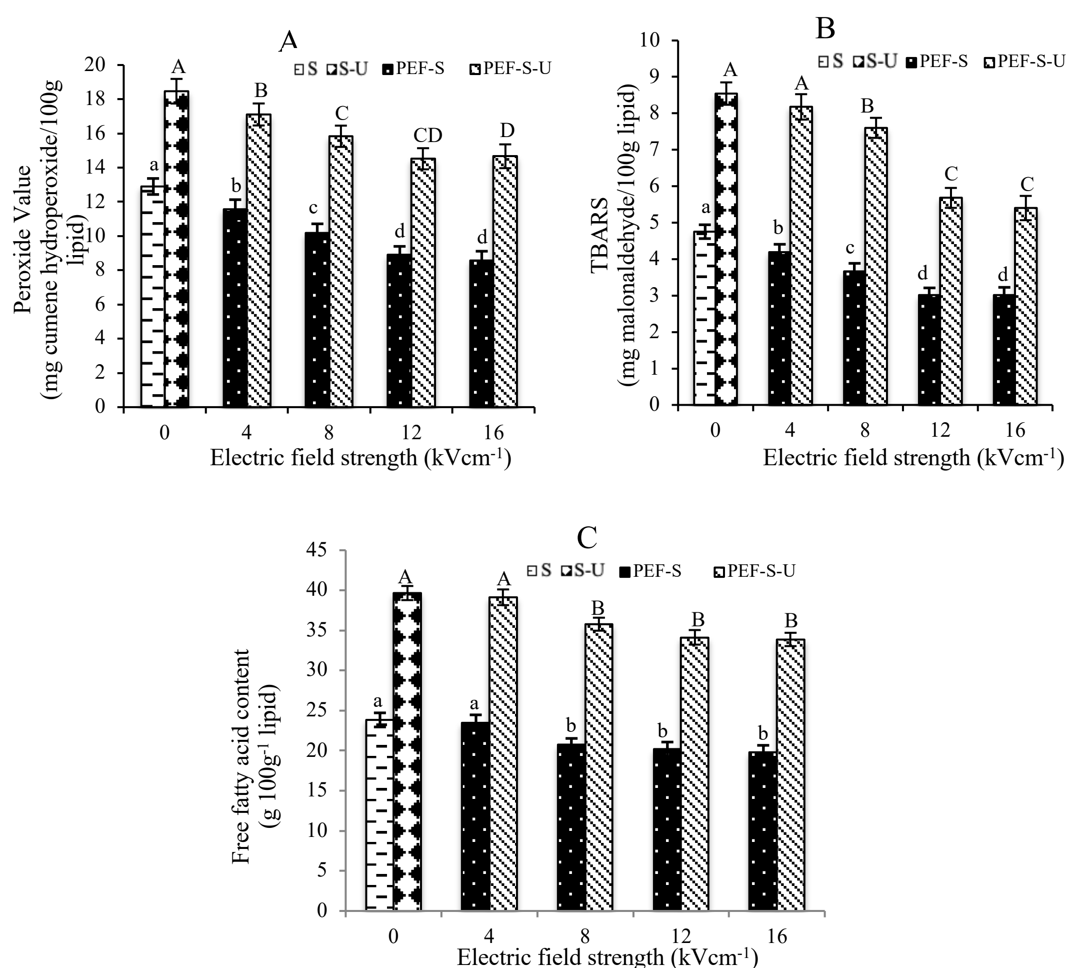

Figure 23. Peroxide value (A), TBARS value (B) and free fatty acid content (C) of lipids extracted from cephalothorax of Pacific white shrimp by different methods. S: lipids extracted by typical solvent extraction; S-U: lipids extracted by UAE process; PEF-S: lipids extracted by PEF treatment at 16$kVcm^{-1}$ and 240 pulses followed by typical solvent extraction; PEF-S-U: lipids extracted by PEF treatment at 16$kVcm^{-1}$ and 240 pulses followed by UAE process. UAE was carried out at 80% amplitude for 25 min in continuous mode. Bars represent the standard deviation (n=3). Different lowercase or uppercase letters on the bars within the same extraction process used denote significant difference ($p<0.05$).

4.5.4 Characteristics of lipids extracted from cephalothorax with the selected extraction conditions

4.5.4.1 Fatty acid profiles

Table 5 shows the fatty acid profiles of lipids extracted by 1) typical solvent extraction (S), 2) ultrasound assisted process (S-U), 3) PEF treatment (16 $kVcm^{-1}$ 240 pulses) followed by solvent extraction (PEF-S) and 4) PEF treatment followed by ultrasound assisted process (PEF-S-U). Overall, palmitic acid (C16:0) was present as the most abundant fatty acid, followed by oleic acid (C18:1) and stearic acid (C18:0), respectively. Similar fatty acid composition of lipids from cephalothorax of Pacific white shrimp was documented by Gulzar and Benjakul (2018). For C18 fatty acids, all samples had similar fatty acid composition except for S-U sample, in which oleic acid (C18:1 n-9) was lower than others, whereas linoleic acid (C18:2 n-6) was lowest in S sample. PEF pre-treatment helped better retention of fatty acids, particularly the unsaturated ones, which were reduced due to UAE. The loss of unsaturated fatty acids during UAE is attributed to cavitation effect and increased oxygen incorporated, which induced oxidation of fatty acid, especially PUFA. The oxidation of these PUFAs could be lowered, plausibly due to the inactivation of oxidative enzymes by the application of PEF treatment. Highest PUFA content was found in the PEF-S-U sample, while the lowest PUFA content was observed in S-U sample. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content was highest in PEF-U sample, whereas lowest content was found in U sample, conforming that PEF treatment played a major role in suppressing the oxidation of PUFAs. When UAE process was used, the cavitation effect associated with the turbulence more likely caused the damage of fat cells or tissues or membrane lipids, thus facilitating the release of these lipids rich in PUFA. Lin *et al.* (2003); Takeungwongtrakul *et al.* (2012); Gulzar and Benjakul (2018) also found PUFAs to be the major fatty acids in Pacific white shrimp lipids. The decrease in PUFAs in S-U sample was attributed to the oxidation as documented by Gulzar and Benjakul (2018). PEF treatment of olive oil slightly increased the MUFA/PUFA and decreased the saturated fatty acid content (Abenoza *et al.*, 2013). Thus, PEF pre-treatment with

UAE process could help in retaining PUFAs in lipid extracted

Table 5. Fatty acid profile of lipids extracted from cephalothorax of Pacific white shrimp using different methods

Fatty acids (g/100g lipids)	S*	S-U*	PEF-S*	PEF-S-U*
C12:0	0.09 ± 0.00b	0.14 ± 0.00a	0.09 ± 0.00b	0.07 ± 0.00c
C14:0	0.67 ± 0.00b	0.89 ± 0.00a	0.65 ± 0.00b	0.36 ± 0.00c
C14:1	0.09 ± 0.00b	0.12 ± 0.00a	0.09 ± 0.01b	0.07 ± 0.00c
C15:0	0.44 ± 0.00b	0.53 ± 0.00a	0.43 ± 0.00b	0.31 ± 0.00c
C16:0	24.20 ± 0.06b	26.81 ± 0.04a	23.20 ± 0.10c	19.37 ± 0.08d
C16:1 n-7	1.42 ± 0.00b	1.74 ± 0.06a	1.39 ± 0.00b	1.06 ± 0.00c
C17:0	0.79 ± 0.00b	0.80 ± 0.00b	0.79 ± 0.00b	1.02 ± 0.00a
C17:1	0.19 ± 0.00a	0.21 ± 0.02a	0.18 ± 0.00b	0.21 ± 0.00a
C18:0	7.96 ± 0.01b	7.44 ± 0.01d	8.31 ± 0.03a	7.72 ± 0.05c
C18:1 n-9	14.80 ± 0.03a	14.00 ± 0.12b	14.67 ± 0.06a	14.16 ± 0.10b
C18:2 n-7	2.83 ± 0.00a	2.76 ± 0.01a	2.82 ± 0.01a	2.82 ± 0.09a
C18:2 n-6	16.06 ± 0.38b	16.22 ± 0.01ab	16.34 ± 0.06ab	16.53 ± 0.07a
C18:3 n-3 (ALA)	0.95 ± 0.00c	1.01 ± 0.02a	0.98 ± 0.00b	0.82 ± 0.00d
C20:1 n-9	1.80 ± 0.01a	1.73 ± 0.00c	1.78 ± 0.00b	1.08 ± 0.00d
C20:2 n-6	2.62 ± 0.00a	2.44 ± 0.03c	2.55 ± 0.02b	1.73 ± 0.00d
C20:3 n-3	0.10 ± 0.00b	ND	0.10 ± 0.00b	0.15 ± 0.00a
C20:4 n-6 (ARA)	0.29 ± 0.00a	0.26 ± 0.00b	0.28 ± 0.00a	0.17 ± 0.00c
C20:5 (EPA)	6.06 ± 0.01b	5.15 ± 0.00c	6.06 ± 0.05b	8.20 ± 0.03a
C22:0	0.51 ± 0.00b	0.53 ± 0.00a	0.50 ± 0.02bc	0.49 ± 0.00c
C22:1	0.63 ± 0.00b	0.64 ± 0.00a	0.63 ± 0.00b	0.11 ± 0.00c
C22:2 n-6	0.20 ± 0.00a	0.18 ± 0.00b	0.20 ± 0.00a	0.16 ± 0.01c
C22:6 n-3 (DHA)	8.75 ± 0.08b	8.34 ± 0.02c	8.59 ± 0.34bc	10.39 ± 0.10a
C23:0	2.44 ± 0.00b	2.06 ± 0.02c	2.43 ± 0.01b	5.82 ± 0.15a
C24:1	0.72 ± 0.00b	0.74 ± 0.00a	0.71 ± 0.00c	0.64 ± 0.01d
Unidentified peak	5.32 ± 0.05c	4.97 ± 0.11c	5.99 ± 0.38b	6.43 ± 0.13a
Saturated fatty acid (SFA)	37.15 ± 0.10b	39.22 ± 0.08a	36.43 ± 0.18c	35.19 ± 0.08d
Monounsaturated fatty acid (MUFA)	19.66 ± 0.06a	19.40 ± 0.15b	19.49 ± 0.09ab	17.36 ± 0.14c
Polyunsaturated fatty acid (PUFA)	37.90 ± 0.16b	36.38 ± 0.07c	37.96 ± 0.53b	40.99 ± 0.34a

Different lowercase letters in the same column indicate significant differences ($p<0.05$).*S: lipids extracted by typical solvent extraction; *S-U: lipids extracted by UAE process; PEF-S*: lipids extracted by PEF treatment at 16kVcm^{-1} and 240 pulses followed by typical solvent extraction; PEF-S-U*: lipids extracted by PEF treatment at 16kVcm^{-1} and 240 pulses followed by UAE process

4.5.4.2 FTIR spectra

FTIR spectra of four different lipid samples extracted using various processes are illustrated in Figure 24. S and S-U samples had a peculiar peak at 3400 cm^{-1}, indicating that hydroperoxides were present in the aforementioned samples. The absorbance peak between 3600 and 3400 cm^{-1} is exhibited by the hydroperoxide moieties due to their –OO–H stretching vibrations (Van De Voort *et al.*, 1994). For the spectra observed between 3100–2800 cm^{-1}, representing C–H stretch vibrations and overlapping with the –OH group in carboxylic acid, PEF-S and PEF-S-U samples were found to have slightly larger peaks. C–H stretching vibration bands between 3100 and 2854 cm^{-1} have been identified to represent the degree of unsaturation of fats and oils (Afran and Newbery, 1991). This was concomitant with the results obtained from fatty acid profile (Table 5), showing that PEF-S and PEF-S-U sample had high levels of PUFAs. The ratio between the absorbance band at 2854 cm^{-1}, and the absorbance band between 3600 and 3100 cm^{-1} ($A_{2854}/A_{3600–3100}$), has been used to monitor the oxidation process. Lower $A_{2854}/A_{3600–3100}$ indicates that higher oxidation takes place (Guillén and Cabo, 2004). PEF-S and PEF-S-U samples had higher $A_{2854}/A_{3600–3100}$ ratio than S and S-U samples. Thus, PEF could help prevent oxidation in lipids. Gulzar and Benjakul (2018) reported that lipids extracted by UAE process had lower $A_{2854}/A_{3600–3100}$ ratio than lipids extracted by typical process. The peaks at wavenumbers of 1736-1745 cm^{-1} represent the ester carbonyl group of triglycerides (Takeungwongtrakul *et al.*, 2015). Similar peaks were observed at 1744 cm^{-1}, indicating that triglycerides were present in all the samples. However, the PEF-S and PEF-S-U showed the slightly larger peaks. The decrease in the aforementioned peak of S-U sample was plausibly due to the hydrolysis of the ester bond induced by lipases. The bands near 1240 and 1201 cm^{-1} with the stretching vibration of the C–O ester groups and with the bending vibration of the CH_2 group represent the proportion of saturated acyl groups in oil. The peaks at approximately 1117 and 1099 cm^{-1} representing the stretching vibration of the C–O ester groups are inversely proportional to saturated acyl groups (Guillén and Cabo, 1997). S and S-U samples had larger peaks at 1240 and 1201 cm^{-1} and smaller peaks at 1117 and 1099 cm^{-1}, compared to PEF-S and PEF-S-U samples, indicating that the presence of more

saturated fatty acids in the formers. FTIR results confirmed that lipid oxidation was suppressed to some degree in PEF treated samples, regardless of extraction processes.

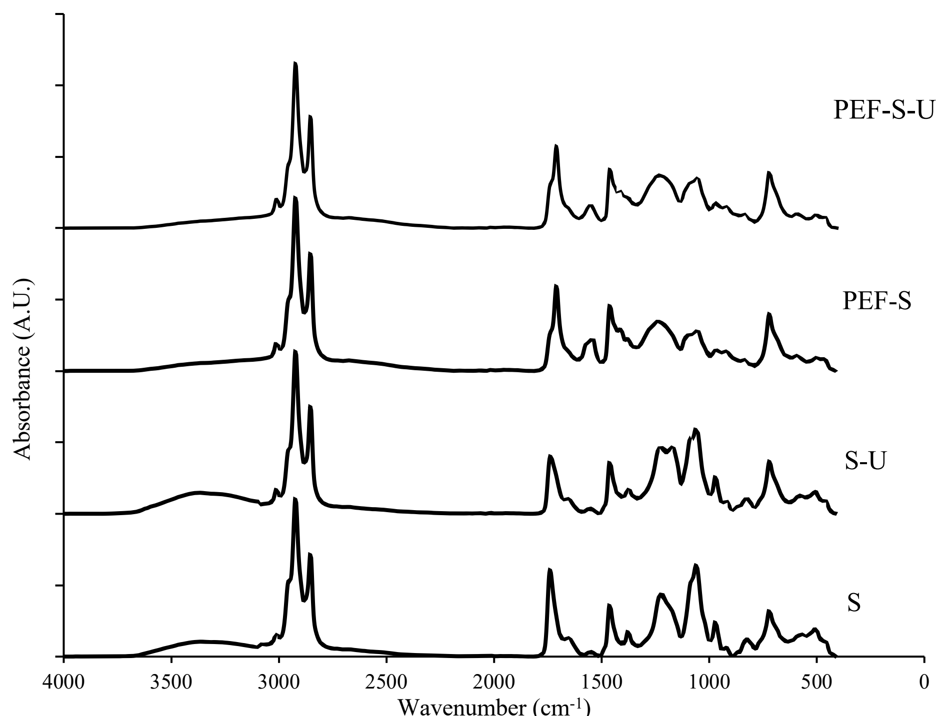

Figure 24. FTIR spectra of lipids extracted from cephalothorax of Pacific white shrimp using different selected extraction methods. S: lipids extracted by typical solvent extraction. S-U: lipids extracted by solvent extraction followed by UAE; PEF-S: lipids extracted by solvent extraction with prior PEF pre-treatment at 16kVcm^{-1} and 240 pulses; PEF-S-U: lipids extracted by solvent extraction with prior PEF treatment at 16kVcm^{-1} and 240 pulses followed by UAE process.

4.5.4.3 Carotenoids

Thin-layer chromatograms (TLC) of different selected samples are illustrated in Figure 25. TLC is a fast and simple separation method for quantitative and qualitative analysis of carotenoids (Sánchez-Camargo *et al.*, 2011). Seven distinctly prominent bands at retention factors (R_f) of 0.35, 0.40, 0.44, 0.68, 0.75, 0.77, 0.80 and one small band at R_f of 0.96 were obtained in the chromatogram. Bands with R_f of 0.35 and 0.96 were identified to be free astaxanthin and β-carotene,

respectively. Free astaxanthin from lipid of Brazilian red spotted shrimp corresponded to R_f of 0.33 (Sánchez-Camargo *et al.*, 2011). The orange bands having R_f of 0.68, 0.75 and at 0.77 corresponded to canthaxanthin, astaxanthin monoester and astaxanthin diester, respectively (Gulzar and Benjakul, 2019). There was a marked difference in the band intensity and color between PEF-S-U and other three samples. PEF-S-U sample showed the bands with higher color intensity, indicating the presence of carotenoids at a higher content than the other samples. This was plausibly due to the fact that PEF treatment could lower the oxidation of carotenoids in the PEF-S-U sample. Additionally UAE effectively increased the extraction efficiency of carotenoids from shrimp cephalothorax.

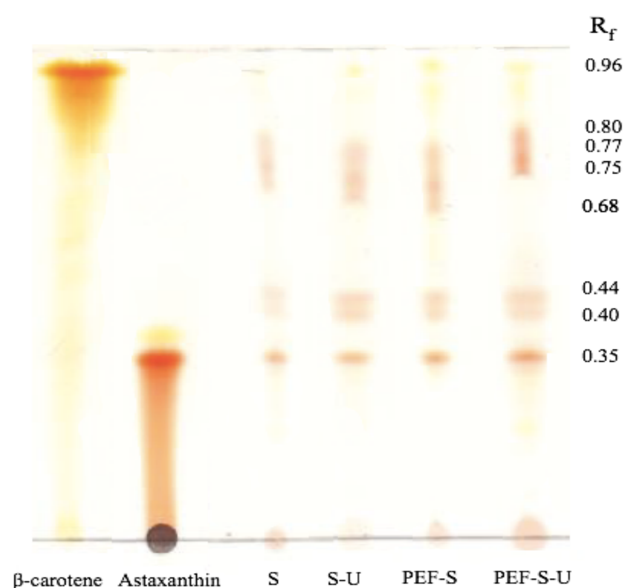

Figure 25. Thin-layer chromatography of lipids extracted from cephalothorax of Pacific white shrimp using different selected extraction methods. Caption see figure 24.

4.6 Conclusion

PEF pre-treatment of cephalothorax of Pacific white shrimp prior to lipid extraction by UAE process was a beneficial process in terms of yield and quality. The yields and carotenoid content in lipids extracted from cephalothorax were increased by PEF pre-treatment. On the other hand, the lipid also suffered less oxidation and

hydrolysis when PEF pre-treatment was applied. Consequently, PUFAs were more retained in the extracted lipids. Overall, the adverse effects on lipid quality caused by UAE could be conquered to some degree by PEF pre-treatment.

4.7 References

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CHAPTER 5

IMPACT OF PRE-TREATMENT AND ATMOSPHERE ON QUALITY OF LIPIDS EXTRACTED FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP BY ULTRASONIC ASSISTED PROCESS

5.1 Abstract

Impacts of different pre-treatment conditions and atmosphere on yield and oxidative stability of lipids from Pacific white shrimp (*Litopenaeus vannamei*) cephalothorax using ultrasonic assisted extraction (UAE) process were studied. Cephalothorax was subjected to vacuum-microwave (VM) heating prior to UAE using a mixture of isopropanol/n-hexane (1:1) as solvent. Nitrogen gas was flushed at two flow rates; low (2.15 L/min) and high (4.35 L/min) into the system during ultrasonication. Vacuum-microwave heating resulted in the increase of lipid yield and highest yield was observed in the samples extracted by a combination of VM and UAE. Tannic acid (TA) was incorporated into cephalothorax at three different levels (0.05, 0.1 and 0.2%) in combination with VM, followed by nitrogen flushing. Nitrogen flushing during ultrasonication process resulted in the increased oxidative stability of lipids as confirmed by low PV, TBARS and FFA. Furthermore, astaxanthin content in the lipid was found to be increased by aforementioned treatments.

5.2. Introduction

Pacific white shrimp is widely consumed seafood and holds enormous value in the market. Overall exports of shrimps worldwide were valued at US$19.3 billion in 2017, out of which Thailand exported an estimated US$950 million. Shrimp exports around the world have seen an upsurge of 6.1% from 2016 to 2017 (Workman, 2018). These numbers reflect the huge potential of the shrimp processing industry but waste management has become challenging, particularly via effective utilization. Wastes from shrimp processing industry comprise cephalothorax, carapace, and tail, which are either dumped back into the farms as aquaculture feed or supplemented as animal feed (Nwanna *et al.*, 2004). Nevertheless, improper disposal poses a major

environmental problem for the industry as well as society. Since cephalothorax contains high-value bioactive compounds, such as omega-3 fatty acids (FA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and carotenoids including β-carotene and astaxanthin, it has been used for extraction of lipids rich in those compounds (Amiguet *et al.*, 2012). Due to the high market value with the increasing demand of these valuable compounds, waste valorization from shrimp industry has picked up some pace in the past few years. Lipids from shrimp cephalothorax have received increasing interest due to the presence of omega-3 fatty acids such as EPA and DHA. These fatty acids are of high importance as they have been shown to possess anti-inflammatory, anti-thrombosis and anti-arrhythmia properties, providing benefits for patients suffering from cardiovascular diseases (Sahena *et al.*, 2009).

Extraction of lipids and carotenoids from the shrimp cephalothorax has been performed principally using the typical solvent extraction process. Other techniques such as supercritical fluid extraction or enzyme treatment have disadvantages due to their high cost, low efficiency and requirement of skilled manpower to handle. Ultrasonic assisted extraction (UAE) has been employed successfully to recover lipid from shrimp cephalothorax (Gulzar and Benjakul, 2018). Ultrasonic extraction process has found numerous applications in chemical and food-based systems due to its advantages such as high yield, low energy costs, simplicity of work-up and less solvent consumption. Ultrasound extraction process was documented to be an efficient, fast, and economical method for extraction of stevioside from *Stevia Rebaudiana* (Rouhani, 2019). Ultrasound irradiation was also proved to be an efficient and convenient procedure for the synthesis of some aryl amides from carboxylic acids and isocyanides with several advantages, such as simple work-up procedure, shorter reaction time and higher yield (Ramazani *et al.*, 2015).

Although the yield of lipid and carotenoid was increased almost by two-folds by UAE, there was a disadvantage, mainly the enhanced oxidation and hydrolysis (Gulzar and Benjakul, 2018). Lipid oxidation has been found to take place during the ultrasonic extraction of lipids and oils (Chemat *et al.*, 2004; Gulzar and Benjakul,

2018; Zhang *et al*., 2017). The action of lipoxygenase on unsaturated fatty acid (Gulzar and Benjakul, 2018), sonolysis of water to produce free radicals (Riesz and Kondo, 1992), and the presence of trace metal ions such as Cu^{2+} and Fe^{2+}(Suslick *et al.*, 1986) have been proposed. Mechanical damage during sonication causes the release of free FA, particularly the unsaturated ones. High dissolved oxygen concentration due to ultrasonication mainly contributes to augmented chances of lipid extracted by UAE. The use of antioxidants such as tannic acid (TA) resulted in the retardation of lipid oxidation in many products (Gulzar and Benjakul, 2019a; Maqsood and Benjakul, 2010b). TA is also an enzyme inhibitor and was found to inhibit lipase in the shrimp cephalothorax (Gulzar and Benjakul, 2019a).

Preheating at 95 ºC and addition of TA were found to lower the quality loss of lipid extracted from shrimp cephalothorax by UAE process (Gulzar and Benjakul, 2019a). Vacuum-microwave extraction (VME) is also emerging as a potential alternative to the typical solvent extraction process due to several advantages, including unique heating mechanism, compromising capital cost and, protection of sensitive compounds (Eskilsson and Björklund, 2000; Howard, 1995). Microwaves are electromagnetic waves that can penetrate into biological tissues. It can interact with polar components and produce heat due to dipole rotation (Eskilsson and Björklund, 2000). With the introduction of vacuum into the microwave heating system, the boiling temperature of the solvents can be reduced, which in turn protects the sensitive compounds. Additionally, heat also promotes the extraction process by disruption of starting material, thus enhancing the mass transfer and diffusion of target compounds.

The presence of oxygen involved in the system during ultrasonication could be conquered by flushing of nitrogen during ultrasonication. Nitrogen is an inert gas and does not react with any active species or radicals. Although the extraction of oils from plant and animal sources using UAE increased the yield tremendously, cavitation effect associated with enhanced lipid oxidation and hydrolysis is still the limitation. Exclusion of oxygen could be a new challenge of maintaining the quality of lipids while increasing the yield simultaneously using UAE.

5.3 Objectives

To investigate the combined effect of vacuum-microwave heating and UAE using a mixture of isopropanol/n-hexane (1:1) on the yield of lipids.

To elucidate the impact of nitrogen flushing and tannic acid incorporation on oxidative stability of the lipids extracted by UAE process.

5.4 Materials and methods

5.4.1 Sample preparation

Shrimp cephalothorax of freshly harvested Pacific white shrimp was gifted from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. The samples (10 kg) kept in a polyethylene bag were imbedded in a Styrofoam container with crushed ice (20 kg) and transported to the seafood chemistry and biochemistry laboratory, Prince of Songkhla University, Hat Yai, within 1 h.

5.4.2 Extraction of lipid using UAE process under different vacuum-microwave pre-treatments and atmospheres

Shrimp cephalothorax samples were ground for 5 min using a blender (National Model MK-K77, Tokyo, Japan). The samples were then separated into four portions. The first portion (50g) was homogenized with 250 mL of isopropanol and n-hexane (1:1) mixture at a speed of 9500 rpm for 2 min at 4 °C using an IKA Labortechnik homogenizer (Selangor, Malaysia) (Gulzar and Benjakul, 2018). For the second portion, the ground sample mixed with the solvent mixture as previously described was subjected to UAE process as described by Gulzar and Benjakul, (2018) using an Ultrasonic Processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA). The input ultrasonic amplitude used was 80% in continuous mode for 25 min. For the third portion, extraction by UAE was conducted under the nitrogen atmosphere at two flow rate levels. During ultrasonication, the samples were flushed with nitrogen gas to continuously replace the air present in the vessel headspace. Samples were ultrasonicated in a three-neck round bottom flask, with openings for ultrasonic probe, nitrogen gas inlet, and outlet respectively (Figure 26).

The velocity of nitrogen gas flushed into the flask was measured using a DIGICON DA-45 anemometer (Digicon S.A, Gravataí/RS, Brazil). The volumetric flow rate of nitrogen gas (Q) was calculated using the following formula

$$Q = v\frac{\pi d^2}{4}$$

where v is the velocity of nitrogen gas (m/s) and d is the internal diameter of nitrogen inlet pipe (m).

To prevent excessive loss of solvents from the dynamic system, a condenser was fitted at the outlet to reflux the solvent back into the flask. The flask was placed in an ice bath to prevent the temperature rise during the ultrasonication process.

For the fourth portion, ground shrimp cephalothorax (50 g) was heated using a SHARP R-220 microwave oven (SHARP Corporation, Japan). The oven was fitted with a VALUE VE125N vacuum pump (VALUE Mechanical & Electrical Products Co. Ltd., Zhejiang, China) to create a vacuum inside the microwave vessel placed in the oven chamber. Heating of shrimp cephalothorax was done using output power of 800 W for 5 min. Final temperature after MW heating was approximately 95 °C. After heating, all the samples were homogenized with solvents and subjected to UAE process, both in absence and presence of nitrogen atmosphere, as explained earlier.

After the extraction, the lipids of all the samples were separated from the solvent by centrifugation and evaporation by the process described by Gulzar and Benjakul (2018). Lipids were collected and transferred into vials, flushed with nitrogen gas to remove traces of solvent and tightly sealed. The samples were kept at −40 °C. Different treatments have been named as follows: S for the typical solvent extraction process; U for the ultrasonic assisted extraction process; LN-U and HN-U for the samples flushed with nitrogen gas at low and high flow rate during the ultrasonication process; VM-U for the sample, in which shrimp cephalothorax was pre-treated with vacuum-microwave and subsequently subjected to UAE process; VM-LN-U and VM-HN-U for the samples, in which shrimp cephalothorax pre-treated

with vacuum-microwave, followed by extraction using UAE under nitrogen flushing at low and high flow rate.

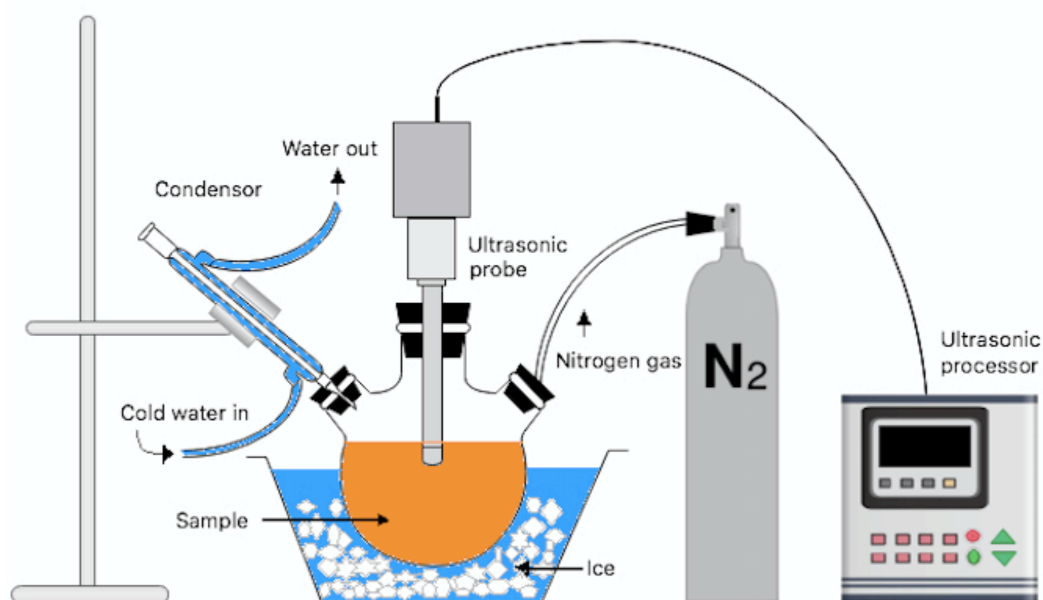


Figure 26. Layout of the UAE process under nitrogen atmosphere of Pacific white shrimp cephalothorax.

5.4.3 Impact of tannic acid incorporation and vacuum-microwave pre-treatment in the absence and presence of nitrogen flushing on lipid oxidation/hydrolysis

To investigate the impact of tannic acid on lipid extraction, 5 mL of tannic acid solution to obtain the final concentration of 0.05, 0.1 and 0.2% (by weight) were added to 50 g ground shrimp cephalothorax. The mixtures were heated using a vacuum-microwave as previously described. Lipid extraction was further conducted using the solvent mixture with the aid of UAE process in the presence of nitrogen gas at the selected flow rate. The obtained lipids were named as 'TA-0.05', 'TA-0.1' and 'TA-0.2', respectively. U, VM-U, and LM-LN-U samples were also prepared as mentioned above. All samples were analyzed.

5.4.4 Analyses

5.4.4.1 Determination of extraction yield of lipid

The extraction yield was determined by weighing the extracted lipid and expressed as g/100g of starting ground shrimp cephalothorax.

5.4.4.2 Determination of oxidation and hydrolysis of lipids

Peroxide value (PV) was measured by the ferric thiocyanate method as described by Chaijan *et al.* (2006). Lipid sample (50 µL) was 10-fold diluted using 75% ethanol, (v/v). To the prepared sample, a mixture of 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µL of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added. The mixture was mixed well and the absorbance was read at 500 nm using a spectrophotometer. The blank was prepared in the same way, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide with a concentration range of 0.5–2 ppm.

Thiobarbituric acid reactive substances (TBARS) were examined by the procedure tailored by Buege and Aust (1978). Lipid sample (0.5g) was mixed with 2.5 mL of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was then heated in boiling water (95–100 °C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600g at 25 °C for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was read at 532 nm using a spectrophotometer. A standard curve was prepared using malonaldehyde (MDA) at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde/ kg lipid.

Free fatty acid (FFA) content was determined as per the method of Lowry and Tinsley (1976). To a lipid sample (0.1 g), 5 mL of isooctane was added and swirled vigorously to dissolve the sample. The mixture was added with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, shaken vigorously for 90 s and allowed to stand for 20 s. The absorbance of the upper layer was read at 715 nm. A standard curve was

prepared using palmitic acid in isooctane at concentrations ranging from 0 to 10 μmol/mL. FFA content was expressed as g FFA/100 g lipid.

5.4.4.3 Characterization of lipids extracted using the selected pre-treatment and atmosphere

5.4.4.3.1 Determination of fatty acid profile

Fatty acid profile was analyzed as fatty acid methyl esters (FAMEs) using gas chromatography (GC) following the method of Gulzar and Benjakul (2019b). Fatty acids were firstly transmethylated by 2 M methanolic sodium hydroxide, along with 2 M methanolic hydrochloric acid. FAMEs were analyzed by gas chromatography (GC) using Agilent 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. FAME dissolved in hexane was determined using Agilent J&W fused silica capillary column (100 m × 0.25 mm × 0.20 μm) (a split ratio of 1:20). The injection port temperature was kept at 250 °C and detector (flame ionization detector, FID) temperature was 270 °C. The oven temperature ranged from 170 to 225 °C with a flow rate of 1 °C /min. The chromatographic peaks of the samples were identified based on retention times, compared to those of standards. Peak area ratio was used for calculation and the content was expressed as g fatty acid/100 g lipid.

5.4.4.3.2 Identification and quantification of astaxanthin

Astaxanthin was analyzed by high-performance liquid chromatography (HPLC), Waters 2475 (Milford, MA, USA) in which a photodiode array detector (Waters 2998, Milford, MA, USA) was equipped. Shrimp oil (100 μL) was mixed with 1 mL of ethanol and vortexed for 1 min. After thorough mixing, the sample was stored at -18 °C for 2 h. The incubated sample was centrifuged for 10 min at 3600g. Fifty μL of the supernatant was taken and dissolved in 1 mL of mobile phase, methanol/acetonitrile (1:1, v/v) and subjected to HPLC analysis, by using a Thermo-scientific column (C18, 150 × 4.6 mm, 5 μm) at a flow rate of 1.2 mL/min under isocratic conditions. The chromatogram was recorded at 470 nm. Astaxanthin peaks were identified using standards

5.4.4.3.3 Analysis of FTIR spectra

Lipid sample (200 µL) was introduced onto the horizontal attenuated total reflectance trough plate crystal cell (45 ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies, Inc., Madison, WI, USA). The cell was mounted to FTIR spectrometer (Bruker Co., Ettlingen, Germany). Spectra of the mid-infrared region of the range of 4000–500 cm^{-1} with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were resized against a background spectrum recorded from the clean empty cell at 25 °C. Spectral data analysis was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

5.4.5 Statistical analysis

A completely randomized design was used throughout the study. All the experiments were done in triplicates and the results were expressed as means ± SD. Analysis of variance (ANOVA) was used for data analysis. Means were compared by Duncan's multiple range test. Statistical analysis was done using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

5.5 Results and discussion

5.5.1 Impact of different pre-treatments and atmosphere on extraction yield

The effect of different pre-treatments including 1) nitrogen flushing and 2) vacuum-microwave heating on the extraction yields of lipids from shrimp cephalothorax is shown in Figure 27. Lowest yield (4.16±0.23 g/100g shrimp cephalothorax) was observed in lipids extracted by normal solvent extraction process (S) ($p<0.05$). UAE was shown to increase the yield of lipids drastically, in which the yield of 9.08±0.31 g/100g cephalothorax was achieved (U). There was a 118% increase in the extraction yield of lipids when UAE was used. Ultrasound waves release enormous energy in the solution, causing a rapid breakdown of cells to liberate the target compounds. UAE increased the extraction yield of lipids from shrimp cephalothorax by two-folds (Gulzar and Benjakul, 2018). Although the UAE

can augment the extraction yield tremendously, it could induce oxidative damages caused during the cavitation effect. To alleviate such a problem, nitrogen gas was used to create an inert atmosphere during sonication. Nitrogen gas was flushed into the samples at 2.15 L/min and 4.65 L/min, so-called LN-U and HN-U, respectively. There was no difference in yield for samples extracted under both nitrogen atmospheres. Also, there was no difference in yield between lipid extracted under air and nitrogen atmosphere ($p>0.05$).

When vacuum-microwave heating of cephalothorax was implemented prior to UAE, the marked increased lipid yield was gained. Vacuum-microwave extraction (VME) has been employed to increase the extraction yield of antioxidants from plants (Xiao *et al.*, 2009). Preheating prior to extraction can result in the better extraction yield, as the heat was able to breakdown cell matrices and further facilitated the extraction process. Lipid yield from whole red hake was increased due to heating (Dubrow *et al.*, 1973). Pre-treatment of shrimp cephalothorax at 95 ºC rendered the highest yield of lipid extracted by UAE (Gulzar and Benjakul, 2019a). Microwaves are high energy electromagnetic waves with frequency, ranging between 300 MHz and 300 GHz. The polarizing nature of microwaves causes the polar molecules like water to vibrate at very high frequency, resulting in the generation of heat by friction (Galema, 1997). Water is present in abundance in living cells both outside and inside the cell matrix. Therefore microwave heating is faster and more penetrative, compared to conventional heating methods (Ayappa *et al.*, 1991).

Heating of cephalothorax using vacuum-microwave in combination with UAE using isopropanol:n-hexane as medium resulted in the highest yield of lipid from shrimp cephalothorax (12.34±0.35 g/100g cephalothorax) (VM-U). VME, when used in combination with UAE, facilitated the extraction of polysaccharides from *Inonotus obliquus* (Chen *et al.*, 2010). Nitrogen flushing of VM treated samples at low (VM-LN-U) and high (VM-HN-U) levels during the UAE did not show any significant difference in the extraction yield ($p>0.05$). Yields of the former and latter were 12.05±0.37 g/100g cephalothorax and 12.09±0.31 g/100g cephalothorax respectively.

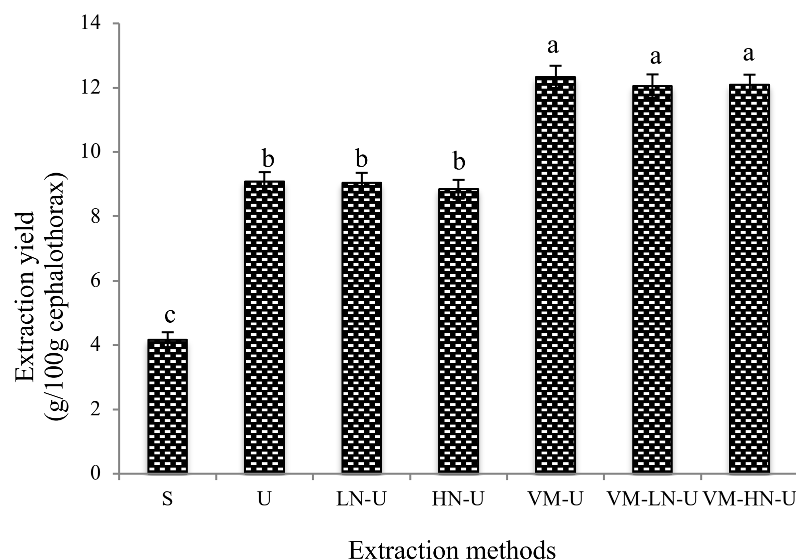


Figure 27. Effect of different pre-treatments and atmosphere on extraction yield of lipids from cephalothorax of Pacific white shrimp. S: lipids extracted by typical solvent extraction; U: lipids extracted by UAE process; LN-U: lipids extracted by UAE process under low nitrogen flow rate; HN-U: lipids extracted by UAE process under high nitrogen flow rate; VM-U: lipids extracted by UAE process under nitrogen atmosphere after prior vacuum-microwave heating of cephalothorax; VM-LN-U: lipids extracted by UAE process under low nitrogen flow rate after prior vacuum-microwave heating of cephalothorax; VM-HN-U: lipids extracted by UAE process under high nitrogen flow rate after prior vacuum-microwave heating of cephalothorax. All samples were extracted using UAE (continuous mode, 80% amplitude) for 25 min. Bars represent the standard deviation (n=3). Different lowercase letters on the bars denote significant difference ($p<0.05$).

5.5.2 Impact of different treatments on oxidation and hydrolysis of lipids extracted by UAE process

5.5.2.1 Peroxide value (PV)

Figure 28A depicts the PV of lipids extracted from shrimp cephalothorax by UAE process under different conditions. Highest PV was found in

the sample extracted using UAE without any treatment (U). PV of lipid extracted under different conditions decreased in the order of samples with following treatments: vacuum-microwave treated ultrasound extracted sample (VM-U), nitrogen flushed vacuum-microwave treated ultrasound extracted sample (VM-LN-U) and VM-LN-U in the presence of TA at different levels. Nitrogen flushing significantly lowered the PV and the addition of TA was found to have added effect. Since VM-LN-U rendered the highest yield and the loss in hexane was minimized, compared to VM-HN-U, in which flow rate of nitrogen was higher and could carry solvent away, VM-LN-U was selected. Effect of nitrogen flow rate at a low and high level on PV showed that nitrogen flow rate below 2.15 L/min showed little effect on lowering the PV and higher-level also had an insignificant effect (data not shown). Therefore, 2.15 L/min was used as the optimum flow rate for nitrogen flushing. Additionally, under nitrogen atmosphere, oxidation of lipid occurred in the extracted lipid at a lower degree, compared with the sample extracted using only UAE (U). Under normal atmospheric conditions, oxygen can be easily dissolved into the system, especially when the system was more turbulent, particularly during ultrasonication. Moreover, the solubility of oxygen in hexane was found to be higher than many other solvents (Quaranta *et al.*, 2013), thus making the system more vulnerable to oxidation. Mechanical effects of cavitation phenomenon enhance the mass transfer in the solution, and the localized high temperature and pressure can promote the formation of singlet oxygen (Davidson *et al.*, 1987). Vacuum-microwave treatment alone was also found to lower the PV of lipid extracted by UAE ($p<0.05$). Rapid microwave heating more likely inactivated lipoxygenase and lipases, which are responsible for oxidative and hydrolytic reactions. Removal of oxygen from headspace in combination with the addition of tannic acid, a potent antioxidant, effectively minimized the lipid oxidation. TA can neutralize the free radicals present in the sample which triggers the lipid oxidation (Maqsood and Benjakul, 2010a). Addition of TA was found to lower PV of lipid from shrimp cephalothorax using UAE process (Gulzar and Benjakul, 2019a). However, no difference in PV was attained between lipid extracted from the sample added with 0.1% and 0.2% TA ($p>0.05$). The action of free radicals such as singlet oxygen on unsaturated fatty acids catalyzed by

lipoxygenase or autooxidation results in the formation of hydroperoxides, the intermediate products of lipid oxidation (Nawar, 1996). PV of lipid depends on several factors involving extraction method, oxygen saturation, fatty acid composition, etc. (Gulzar and Benjakul, 2018). Pertinently, vacuum-microwave treatment, nitrogen flushing and addition of 0.1% TA could lower PV of resulting lipids.

5.5.2.2 TBARS

Figure 28B shows TBARS values of lipids from shrimp cephalothorax under different conditions by UAE process. U-sample showed highest TBARS value, followed by VM-U, VM-LN-U, and VM-LN-U containing TA at various levels, respectively. Nitrogen flushing significantly ($p<0.05$) reduced lipid oxidation as seen by the lower levels of TBARS. Additionally, the incorporation of TA further suppressed lipid oxidation. TA at levels of 0.05 - 0.1% yielded lipid with similar TBARS values ($p>0.05$). Overall, TA significantly lowered the TBARS of lipids extracted from shrimp cephalothorax by UAE process (Gulzar and Benjakul, 2019a). Lipid oxidation in striped catfish was reduced significantly by adding TA and TBARS were increased at a lower rate during refrigerated storage (Maqsood and Benjakul, 2010b). TBARS indicate the presence of polar and aromatic hydrocarbons such as aldehydes and ketones, etc. as the secondary lipid oxidation products in the lipid oxidation process (Nawar, 1996). PV and TBARS are major indicators of lipid oxidation in fats and oils. In general, hydroperoxides are further decomposed into dimers and oligomers, particularly off-flavor volatiles (Shahidi, 1998). In addition to the prevention of oxidation by creating an oxygen-deficient system by nitrogen flushing, TA could act as a potential radical scavenger and suppressed the lipid oxidation in lipids extracted by UAE mediated by cavitation effect. Vacuum-microwave heating was also found to significantly decrease the TBARS value of extracted lipids ($p<0.05$). TBARS value of lipids extracted from Pacific white shrimp hepatopancreas decreased with increasing temperature (Senphan and Benjakul, 2012). The decrease in TBARS value of extracted lipids was concomitant with lower levels of PV, indicating that lesser hydroperoxides were further oxidized to give rise to

secondary oxidation products. The results, therefore, established the fact that lipid oxidation was markedly lowered when cephalothorax was vacuum-microwave preheated in the presence of TA and nitrogen flushing during UAE process.

5.5.2.3 Free fatty acid (FFA) content

FFA content is usually associated with the acid value of lipid, indicating the formation of free fatty acids. Figure 28C shows the FFA content of lipids from shrimp cephalothorax under different conditions. UAE process is known to increase FFA content of the oils (Gulzar and Benjakul, 2018; Zhang *et al.*, 2017). Mechanical effects of cavitation during UAE can cause the lysis of the ester bond of lipids. Furthermore, the lipase released into the system from the cells and temperature rise during sonication facilitates the hydrolysis process (Gulzar and Benjakul, 2019a). FFA content is augmented, mainly triggered by the action of lipase or phospholipase, which hydrolyzes the ester bond of lipid and phospho-ester bond of phospholipid (Pacheco-Aguilar *et al.*, 2000). These FFAs are prone to oxidation. When FFA was accumulated, off-odor could be formed (Mackie, 1993). PUFAs, arisen from phospholipid hydrolysis, are more sensitive to oxidation (Gulzar and Benjakul, 2018). Vacuum-microwave treatment prior to UAE was shown to prevent hydrolysis ($p<0.05$). Conversely, nitrogen flushing did not show the profound effect ($p>0.05$) on FFA content. Addition of TA drastically reduced FFA content of lipids extracted. It was noted that TA was proven to exhibit the inhibitory effect on lipases as shown by Gulzar and Benjakul (2019a). Thus TA was found to reduce the FFA content of the lipid from shrimp cephalothorax using UAE process. Inhibition of lipase might be plausibly due to the formation of a substrate-tannin complex, which could not be hydrolyzed by lipase (Kumar and Singh, 1984). Furthermore, TA might bind some enzymes, especially lipase or phospholipase, thus lowering their activities. Moreover, microwave heating can also cause the aforementioned enzymes to denature. TA at 0.1 and 0.2% were found to be more effective in reducing FFA content than 0.05% ($p<0.05$).

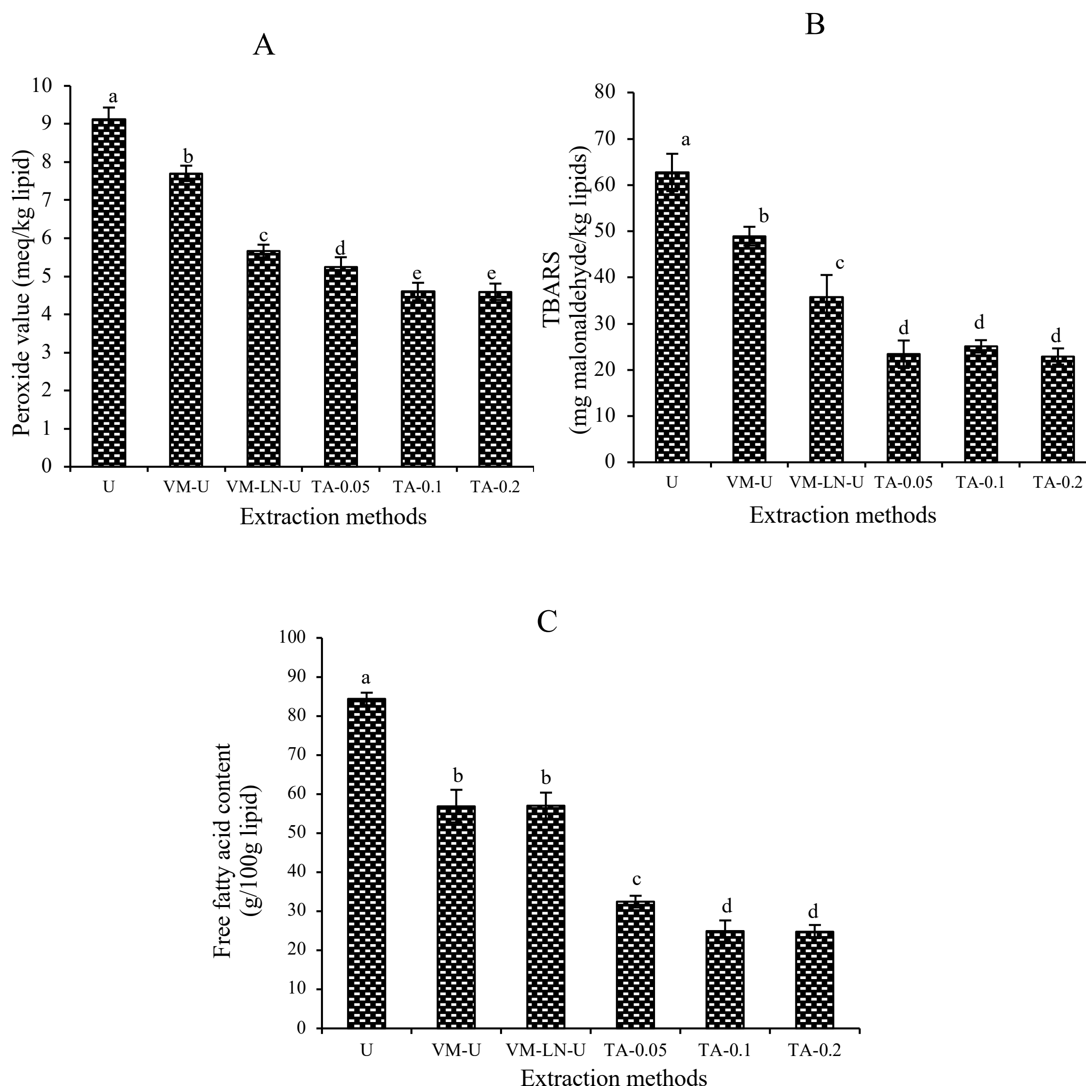


Figure 28. Peroxide value (A), TBARS value (B) and free fatty acid content (C) of lipids from cephalothorax of Pacific white shrimp extracted by UAE under different conditions and treatments. U: lipids extracted by UAE; VM-U: lipids extracted by UAE after prior vacuum-microwave heating of cephalothorax; VM-LN-U: lipids extracted by UAE under nitrogen atmosphere after prior vacuum-microwave heating of cephalothorax. TA-0.05, TA-0.1, TA-0.2: lipids extracted by UAE under nitrogen atmosphere after prior vacuum-microwave heating of cephalothorax added with 0.05, 0.1 and 0.2% tannic acid, respectively. All samples were extracted using UAE (continuous mode, 80% amplitude) for 25 min. Bars represent the standard deviation (n=3). Different lowercase letters on the bars denote significant difference ($p<0.05$).

5.5.3 Characteristics of lipids extracted from cephalothorax with different extraction conditions

5.5.3.1 Fatty acid profiles

Fatty acid profiles of the lipids from shrimp cephalothorax under different conditions and treatments are tabulated in Table 6. For all the samples, palmitic acid was found to be the most abundant fatty acid. Among the groups of fatty acids, PUFAs were found as the majority, particularly when TA was added during UAE process. Generally, marine lipids are known to contain high amounts of PUFAs (Sargent *et al.*, 1997). Lipids from shrimp cephalothorax are also known to have high amounts of PUFAs (Takeungwongtrakul *et al.*, 2012). PUFAs are not synthesized by shrimps themselves, but are mainly from the dietary sources such as marine micro- and macroalgae, which are known to be rich sources of PUFAs (Zhukova and Aizdaicher, 1995). Lipids extracted by UAE process resulted in the loss of EPA and DHA, as they are prone to oxidation (Gulzar and Benjakul, 2018). With the addition of TA along with the nitrogen flushing, higher retention of these valuable fatty acids was obtained. Highest EPA and DHA contents were found in the sample added with 0.2% TA along with nitrogen flushing. Thus TA was able to retard the oxidation significantly ($p<0.05$), particularly at a higher level. Highest DHA was found in TA-0.2 sample ($p<0.05$). Polyene index of the lipids subjected to different treatments reconfirmed their oxidative stability. Polyene index (DHA+EPA/C16:0 ratio) of TA-0.2 sample was found to be higher than the control UAE sample (U) by a 41.86%. The saturated fatty acids seemed to be similar since they possess a stable structure. Therefore, the oxidation of TA at a high level in combination with nitrogen flushing effectively prevented the oxidation associated with the loss in PUFA, particularly omega-3 fatty acids.

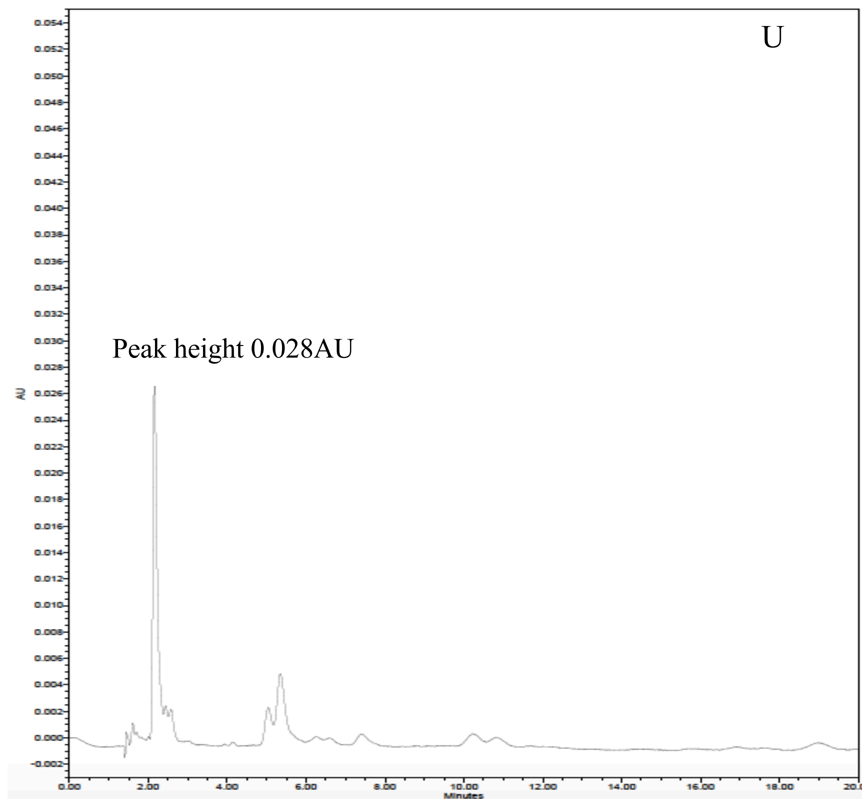
Table 6. Fatty acid profile of lipids extracted from cephalothorax of Pacific white shrimp under different conditions and pre-treatments

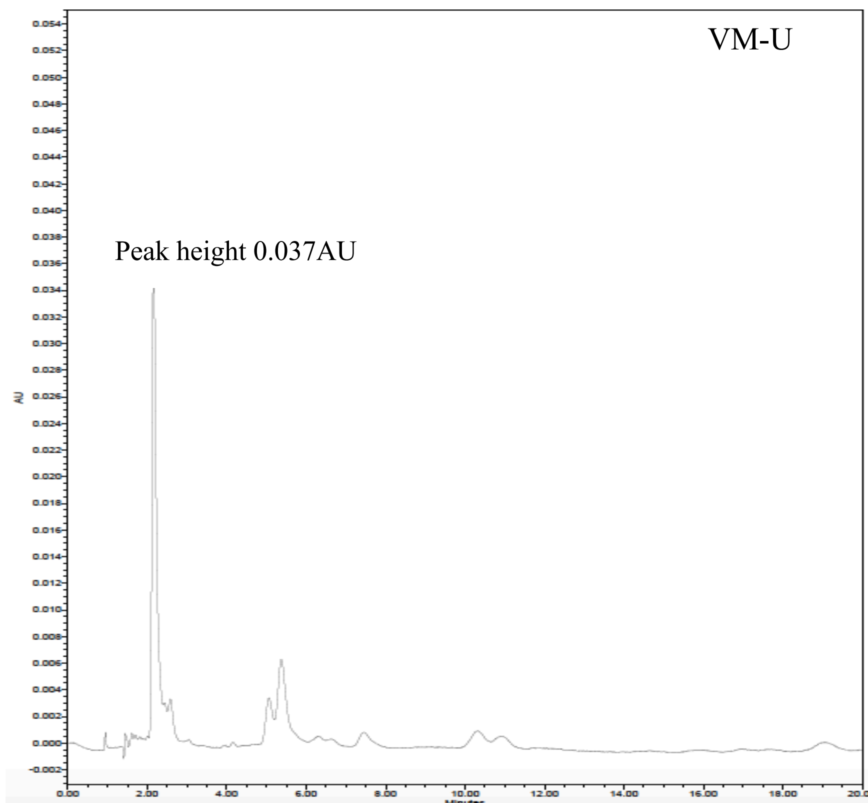
Fatty acids (g/100 g lipids)	U*	VM-U*	VM-LN-U*	TA-0.05*	TA-0.1*	TA-0.2*
C14:0	0.71 ± 0.00c	0.64 ± 0.00d	0.65 ± 0.00d	0.75 ± 0.00b	0.72 ± 0.00c	0.83 ± 0.03a
C14:1	0.21 ± 0.00c	0.19 ± 0.00d	0.20 ± 0.00cd	0.23 ± 0.01b	0.31 ± 0.00a	0.18 ± 0.00d
C15:0	0.41 ± 0.00b	0.43 ± 0.00a	0.14 ± 0.01c	ND	ND	ND
C16:0	22.69 ± 0.02a	22.63 ± 0.26a	22.34 ± 0.08a	21.24 ± 0.02b	20.59 ± 0.17c	21.31 ± 0.91b
C16:1 n-7	1.53 ± 0.04b	1.49 ± 0.12b	2.02 ± 0.04a	1.21 ± 0.00d	1.40 ± 0.02bc	1.28 ± 0.05cd
C17:0	0.89 ± 0.01b	0.97 ± 0.01ab	1.09 ± 0.01a	0.89 ± 0.12b	0.93 ± 0.02b	0.92 ± 0.08b
C17:1	0.15 ± 0.00b	ND	0.14 ± 0.01b	0.29 ± 0.00a	ND	0.21 ± 0.08ab
C18:0	9.33 ± 0.17a	10.25 ± 0.03a	9.35 ± 0.00a	9.62 ± 0.01a	9.09 ± 0.10a	9.07 ± 0.35a
C18:1 n-9	14.78 ± 0.17a	14.25 ± 0.04b	14.29 ± 0.05b	14.63 ± 0.01ab	14.76 ± 0.18a	14.52 ± 0.37ab
C18:2 n-7	2.83 ± 0.01c	2.82 ± 0.02c	3.17 ± 0.17b	3.96 ± 0.00a	3.28 ± 0.03b	3.34 ± 0.06b
C18:2 n-6	15.69 ± 0.20a	15.64 ± 0.01a	14.47 ± 0.12a	14.31 ± 0.00b	15.17 ± 0.02a	13.31 ± 0.60c
C20:1 n-9	1.85 ± 0.03b	1.83 ± 0.01b	1.58 ± 0.03c	1.63 ± 0.01c	2.19 ± 0.14a	1.26 ± 0.00d
C20:2 n-6	1.59 ± 0.00a	1.56 ± 0.03ab	1.41 ± 0.01bc	1.32 ± 0.03cd	1.52 ± 0.02ab	1.16 ± 0.16d
C20:4 n-6 (ARA)	0.66 ± 0.04a	0.49 ± 0.01d	0.53 ± 0.01cd	0.61 ± 0.01abc	0.65 ± 0.04ab	0.55 ± 0.07bcd
C20:5 (EPA)	8.00 ± 0.03b	8.13 ± 0.00b	8.85 ± 0.03a	8.17 ± 0.02b	8.83 ± 0.05a	8.88 ± 0.25a
C22:0	1.68 ± 0.02a	0.74 ± 0.02d	0.72 ± 0.04d	1.09 ± 0.02b	0.84 ± 0.01c	0.81 ± 0.02c
C22:1	0.46 ± 0.02d	0.43 ± 0.00d	0.96 ± 0.01a	0.62 ± 0.00b	0.56 ± 0.04c	0.36 ± 0.01e
C22:2 n-6	0.31 ± 0.00e	ND	0.38 ± 0.03d	0.43 ± 0.00c	0.59 ± 0.00b	1.22 ± 0.01a
C22:6 n-3 (DHA)	8.67 ± 0.06d	8.74 ± 0.01d	8.77 ± 0.05d	12.01 ± 0.05b	11.45 ± 0.11c	13.33 ± 0.48a
C23:0	3.46 ± 0.20b	3.61 ± 0.03b	3.05 ± 0.01c	3.22 ± 0.00c	3.94 ± 0.06a	2.83 ± 0.07d
C24:1	0.75 ± 0.03ab	0.75 ± 0.02ab	0.70 ± 0.00bc	0.70 ± 0.00bc	0.78 ± 0.00a	0.66 ± 0.03d
Saturated fatty acid (SFA)	39.17 ± 0.38a	39.27 ± 0.17a	37.33 ± 0.06b	36.81 ± 0.07b	36.11 ± 0.01b	35.77 ± 0.04b
Monounsaturated fatty acid (MUFA)	19.72 ± 0.25a	18.94 ± 0.08c	19.88 ± 0.15a	19.31 ± 0.01b	20.00 ± 0.01a	18.47 ± 0.19d
Polyunsaturated fatty acid (PUFA)	37.75 ± 0.07cd	37.35 ± 0.09d	38.25 ± 0.04c	40.81 ± 0.05b	41.48 ± 0.17ab	41.80 ± 0.81a

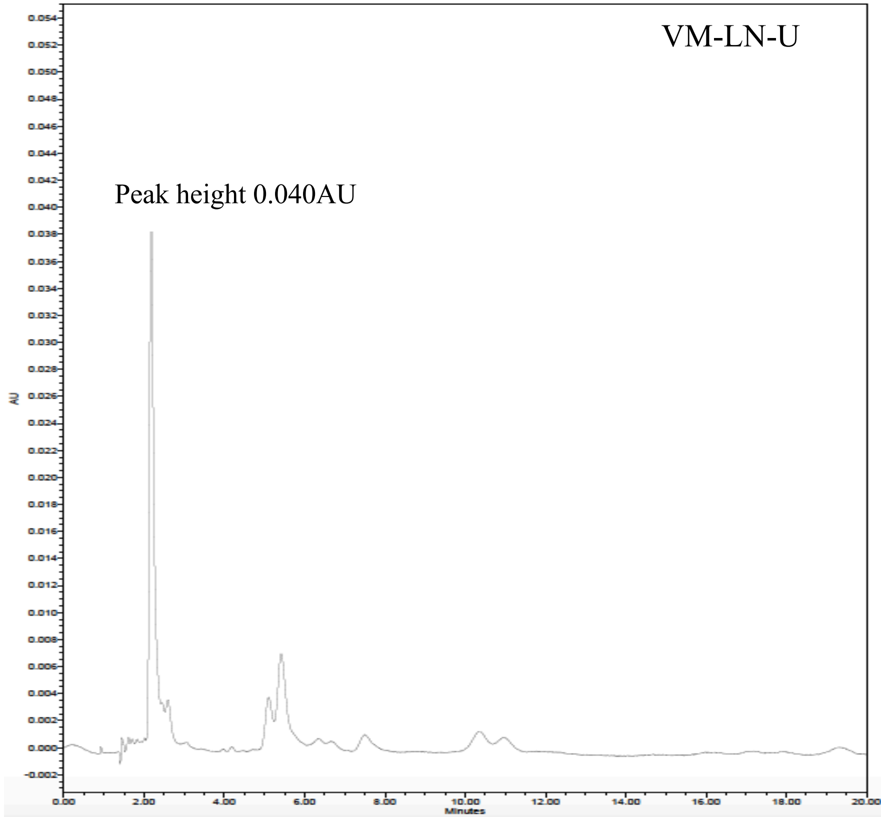
*U: lipids extracted by UAE process; *VM-U: lipids extracted by UAE process after prior vacuum-microwave heating of cephalothorax; VM-LN-U*: lipids extracted by UAE process under nitrogen atmosphere after prior vacuum-microwave heating of cephalothorax; *TA-0.05, *TA-0.1, *TA-0.2: lipids extracted by UAE process under nitrogen atmosphere after prior vacuum-microwave heating of cephalothorax added with 0.05, 0.1 and 0.2% tannic acid, respectively.

5.5.3.2 Astaxanthin

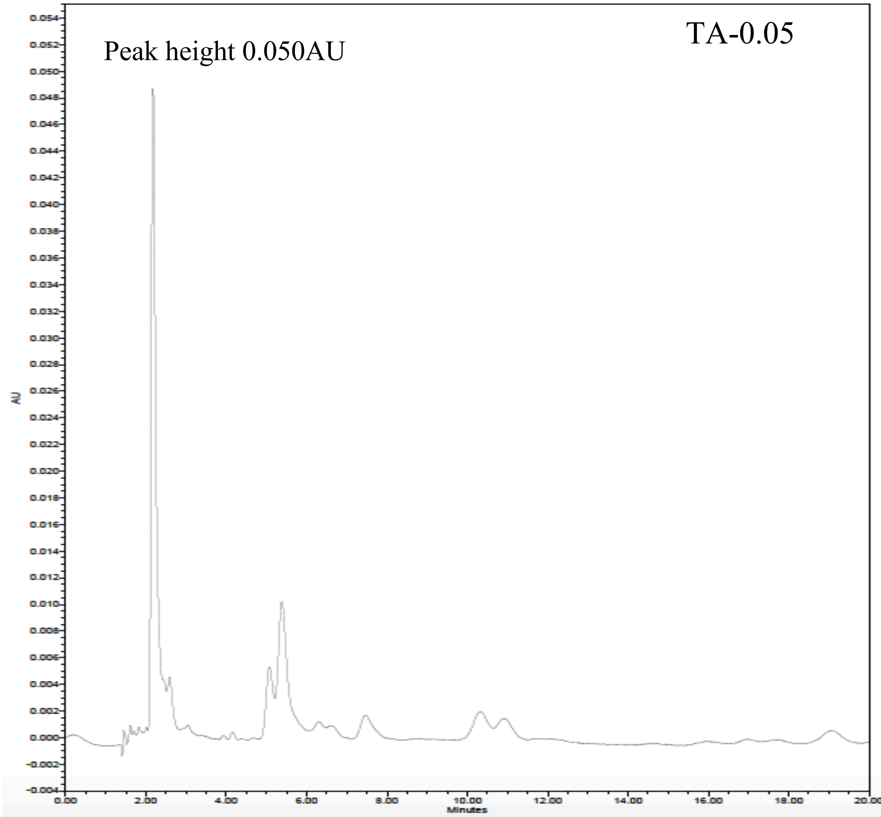
Figure 29 depicts the chromatograms of astaxanthin in lipid from shrimp cephalothorax using UAE process under different conditions and treatments. Lipids extracted from vacuum-microwave treatment alone (VM-U) and in combination with nitrogen flushing (VM-LN-U) showed higher astaxanthin content than U sample. However, the highest astaxanthin content was found in the samples treated with TA at the highest level (TA-0.2) ($p<0.05$). This clearly indicates that apart from retarding lipid oxidation, TA and nitrogen flushing were also able to prevent oxidation of astaxanthin. The results were in line with Gulzar and Benjakul, (2019a) who documented that lipid samples extracted from shrimp cephalothorax using UAE process and pre-treated with TA showed larger bands of astaxanthin as determined by TLC, compared to the non-pre-treated samples. Lowest astaxanthin content was found in the samples extracted with ultrasound without any pre-treatment or TA addition. The results were in line with the findings of Zhao *et al.*, (2006) who demonstrated that the concentration of all the astaxanthin esters decreased with ultrasound treatment. With the application of ultrasound, astaxanthin underwent degradation into colorless compounds. Astaxanthin is a carotenoid present in crustaceans, which gives them a characteristic reddish-orange color. Astaxanthin is also a powerful antioxidant with superior health benefits like anticancer, prevention of cardiovascular diseases, immunity booster and anti-inflammatory properties (Félix-Valenzuela and Goycoolea, 2006). Astaxanthin is also shown to exhibit antioxidative effect to prevent lipid oxidation during storage (Gómez-Estaca *et al.*, 2017). When the UAE, a harsh condition, was applied, astaxanthin levels are found to decrease. With the incorporation of TA and inert nitrogen atmosphere during extraction, the decomposition of astaxanthin could be significantly reduced ($p<0.05$).


U
Peak height 0.028AU


VM-U
Peak height 0.037AU



VM-LN-U
Peak height 0.040AU
AU
Minutes



TA-0.05
Peak height 0.050AU
AU
Minutes

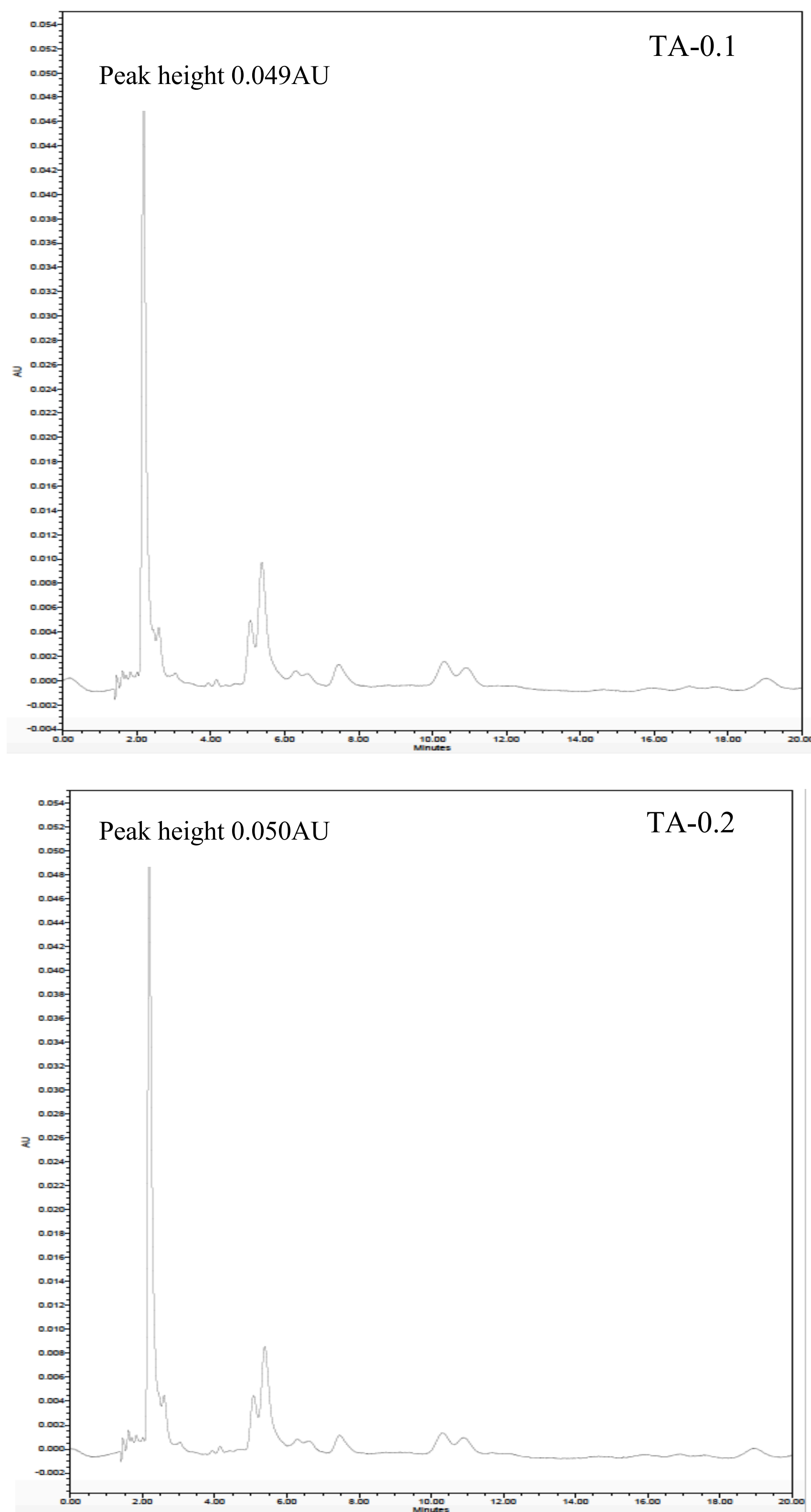


Figure 29. Chromatographs (HPLC) of astaxanthin in lipids extracted from cephalothorax of Pacific white shrimp extracted under different conditions and treatments. Caption see Figure 28.

5.5.3.3 FTIR spectra

FTIR spectra of 3 selected samples including U, VM-LN-U, and TA-0.2 are shown in Figure 30. The use of FTIR spectra in food systems is especially used to identify the functional groups of compounds present in biological samples as it is simple, rapid, and offers nondestructive measurements of chemical and physical components (Rodriguez-Saona and Allendorf, 2011). At 3400 cm^{-1}, the samples had a peculiar peak, in which higher amplitude was found in UAE only treated sample (U). Absorbance peak between 3600-3400cm^{-1} represents hydroperoxide moieties owing to their –OO–H stretching vibrations (Van De Voort *et al.*, 1994). The results were in line with the findings of PV (Figure 28A), indicating the presence of intermediate lipid oxidation products. Absorbance bands observed between 3100-2800 cm^{-1} represent the C–H stretch vibrations and overlapping with the –OH group in a carboxylic acid. These bands have been identified to represent the degree of unsaturation in fats and oils (Afran and Newbery, 1991). The highest amplitude in this absorbance band was noticeable in the TA-0.2 sample, followed by VM-LN-U. To monitor the lipid oxidation, Guillén and Cabo (2004) used the ratio between the absorbance band at 2854 cm^{-1}, and 3600 - 3100 cm^{-1} ($A_{2854}/A_{3600–3100}$). Lower $A_{2854}/A_{3600–3100}$ values indicated more intense lipid oxidation. Generally, the unsaturation degree to hyperoxide ratio would be less in more oxidized samples. U samples showed the lowest $A_{2854}/A_{3600–3100}$ values, compared to VM-LN-U and TA-0.2 samples. The peaks at 1730-1745 cm^{-1} represent the ester carbonyl group of triglycerides (Takeungwongtrakul *et al.*, 2015). Similar peaks were observed at 1732 cm^{-1}, indicating the presence of triglycerides in all the samples. Nevertheless, VM-LN-U and TA-0.2 samples showed higher amplitude. The decrease in the aforementioned peak of U sample was plausibly associated with the hydrolysis of the ester bond as evidenced by higher FFA content (Figure 28C). Lipids extracted by UAE process and subjected to heat pre-treatment at 95 ºC and addition of TA showed larger peaks at 1744 cm^{-1} (Gulzar and Benjakul, 2019a). The band around 1100 cm^{-1} representing the stretching vibration of the C–O ester groups are inversely proportional to saturated acyl groups (Guillén and Cabo, 1997). Evidently, TA-0.2 and VM-LN-U samples showed larger peaks due to the lesser presence of saturated

acyl groups, compared to U sample. Thus, lipid oxidation was retarded by nitrogen flushing along with TA addition during the UAE process.

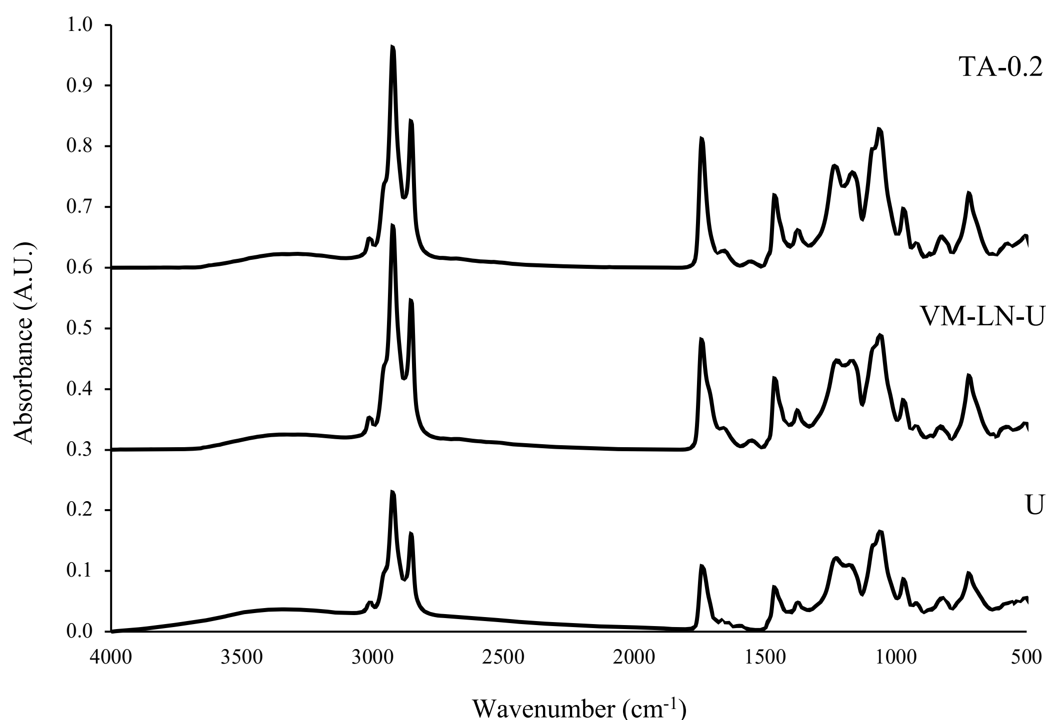


Figure 30. FTIR spectra of lipids extracted from cephalothorax of Pacific white shrimp using the selected extraction conditions and treatments. Caption see Figure 28.

5.6 Conclusion

Vacuum-microwave treatment in combination with UAE resulted in the increase in extraction yield of lipid from shrimp cephalothorax. Lipid oxidation and hydrolysis were significantly suppressed by nitrogen flushing the overhead of ultrasonication vessel and addition of tannic acid. The combination of all these treatments led to the lowered oxidation and hydrolysis of lipid. Also omega-3 fatty acid and astaxanthin were more retained. Overall, the adverse effects of UAE on quality of lipids could be suppressed to some degree by incorporating these treatments into the extraction process.

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CHAPTER 6

CHARACTERISTICS AND STORAGE STABILITY OF NANOLIPOSOMES LOADED WITH SHRIMP OIL AS AFFECTED BY ULTRASONICATION AND MICROFLUIDIZATION

6.1 Abstract

Shrimp oil, a rich source of n-3 fatty acids and astaxanthin, was encapsulated in nanoliposomes, prepared using ultrasonication (US) and microfluidization (MF) methods, to prevent oxidation during storage. Nanoliposomes prepared by US and MF were characterized based on particle size, structure and stability. The particle size of US nanoliposomes ranged between 40 and 284 nm, while MF nanoliposomes ranged from 214 – 928 nm. US nanoliposomes exhibited better centrifugal stability than MF counterparts ($p<0.05$). Nanoencapsulation efficiency (NEE) of US nanoliposomes was higher (93.64%) than that of MF (75.18) and remained constant over the storage of 8 weeks at 30 °C. Nanoliposomes showed higher oxidative stability during the storage than unencapsulated oil ($p<0.05$) with better retention of EPA and DHA, particularly in US nanoliposomes. Overall, encapsulation of shrimp oil in nanoliposomes proved to be an effective method to prevent oxidation of oil during storage and mask the undesirable fishy odor.

6.2 Introduction

Shrimp processing industry has grown enormously, capturing the market worldwide. The oil obtained from the shrimp is majorly present in the head (cephalothorax), which is an inedible part and ends up as a discard from shrimp processing industry. Shrimp oil has gained increasing attention for consumers because of the presence of n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The aforementioned fatty acids are of high importance as they possess several health benefits, e.g. preventive effect on cardiovascular diseases. These fatty acids also possess anti-inflammatory properties and help reduce the risk of Alzheimer's disease (Swanson *et al.*, 2012). Shrimp oil also contains

carotenoids, e.g. β-carotene and astaxanthin. Astaxanthin is a coloring pigment found in crustaceans and has superior antioxidant activity to other carotenoids including canthaxanthin, zeaxanthin, and lutein (Naguib, 2000).

Shrimp oil contains a large amount of polyunsaturated fatty acids (PUFAs), which are prone to oxidative deterioration, resulting in the formation of undesirable off-flavor (Gulzar and Benjakul, 2018). Oils can undergo oxidation when catalysts such as transition metals (Fe, Cu), enzymes (lipoxygenases) and some chemical oxidizers are present. Environmental factors such as heat as well as light (UV) also accelerate lipid oxidation (Kolanowski *et al.*, 2007). To conquer the oxidation of oils, several techniques have been employed. Those involve modified atmospheric packaging, usage of antioxidants, micro-encapsulation (Raei and Jafari, 2013; Takeungwongtrakul *et al.*, 2014), etc. Encapsulation method has been used successfully in retarding or suppressing oxidation process and masking undesirable odors and flavors of oils (Takeungwongtrakul *et al*., 2014). However, conventional encapsulation with different wall materials may not ensure the protection efficacy towards oxidation of marine oils. Fish oil encapsulated with trehalose showed lower oxidation for 14 days, but the oxidation was increased rapidly afterward (Drusch *et al.*, 2006).

One of the most recent and reliable techniques used for encapsulation of bioactive molecules is the nanoliposome technology (Munin and Edwards-Lévy, 2011). Liposomes are spherical vesicles consisting of a phospholipid bilayer and an aqueous core with sizes ranging from nanometer to micrometer. Liposomes with the size less than 1000 nm are referred to as nanoliposomes. Colloidal particles ranging in the size of 1-1000 nm are generally referred to as nanoparticles (Hallaj-Nezhadi *et al.*, 2013). Numerous procedures have been introduced to prepare nanoliposomes including thermal and non-thermal processes to incorporate bioactive and functional components (Mozafari *et al.*, 2008). Depending upon the size, liposomes can be single-layered (unilamellar) or multiple layered (multilamellar). Encapsulation in liposomes has been widely used to incorporate water-soluble or lipid-soluble components for targeted delivery (Dias *et al.*, 2017). Nanoliposomes have been used

to encapsulate fish oils due to advantages such as better oxidative stability, better retention of n-3 fatty acids, masking of fishy odor, etc. (Ghorbanzade *et al.*, 2017). Encapsulation of unsaturated fatty acids into liposome capsules creates a barrier to prevent the oxidation of these compounds (Hadian, 2016).

Shrimp oil, especially from processing by-products has drawn attention owing to its abundance in n-3 fatty acids and astaxanthin. Nevertheless, it is prone to oxidation. Microencapsulation for shrimp oil using different wall materials along with spray drying was performed (Takeungwongtrakul *et al.*, 2015). However, the process has disadvantages of using high temperature during the drying process that likely accelerates lipid oxidation (Jafari *et al.*, 2008). Shrimp oil encapsulation, therefore requires a process which uses relatively lower temperature for processing and provides better oxidative stability and masks the fishy odor. Consequently, liposome technology could be implemented in the encapsulation of shrimp oil to conquer the stability issues.

6.3 Objectives

To investigate the incorporation of shrimp oil in nanoliposomes using different preparation methods.

To monitor the stability of shrimp oil nanoliposomes during the prolonged storage.

6.4 Materials and methods

6.4.1 Chemicals

Soy lecithin (L-α-Phosphatidylcholine) was purchased from Sigma (St. Louis. MO, USA). Shrimp oil was extracted from locally purchased Pacific white shrimp (*Litopenaeus vannamei*) cephalothorax using the method of (Gulzar and Benjakul, 2020). Shrimp cephalothorax were preheated at 95 °C for 15 min. Thereafter, preheated samples were ground for 5 min using a blender (National Model MK-K77, Tokyo, Japan). Fifty gram (50 g) shrimp cephalothorax was homogenized with 250 mL of isopropanol and n-hexane (1:1) mixture at a speed of 9500 rpm for 2

min at 4 °C using an IKA Labortechnik homogenizer (Selangor, Malaysia). Ten mL of 1% (v/v) tannic acid solution was added to the homogenized sample. The samples were subsequently ultrasonicated using an Ultrasonic Processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA), at ultrasonic amplitude of 80% in continuous mode for 25 min. During ultrasonication, the samples were flushed with nitrogen gas at 2.15 L/min to continuously replace the air present in the vessel headspace. These conditions of shrimp oil extraction were chosen since they rendered the shrimp oil with the highest oxidative quality and extraction yield.

6.4.2 Preparation of nanoliposomes by different methods

6.4.2.1 Ultrasonication method for nanoliposome preparation

Nanoliposomes were prepared by the method of Rasti *et al*. (2012) with modifications. Lecithin was firstly dissolved in ethanol to attain 5% (w/v) and heated up to 45 °C to ensure complete dissolution. Subsequently, 5 mL of shrimp oil (preheated to 30 °C) was dropped gradually into 100 mL of prepared lecithin solution. The mixture was stirred at 1,000 rpm on a hotplate magnetic stirrer at 30 °C. Deionized water (100 mL) and glycerol (2% v/v) was added to hydrate the solution and homogenized for 10 min by an IKA Labortechnik homogenizer (Selangor, Malaysia). The liposomal dispersions (approximately 200 mL) were subjected to ultrasonication (10 min; 1 s on and off pulse) at 25 °C using an ultrasonic processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude. Ethanol was removed using EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 30 °C. After preparation, final nanoliposomes were placed under nitrogen atmosphere at 25 °C for 1 h for stabilization. The sample was labeled as ‘US’.

6.4.2.2 Microfluidization method for nanoliposome preparation

Liposomal dispersions were prepared as per the method discussed above. The freshly prepared liposomes (200 mL) were forced through a high-pressure microfluidizer (Microfluidics, Model HC 5000, Stanwood, WA, USA) at 7000 psi for ten times. Ethanol was removed using EYELA rotary evaporator N-1000 (Tokyo

Rikakikai, Co., Ltd., Tokyo, Japan) at 30°C. The obtained nanoliposomes were placed under nitrogen atmosphere at 25 °C for 1 h and referred to as 'MF'.

6.4.3 Characterization of nanoliposomes prepared by ultrasonication and microfludization

6.4.3.1 Determination of particle size

Particle size distribution and mean particle size of nanoliposome samples, both US and MF, were determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) following the procedure of Castellani *et al.*, (2006). Five mL of each sample were diluted with 20 mL of 1% (v/v) sodium dodecyl sulfate (SDS) solution to dissociate the flocculated vesicles. The volume-weighted mean particle diameters (d_{43}) of the nanoliposome vesicles were measured.

6.4.3.2 Centrifugal stability measurement

Stability of nanoliposome samples, US and MF, was examined as per the method of Sciarini *et al.* (2009). Five mL of nanoliposomes were subjected to centrifugation at 3500 g for 15 min. Nanoliposome stability (NS) was calculated as follows:

$$NS = \frac{f_{ev}}{i_{ev}}$$

where f_{ev} is the final volume of the bottom phase and i_{ev} is the initial volume of liposomal dispersion.

6.4.3.4 Transmission electron microscopy

Transmission electron microscopy of nanoliposomes was carried out following the method of Yarnpakdee *et al.* (2009). Nanoliposomal dispersions (US and MF) were heated up to 75 °C and then cooled immediately. Potassium phosphate buffer (50 mM) containing 0.6 M KCl (pH 7) was used to dilute the samples to 0.2 mg/mL. The samples were fixed on a carbon-coated grid, stained negatively with 4% uranyl acetate for 5 min and subsequently washed using distilled water. To visualize

the specimens, a JEOL JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) was used with an accelerating voltage of 160 kV.

6.4.3.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter (Perkin Elmer, Model DSC7, Norwalk, CA, USA) following the method of Rochdi *et al.* (2000). The samples were rehydrated by adding deionized water or 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C. The temperature of samples was cooled down to -40 °C. Temperature calibration was run using the Indium standard. The samples were accurately weighed into aluminium pans and sealed. The samples were scanned at 10 °C/min over the range of -40–150 °C. An empty pan was used as the reference. Total denaturation enthalpy (ΔH) was estimated by measuring the area of the DSC thermogram.

6.4.4 Stability of nanoliposomes during storage

Nanoliposome samples, both US and MF, were kept at 30 °C and randomly taken every 2 weeks for totally 8 weeks for analyses. Shrimp oil without nanoliposome encapsulation was also used as the control.

6.4.4.1 Nanoencapsulation efficiency of nanoliposomes

The efficiency of nanoliposomes, both US and MF, to encapsulate the shrimp oil was evaluated using the method of Ghorbanzade *et al.* (2017) with some modifications. Nanoliposome dispersions were subjected to centrifugation at 4200 g for 15 min (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA) to separate non-encapsulated shrimp oil. The supernatant was collected and mixed with the chloroform/methanol (2:1) mixture at a ratio of 1:5 (v/v). To allow complete extraction, samples were left for 24 h. Shrimp oil at the same amount used for liposome preparation was mixed with the mixed solvents and extracted in the same manner. The absorbance of both samples, total oil, and unencapsulated oil, was read at 468 nm using a spectrophotometer. Encapsulation efficiency (%EE) was calculated according to the following equation (Hill *et al.*, 2013)

$$\%EE = \frac{(OD_{468}\text{ total oil}) - (OD_{468}\text{ unencapsulated oil})}{(OD_{468}\text{ total oil})} \times 100$$

For determination of PV, TBARS and fatty acid profile, nanoliposomes were firstly extracted using the mixture of chloroform and methanol (2:1) using the sample/solvent ratio of 1:5 (v/v) and the lipid samples were subjected to the analyses.

6.4.4.2 Determination of oxidation

Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were used as indicators for lipid oxidation. PV and TBARS were determined following the method of Gulzar and Benjakul (2018). For the determination of PV, lipid sample (50 µl) was 10-fold diluted using 75% ethanol, (v/v). To the prepared sample, a mixture of 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µL of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added. The mixture was mixed well and the absorbance was read at 500 nm using a spectrophotometer. The blank was prepared in the same way, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

For TBARS determination, lipid sample (0.5g) was mixed with 2.5 mL of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was then heated in boiling water (95–100°C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600g at 25°C for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was read at 532 nm using a spectrophotometer. A standard curve was prepared using malonaldehyde (MDA) at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as malonaldehyde equivalent.

6.4.4.3 Fatty acid profile

Fatty acid profile was analyzed as fatty acid methyl esters (FAMEs) using gas chromatography (GC) following the method of (Gulzar and Benjakul, 2019c). Fatty acids were firstly transmethylated by 2 M methanolic sodium

hydroxide, along with 2 M methanolic hydrochloric acid. FAMEs were analyzed by gas chromatography (GC) using Agilent 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. FAME dissolved in hexane was determined using Agilent J&W fused silica capillary column (100 m × 0.25 mm × 0.20 μm) (a split ratio of 1:20). The injection port temperature was kept at 250 °C and detector (flame ionization detector, FID) temperature was 270 °C. The oven temperature ranged from 170 to 225 °C with a flow rate of 1 °C /min. The chromatographic peaks of the samples were identified based on retention times, compared to those of standards. Peak area ratio was used for calculation and the content was expressed as g fatty acid/100 g lipid.

6.4.4.4 Fishy odor intensity

The fishy odor intensity of nanoliposomes and shrimp oil over the storage period of 8 weeks was evaluated using the modified method of Sae-leaw *et al.* (2013). The samples were tested for fishy odor by 8 trained panelists with the ages of 25–35. Panelists were trained twice a week for a total of 8 weeks. The intensity of fishy odor of the samples was evaluated using a linear scale anchored at 'no fishy odor' and 'high intensity of fishy odor'. Nanoliposomes without shrimp oil was used as a reference for 'no fishy odor' whereas oxidized shrimp oil kept at room temperature and subjected to oxygen flushing for 24 h was chosen as a reference for 'high-intensity fishy odor'. A scale from 0 to 5 was employed; 0 corresponding to no fishy odor; 5 to the high intensity of the fishy odor. The samples were placed in the sealable plastic cups. The panelists were asked to sniff the headspace of the cup to determine the fishy intensity of the nanoliposomes and shrimp oil.

6.4.4.5 Measurement of volatile compounds

The solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) was used (Iglesias and Medina, 2008). To extract volatile compounds, 1 g of samples was mixed with 4 mL of deionized water and stirred gently to disperse the sample. The mixture was heated at 60°C in 20 mL headspace vial with equilibrium time of 10 h. The SPME fibre (50/30 lm DVB/Carboxen™/PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was

conditioned at 270°C for 15 min before use and then exposed to the headspace. The 20 mL-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60°C for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270°C. GC–MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m± 0.25 mm ID, with film thickness of 0.25 μm). Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

6.4.5 Statistical analysis

All the experiments were done in triplicates and the data were expressed as means ± SD. Completely randomized design (CRD) was used throughout the study. Randomized complete block design (RCB) was used for sensory analysis study. Analysis of variance (ANOVA) was performed and means were compared by Duncan's multiple range test (Steel and Torrie, 1980). Analysis of data was carried out using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

6.5 Results and discussion

6.5.1 Effect of different preparation methods on the structure and characteristics of nanoliposomes

6.5.1.1 Particle size

Figure 31 represents the size distribution of US and MF nanoliposomes loaded with shrimp oil. The results indicated that the particle size of US

nanoliposomes ranged between 40 and 284 nm and those of MF nanoliposomes ranged between 214 and 928 nm. The mean particle sizes of US and MF nanoliposomes were 104.77 and 512.80 nm, respectively. Nanoliposomes prepared from fish oil as reported by other studies were in a similar range (Liu and Park, 2009; Wang *et al.*, 2015). US nanoliposomes showed a unimodal distribution, while MF counterparts presented a bimodal distribution. Particles distributed in both samples were less than 1000 nm in size. Ultrasonication resulted in more homogeneity of vesicles, whereas microfluidization reduced the size of vesicles over a wide range. The variations in the size of nanoliposome diameters were influenced by several factors including emulsifier types, homogenization, and type of wall materials used (Ghayempour and Mortazavi, 2015). The size of liposomes prepared by sonication was smaller than those prepared from the extrusion method and was controlled by the duration of sonication (Maulucci *et al.*, 2005). For sonication, the induced pressure stress breaks up the large and multilamellar vesicles into small unilamellar vesicles (Small, 1996).

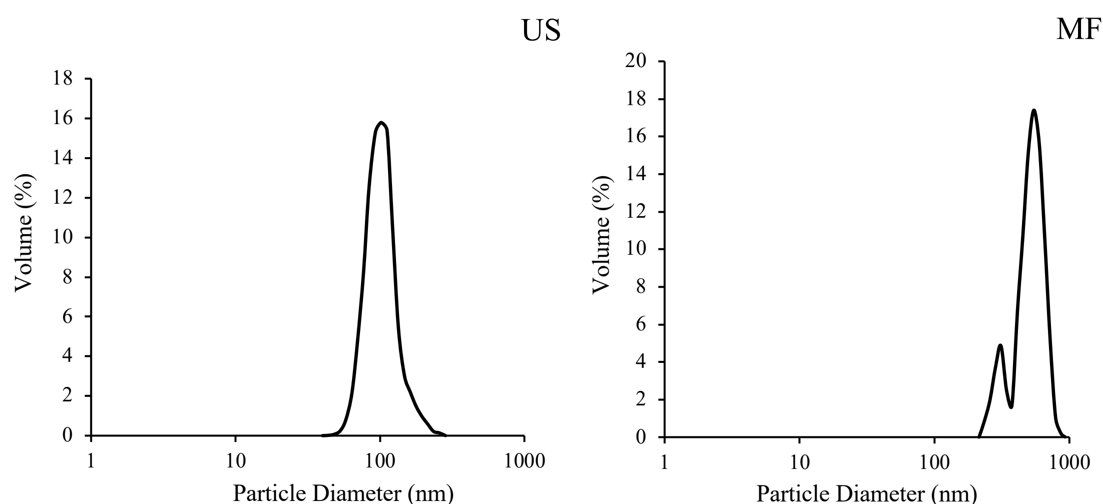


Figure 31. Size distribution (d_{43}) of nanoliposomes containing shrimp oil prepared by different methods. US: Nanoliposomes prepared by ultrasonication method MF: Nanoliposomes prepared by microfluidization method.

6.5.1.2 Centrifugal stability

The centrifugal stability of US and MF nanoliposomes were 94.5% and 79.3% respectively. The results indicated that unencapsulated shrimp oil was separated from the liposomal dispersion after being subjected to centrifugal force. It was observed that US nanoliposomes had higher centrifugal stability than MF counterpart. The stability of liposomes is primarily influenced by factors such as size, the number of phospholipid bilayers, structure, method of preparation and environmental conditions (Laridi *et al.*, 2003). Generally, liposomes with smaller size were more stable is the dispersion. This confirmed higher stability of US nanoliposomes over MF ones. Coincidentally, the mean particle size of US nanoliposomes was smaller than that of MF counterpart (Figure 31). Due to some random movements (Brownian movement) or superimposed convection, the vesicles tend to collide with each other and form larger vesicles (Taylor *et al.*, 2007). Owing to the higher mass of larger vesicles, they tend to phase out from the dispersion once the centrifugal force is applied. The instability of liposomes is caused by the random collisions and eventual merging of membranes of two or more liposomes (Taylor *et al.*, 2005). Thus, preparation methods played a major role in the stability of liposomes.

6.5.1.3 Transmission electron microscopic images

Transmission electron micrographs of US and MF nanoliposomes are shown in Figure 32. Nanoliposomes prepared using ultrasonication (US) and microfluidization (MF) were typically spherical. The micrographs showed that the size of both US and MF nanoliposomes ranged between 100 nm to 500 nm as measured by LPSA (Figure 31). TEM images are used for evaluating the shape and sizes of nanoliposomes. However, for evaluating absolute sizes, TEM may not be ideal, since liposomes can get distorted by the vacuum and the high energy electron beam used during imaging. Consequently, some vesicles appeared distorted or were not completely spherical. TEM images indicated that the vesicles were unilamellar as well as multilamellar in structure. US nanoliposomes were mostly unilamellar, while the large-sized multilamellar vesicles were majorly found in MF nanoliposomes. The

lamella around MF nanoliposomes can be seen to have more than one phospholipid bilayer, whereas US nanoliposomes can be seen to have only a single bilayer. Consequently, the size of MF nanoliposomes was bigger than US counterparts. The results were in agreement with Maulucci *et al*. (2005) who documented that sonication resulted in a decreased radius of vesicles and breakage into unilamellar structures. During ultrasonication, the induced pressure stress caused by cavitation breaks up the large and the multilamellar vesicles into small unilamellar vesicles (Maulucci *et al.*, 2005). Ultrasonication has been reported to reduce the size of liposomes since energy is disseminated from all directions continuously for a prolonged period in the lipid suspension (Rudra *et al.*, 2010). Ultrasonication induces mechanical shear by ultrasonic cavitation, to yield emulsion with a narrow size distribution of droplets. Microfluidizer, on the other hand, uses high pressures (up to l0,000 psi) to guide the flow stream through microchannels toward the impingement area in which the size of the droplets are reduced to the tune of 1 micron and above (Mozafari, 2010). The images also revealed that the shrimp oil was localized in the middle of the nanoliposomal structures. Some lipids might also be located at bilayer. Thus, shrimp oil could be encapsulated as the core in nanoliposomes produced.

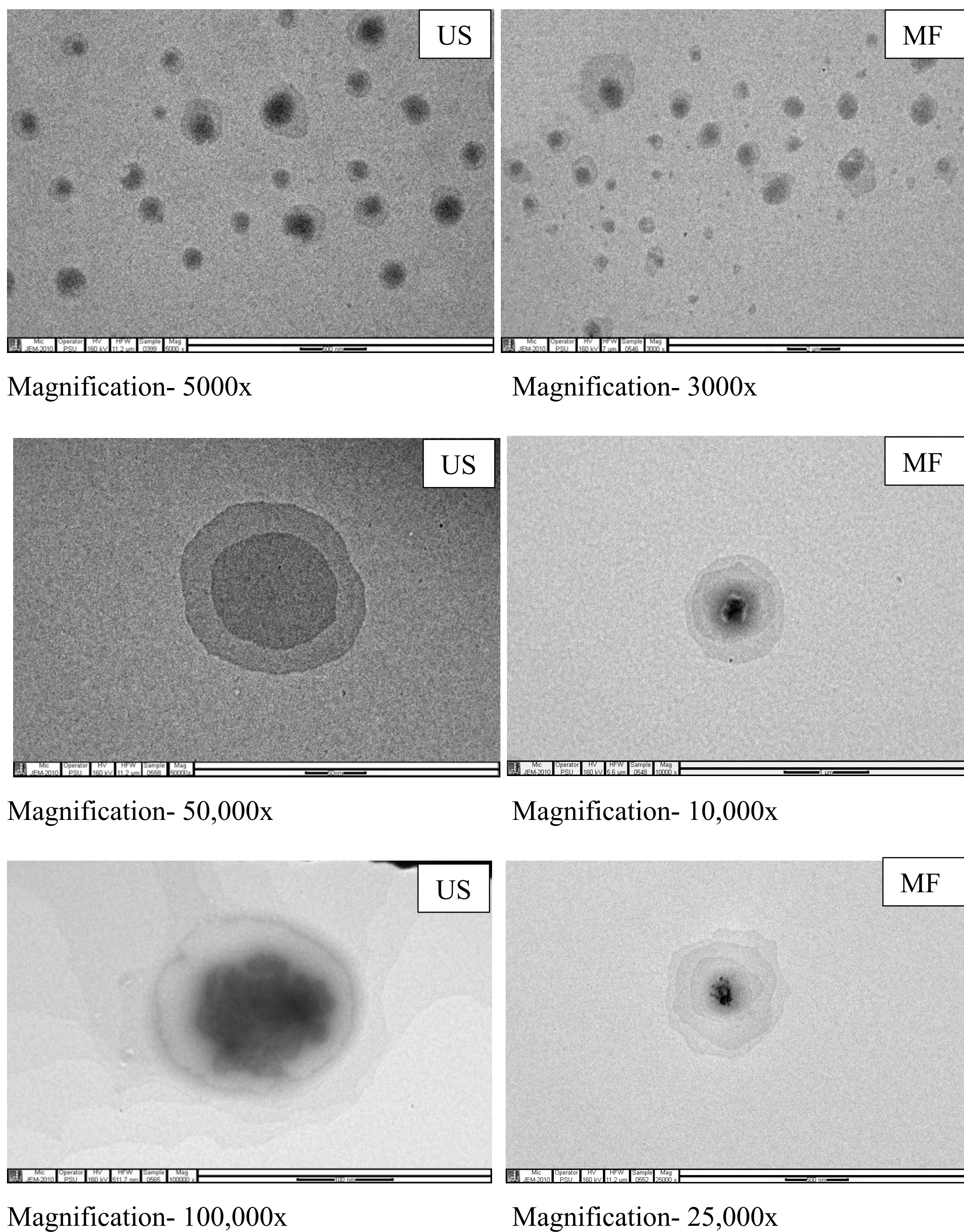


Figure 32. Transmission electron micrographs of nanoliposomes containing shrimp oil prepared by different methods. Caption see Figure 31.

6.5.1.4 Differential scanning calorimetry

DSC thermograms of US and MF nanoliposomes are shown in Figure 33. The phase transition of US nanoliposomes from orderly to disorderly state has taken place at 57.31 °C (peak) and 108.65 °C, whereas for MF nanoliposomes the transition has taken place at 107.03 °C (peak). The phase transition of liposomes occurs at a defined temperature. With calorimetry, as thermal energy is added, the phospholipid bilayers transit from a gel (orderly) state to a fluid (disorderly) state. This affects the Van der Waals interactions between the hydrocarbon chains and increases their mobility (Oh *et al.*, 2012). The phase transition due to addition of thermal energy is also dependent on the size of liposomes. Liposomes that were derived from DMPE having larger hydrodynamic diameters than the other liposomes, had higher phase transition temperature (Oh *et al.*, 2012). Therefore, US nanoliposomes with smaller mean particle size (Figure 31) had a lower phase transition enthalpy compared to MF nanoliposomes. The amount of energy provided is associated with the phase transition, and the conformation of the phospholipids is related to the stability. Multilamellar liposomes have higher phase transition enthalpies and the phase transition temperature is critically dependent on vesicle size (Biltonen and Lichtenberg, 1993). TEM images (Figure 32) confirmed that MF nanoliposomes had multiple lamella, therefore much higher phase transition enthalpies were required for the phase transition.

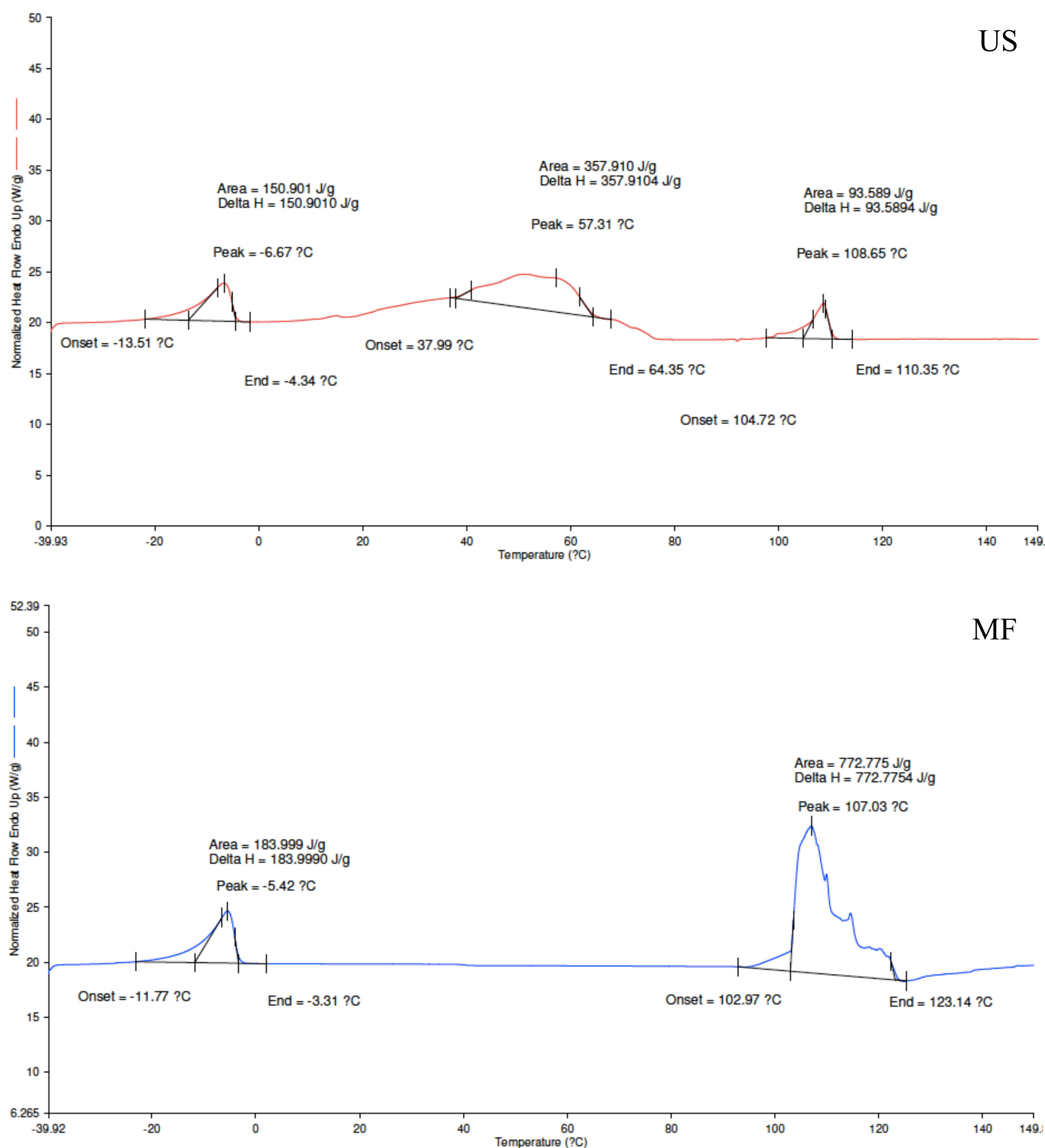


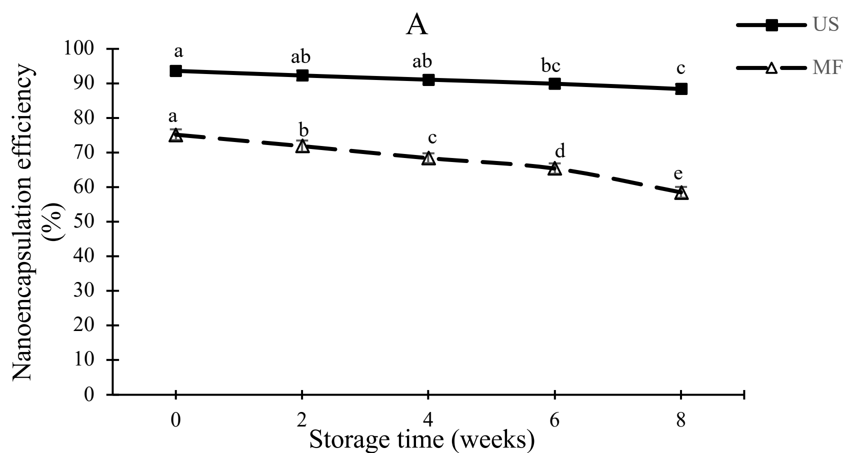
Figure 33. Differential scanning calorimetry thermograms of nanoliposomes containing shrimp oil prepared by different methods. US: Nanoliposomes prepared by ultrasonication method MF: Nanoliposomes prepared by microfluidization method.

6.5.2 Stability of nanoliposomal dispersions prepared by ultrasonication and microfluidization during storage

6.5.2.1 Nanoencapsulation efficiency

Figure 34A depicts the nanoencapsulation efficiency (NEE) of US and

MF nanoliposomes during the storage of 8 weeks at 30 °C. NEE is the indicator of how much shrimp oil was encapsulated in the liposome. US nanoliposomes showed higher NEE, compared to MF samples. NEE of freshly prepared US and MF nanoliposomes was calculated to be 93.64% and 75.18%. Ghorbanzade *et al*. (2017) documented that nanoencapsulation efficiency of fish oil within nanoliposomes was 92.22%. Based on preliminary studies, the amount of oil and lecithin had an impact on the encapsulation efficiency. Lecithin to shrimp oil ratio of 1:1 (w/w) and shrimp oil to water ratio of 1:20 (v/v) was appropriate to enhance nanoliposome stability and NEE, using both methods of preparation. Stability and NEE of active compounds within liposomes are influenced by numerous factors such as the ratio of lipid phase (Mozafari and Mortazavi, 2005), size and/or specific surface areas of the liposomes (Rasti *et al.*, 2012) and method of liposomal preparation (Elizondo *et al.*, 2012). The higher NEE of US nanoliposomes compared to MF nanoliposomes could be attributed to the increased surface area of nanoliposomes due to smaller mean size of the former. This could be also explained by the increased bonds between hydrophobic fatty acids in shrimp oil and phospholipid bilayer. As the surface area of the particles increased, there was an increased possibility of contact and bonding between fatty acids and phospholipids in the bilayers (Heurtault *et al.*, 2003). Over 8 weeks, NEE of MF liposomes was decreased continuously ($p<0.05$), while that of US nanoliposomes dropped significantly only after week 6. The results were further confirmed by the change in color of liposomes as shown in Figure 34B. The color (redness) of US samples was almost constant over 8 weeks of storage, while redness was slightly decreased for the MF samples, particularly with increasing storage time. Decrease in redness was concomitant with the increase in the whiteness of the nanoliposomes. US nanoliposomes showed lesser decrease in the redness over the storage period compared to MF counterparts. The red color of the nanoliposomes was due to the presence of astaxanthin in the shrimp oil. The total carotenoid content of shrimp oil was 2.45±0.09 mg/g lipid. Additionally, US nanoliposomes appeared to be more reddish since more oil-rich in astaxanthin was encapsulated in US, compared to MF.



B

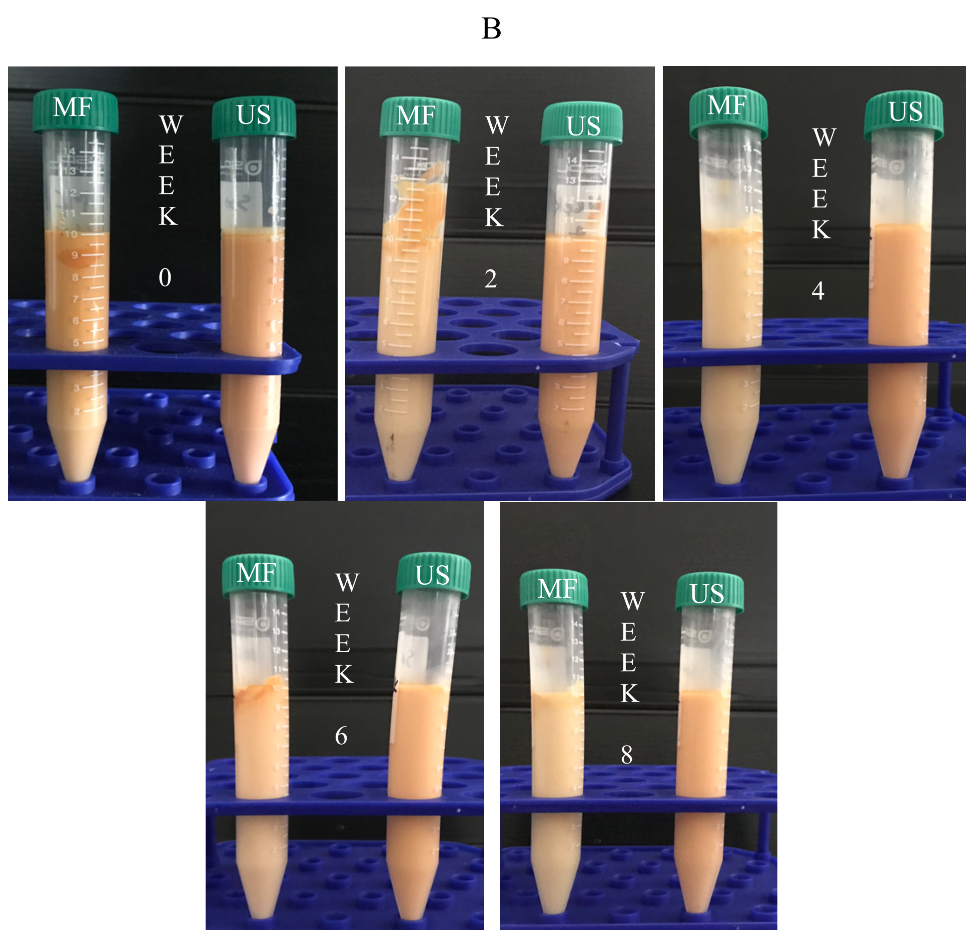


Figure 34. Nanoencapsulation efficiency (A) and appearance during the storage of 8 weeks (B) at 30 °C of nanoliposomes containing shrimp oil prepared by different methods. Bars represent the standard deviation (n=3). Different lowercase letters on the bars denote significant difference ($p<0.05$). Caption see Figure 32

6.5.2.2 Peroxide value

Figure 35A shows the PV of US and MF nanoliposomes in comparison with non-encapsulated shrimp oil (NE) over 8 weeks. There was a sharp increase in PV of unencapsulated shrimp oil, compared to the nanoliposomes up to week 4 ($p<0.05$). Among the nanoliposome samples, US nanoliposomes showed more oxidative stability than MF samples as shown by the lower increase in PV. There was a slight increase in PV of US samples up to 6 weeks of storage ($p<0.05$). However, for MF nanoliposomes, PV increased significantly throughout the storage. The rate of increase in the PV of MF sample was higher than that of US sample. The results were in agreement with Ghorbanzade *et al*. (2017) who showed that PV of unencapsulated fish oil was higher than nanoencapsulated fish oil and the increase in PV was caused by the oxidation of PUFAs. Ghorbanzade *et al*. (2017) reported that the PV of fish oil nanoencapsulated in liposomes was almost constant during storage of 21 days. Shrimp oil is rich in PUFAs, and PUFAs are highly susceptible to oxidation (Gulzar and Benjakul, 2018). Higher PV of MF nanoliposomes could be related to less centrifugal stability and lower NEE. The unencapsulated oil in MF samples was prone to oxidation. The results clearly indicated that encapsulation of shrimp oil in nanoliposomes protected the oil from oxidation. The results posted by Ye *et al.* (2009) demonstrated that the addition of encapsulated fish oil in cheese decreased peroxide and thiobarbituric acid values after 35 days of storage. There was a drop in PV of unencapsulated oil after the 6th week. This was probably owing to the decomposition of hydroperoxides to volatile secondary oxidation compounds (Boselli *et al.*, 2005). Another effect of oxidation on liposomes could be seen in Figure 34B. Oxidation of astaxanthin could also lead to discoloration (loss in redness) of the MF nanoliposomes. Astaxanthin being an antioxidant is prone to oxidation and astaxanthin content in shrimp oil decreased with increased oil oxidation (Gulzar and Benjakul, 2019a).

6.5.2.3 TBARS

Figure 35B represents the TBARS values of US, MF and NE samples over 8 weeks. Unencapsulated shrimp oil had the highest TBARS, which increased

drastically over the storage period (p<0.05). TBARS value of US nanoliposome was the lowest among all the samples at all storage time tested. For the MF nanoliposomes, there was a continuous increase in TBARS value over the storage (p<0.05). The trend in the increase in TBARS values was similar to that of PV, except for the unencapsulated oil, in which PV was decreased after the 6th week. TBARS indicated the presence of polar and aromatic hydrocarbons such as aldehydes and ketones, etc. as the secondary lipid oxidation products in lipid oxidation process (Nawar, 1996). Hydroperoxides formed in the primary oxidation are further oxidized to produce an off-flavor volatile compound (Frankel, 1998). Nanoliposomes, particularly US, could significantly prevent oxidation of shrimp oil and could act as a carrier of shrimp oil in human food systems.

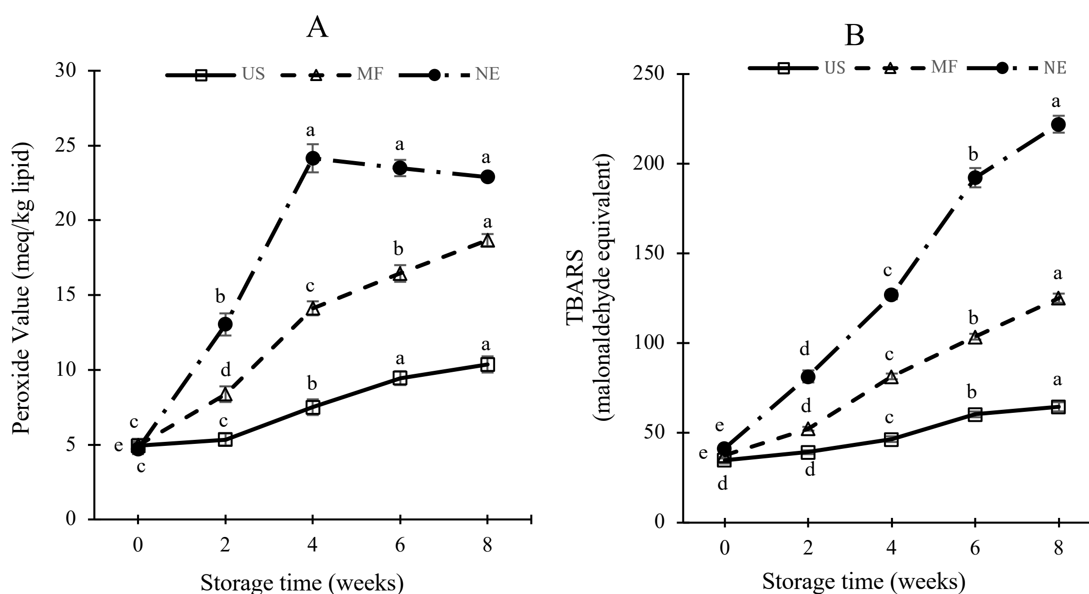


Figure 35. Peroxide value and (A) TBARS value (B) of nanoliposomes containing shrimp oil prepared by different methods and unencapsulated shrimp oil during the storage of 8 weeks at 30 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars in the same samples denote significant difference (p<0.05). US: Nanoliposomes prepared by ultrasonication method MF: Nanoliposomes prepared by microfluidization method. NE: Unencapsulated shrimp oil.

6.5.2.4 Fatty acid profile

Fatty acid profile of nanoliposomes (US, MF) and unencapsulated shrimp oil (NE) at week 0 is illustrated in Table 7. Shrimp oil contained 18.43±1.08 % monoglycerides, 13.87±0.87 % diglycerides, 23.62±1.22 % triglycerides, 16.22±0.72 % free fatty acids and 26.25±1.32 phospholipid. All the lipid samples contained PUFAs as major fatty acids. Linoleic acid (LA) was found to be the major unsaturated fatty acid, followed by DHA and EPA. The change in the EPA, DHA and LA contents over the storage period has been shown in Figure 36A, 36B and 36C. There was a continuous decrease in the PUFA content of unencapsulated oil, particularly LA, EPA, and DHA, resulting in the subsequent increase in the saturated fatty acid (SFA) content (data not shown). Lipids extracted from nanoliposomes also showed a slight decrease in PUFA content. Oxidation of PUFAs resulted in an increase in SFA content, expressed by the increase in polyene index of oils (Gulzar and Benjakul, 2019a). There was a sharp decrease in the EPA, DHA, and LA contents of NE shrimp oil, compared to the nanoliposomes, in which these fatty acids were more retained. By the end of storage time, PUFAs in NE, MF and US samples were decreased by 67.15%, 39.11%, and 15.79% respectively. Encapsulation of shrimp oil in nanoliposomes provided a protective covering to the oil against deteriorative oxidation process. EPA and DHA are polyunsaturated fatty acids and are highly susceptible to oxidation (Miyashita *et al.*, 1993). US nanoliposomes showed higher retention of EPA, DHA, and LA than MF counterparts, as the latter also exhibited higher PV and TBARS values than the former (Figure 35A and 35B), confirming that the oxidation of PUFAs had taken place. The results were in agreement with Ghorbanzade et al. (2017) who showed that nanoencapsulation of fish oil resulted in maximum retention of n-3 fatty acids (DHA + EPA) compared to unencapsulated fish oil fortified into yogurt. Oleic acid content remained almost similar in the encapsulated oil but was higher in the unencapsulated oil. Oil extracted from shrimp cephalothorax stored in ice for 0 and 6 days had similar oleic acid content (Takeungwongtrakul *et al.*, 2012). Shrimp oil extracted using ultrasonic-assisted extraction and typical solvent extraction had similar oleic acid content (Gulzar and Benjakul, 2018), indicating that extraction methods or storage conditions of shrimp

cephalothorax prior to extraction doesn't have any effect on the oleic acid content of shrimp oil. Oleic acid, although a monounsaturated fatty acid, is not much affected by the oxidation as much as LA, EPA or DHA, probably due to its lower degree of unsaturation. Overall, the nanoliposomes were effective in protecting the shrimp oil against loss of n-3 fatty acids.

Table 7. Fatty acid profile of shrimp oil and shrimp oil nanoliposomes prepared by different methods at week 0.

Fatty acids (g/100 g lipids)	NE	MF	US
C14:0	0.71 ± 0.00a	0.69 ± 0.00a	0.66 ± 0.00a
C16:0	25.19 ± 0.02a	24.47 ± 0.22a	24.34 ± 0.19a
C16:1 n-7	1.67 ± 0.02b	1.64 ± 0.08b	1.98 ± 0.06a
C18:0	10.34 ± 0.25a	10.25 ± 0.11a	9.98 ± 0.12b
C18:1 n-9	14.81 ± 0.11a	14.17 ± 0.05b	14.27 ± 0.06b
C18:2 n-7	2.99 ± 0.01b	3.13 ± 0.01a	3.18 ± 0.09a
C18:2 n-6 (Linoleic acid)	15.08 ± 0.11a	15.34 ± 0.09a	15.47 ± 0.12a
C20:1 n-9	1.75 ± 0.03b	1.93 ± 0.05a	1.76 ± 0.03b
C20:2 n-6	1.48 ± 0.00a	1.47 ± 0.03a	1.42 ± 0.01a
C20:4 n-6 (ARA)	0.68 ± 0.03a	0.62 ± 0.01a	0.63 ± 0.01a
C20:5 (EPA)	6.48 ± 0.26a	6.23 ± 0.15a	6.39 ± 0.07a
C22:0	1.36 ± 0.02a	1.44 ± 0.02a	1.42 ± 0.04a
C22:6 n-3 (DHA)	9.06 ± 0.09a	9.11 ± 0.14a	9.07 ± 0.25a
C23:0	3.97 ± 0.14a	3.81 ± 0.03a	3.95 ± 0.01a
Saturated fatty acid (SFA)	41.57 ± 0.22a	40.66 ± 0.13a	40.35 ± 0.34a
Monounsaturated fatty acid (MUFA)	18.23 ± 0.15a	17.74 ± 0.08a	18.01 ± 0.11a
Polyunsaturated fatty acid (PUFA)	35.77 ± 0.27a	35.9 ± 0.12a	36.16 ± 0.18a

Different lowercase letters in the same row indicate significant differences ($p<0.05$). US: Nanoliposomes prepared by ultrasonication method; MF: Nanoliposomes prepared by microfluidization method; NE: Unencapsulated shrimp oil.

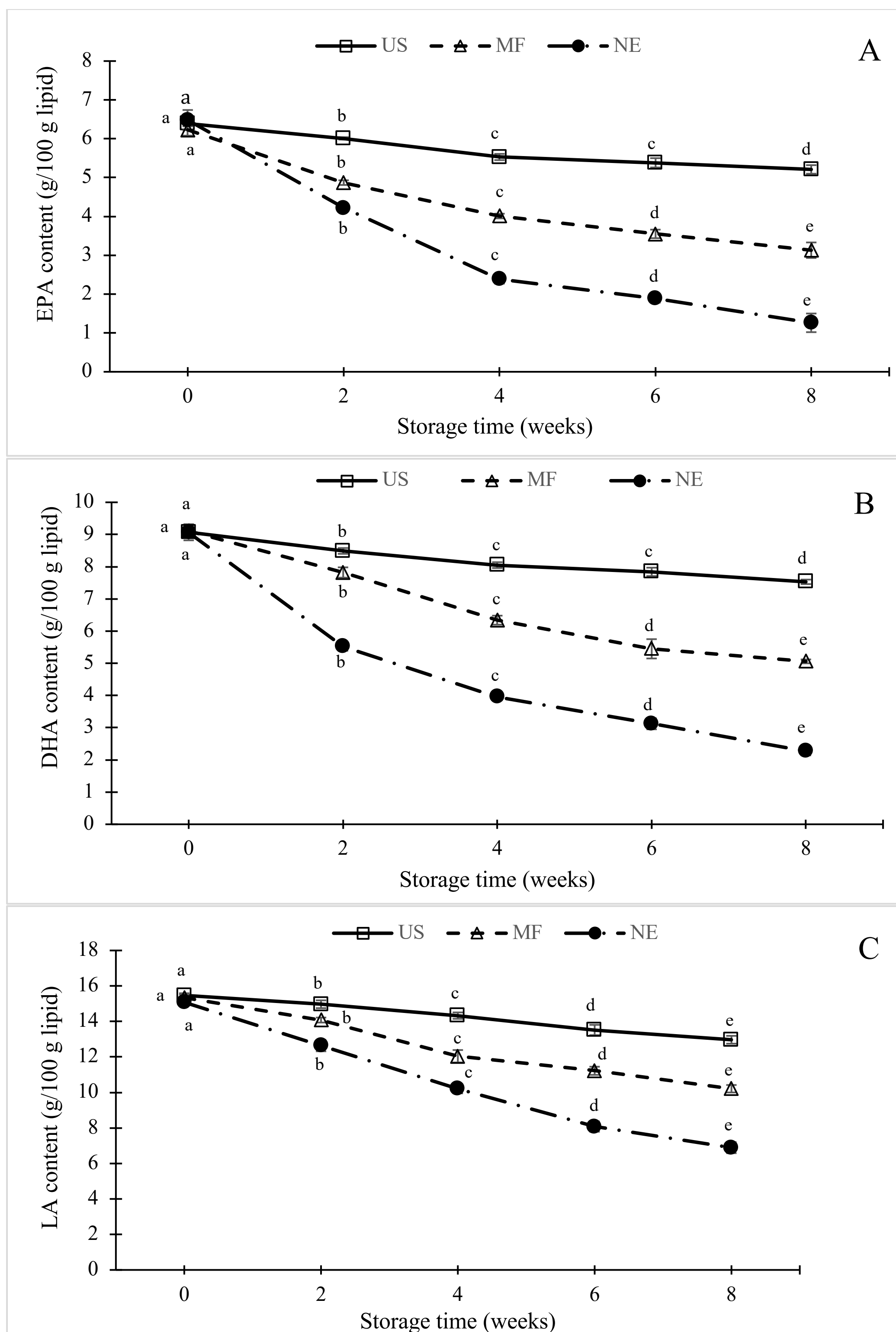


Figure 36. EPA, (A) DHA, (B) and LA (C) content of nanoliposomes containing shrimp oil prepared by different methods and unencapsulated shrimp oil during the storage of 8 weeks at 30 °C. Caption see Figure 35.

6.5.2.5 Fishy odor

Development of fishy odor in US, MF and NE samples over 8 weeks is tabulated in Table 8. There was no detectable fishy odor in both the nanoliposome samples at week 0. However, the fishy odor increased over the storage of 8 days in the nanoliposomes. MF nanoliposomes showed a greater increase in fishy intensity, compared to US samples. NE sample showed the highest fishy odor among all the samples with a sharp increase at week 2. Fishy odor is a characteristic undesirable smell found in marine oils, which had undergone lipid oxidation. It is principally caused by the secondary oxidation of PUFAs to produce volatile off-odor compounds such as aldehydes and ketones (Thiansilakul *et al.*, 2010). These volatile possess low organoleptic thresholds (McGill *et al.*, 1977). Increased fishy odor intensity in NE sample was coincidental with higher TBARS value (Figure 35B). Since lipids rich in n-3 fatty acids are vulnerable to oxidation, there was some detectable fishy odor present even in freshly extracted marine oils. However, encapsulation of shrimp oil in nanoliposomes resulted in almost complete masking of the fishy odor.

Table 8. Development of fishy odor intensity in shrimp oil and shrimp oil nanoliposomes prepared by different methods during storage of 8 weeks at 30 °C.

Week	US	MF	NE
0	0.25 ± 0.11c	0.28 ± 0.10d	1.59 ± 0.28d
2	0.44 ± 0.18bc	1.01 ± 0.12c	3.14 ± 0.41c
4	0.70 ± 0.22b	2.05 ± 0.24b	4.26 ± 0.27b
6	1.44 ± 0.25a	2.25 ± 030ab	4.73 ± 0.30a
8	1.58 ± 0.39a	2.38 ± 0.29a	4.88 ± 0.04a

Different lowercase letters in the same column indicate significant differences ($p<0.05$). US: Nanoliposomes prepared by ultrasonication method; MF: Nanoliposomes prepared by microfluidization method; NE: Unencapsulated shrimp oil.

6.5.3 Volatile compounds

Volatile compounds found in US, MF and NE samples are tabulated in Table 9. Hydrocarbons were majorly present in US and MF samples. Most of the aldehydes and ketones were present in unencapsulated shrimp oil (NE), whereas in these aromatic compounds were mostly not detected in nanoliposome. Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to follow lipid oxidation in a number of foods (Takeungwongtrakul and Benjakul, 2013). Most of the aromatic compounds, including octenal, heptenal and hexadecanal, 6-hepten-3-one, hexanol, pentanol were found in NE sample, indicating that lipid oxidation had taken place to higher extent. Those compunds more likely contributed to fishy odor detected in NE. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acids (Varlet *et al.*, 2006). Aromatic compounds including 3-methoxy-1-(4-methoxyphenyl)-2(E)-propen-1-one, hexadecanal, 2,4-ditert-butylphenol and octadecanal were formed during lipid oxidation (Takeungwongtrakul and Benjakul, 2013). Gas chromatography-olfactometry (GC-O) coupled with frequency-of-detection (FDT) and time-intensity methods revealed that heptenal, octenal and 2,4-decadienal mainly contributed to fishy smell in unsmoked salmon (Varlet *et al.*, 2006). NE sample also contained 2-ethyl furan, 2-penty furan and cis-2-(2-pentenyl) furan. Furans are formed as a result of lipid hydrolysis (Navaneethan and Benjakul, 2019), indicating that lipid hydrolysis had also taken place in unencapsulated shrimp oil. The results clearly indicated that encapsulation of shrimp oil in nanoliposomes prevented lipid oxidation and hydrolysis. Also, liposome could effectively mask the fishy odor of shrimp oil.

Table 9. Volatile compounds in shrimp oil and shrimp oil nanoliposomes.

Compounds	Peak area (abundance) × 10^7		
	NE	MF	US
Hydrocarbons			
Decane	4	10	3
Dodecane	ND	12	9
Hexadecane	ND	12	10
Nonane	ND	39	25
Octane	ND	1	ND
Pentadecane	ND	3	ND
Pentane	ND	3	ND
Aldehydes			
2,4-Decadienal	14	ND	ND
2,4-Heptadienal, (E,E)-	9	ND	ND
Heptadecanal	2	ND	ND
Heptenal	18	ND	ND
Hexanal	10	5	2
Hexadecanal	15	3	2
Octadecanal	4	ND	ND
Octenal	25	5	1
Pentadecanal-	5	ND	ND
Pentanal	1	ND	ND
Tetradecanal	4	ND	ND
Ketones			
6-Hepten-3-one	34	ND	ND
2-Octanone	2	ND	ND
3-Octanone	4	5	1
3-Pentanone	1	ND	ND
3,5-Octadien-2-one	8	ND	ND
Furans			
2-Ethyl furan	3	2	ND
2-pentyl furan	47	ND	ND
cis-2-(2-Pentenyl)furan	43	ND	ND
Alcohols			
1-Tetradecanol	9	ND	ND
1-Dodecanol	3	ND	ND
1-Hexadecanol	31	8	8
1-Hexanol	17	4	3
1-Octen-3-ol	13	5	2
Other compounds			
(Z)-Ethyl heptadec-9-enoate	2	ND	ND
Ethyl 13-methyl-tetradecanoate	12	ND	ND
Tetradecanoic acid, ethyl ester	1	ND	ND

US: Nanoliposomes prepared by ultrasonication method; MF: Nanoliposomes prepared by microfluidization method; NE: Unencapsulated shrimp oil.

6.6 Conclusion

Shrimp oil was effectively encapsulated into nanoliposomes. Nanoliposomes produced using ultrasonication method were more stable, smaller in size and showed better nanoencapsulation efficiency than those produced using microfluidization. The former showed better oxidative stability over the storage of 8 weeks at 30 °C as shown by lower increases in peroxide value, TBARS and more retention of EPA and DHA. Confinement of shrimp oil in nanoliposomes also masked the characteristic fishy odor of the oil. Overall, encapsulation of shrimp oil in nanoliposomes was promising for maintaining quality of shrimp oil and liposomes could be used for the fortification in foods for n-3 fatty acids or astaxanthin enrichment.

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CHAPTER 7

FORTIFICATION OF SKIM MILK WITH NANOLIPOSOMES LOADED WITH SHRIMP OIL: PROPERTIES AND STORAGE STABILITY

7.1 Abstract

Shrimp oil nanoliposomes (SONL) were fortified into skim milk at various levels (2 – 10% v/v), followed by pasteurization at 63 °C for 30 min. Skim milk showed lowered whiteness but increased redness and yellowness as SONL levels added increased ($p<0.05$). Viscosity of fortified samples was also augmented with increasing levels of SONL ($p<0.05$). Sensory analysis indicated that fortified milk skim samples had no perceivable fishy odor and were organoleptically acceptable. When skim milk fortified with 10% SONL was stored up to 15 days at 4 °C, microbial load was less than 2.54 log CFU/mL. pH and acidity values were also within the acceptable limits. As measured by peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), shrimp oil in SONL did not undergo oxidation during the extended storage. Fatty acid profile of shrimp oil revealed no loss of polyunsaturated fatty acids taken place during storage of fortified milk. Therefore, nanoliposomes could be an effective carrier for shrimp oil to be fortified in skim milk.

7.2 Introduction

Oils from marine sources have gained interest to be fortified or supplemented in the human diet in several ways including capsules and fortified products such as bread, juices, biscuits, cakes, etc. Marine oils have been known as the rich source of polyunsaturated fatty acids (PUFAs) with potential health promoting effects on human (Ruxton *et al.*, 2004). The most well-known long chain n-3 PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found to be beneficial for people suffering from many ailments including coronary heart diseases, hypertension, Alzheimer's disease etc. (Cole *et al.*, 2005; Sidhu, 2003). PUFAs are also known to prevent pre-term birth, improve mood and behavior and act as anti-inflammatory agent to improve joint health (Ulu *et al.*, 2013).

Shrimp oil is one of crustacean oils rich in both PUFAs and carotenoids (Higuera-Ciapara *et al.*, 2006). It can be extracted from the head (cephalothorax) or hepatopancreas of shrimp, which are inedible part, commonly discarded as wastes from shrimp processing industry. Over the years, shrimp processing industry has seen an upsurge in the demand, associated with generation of huge amount of by-products. Shrimp cephalothorax is therefore one of the cheapest source of PUFAs. Another valuable compound present in shrimp oil is astaxanthin, a pigment found in crustaceans, known to have exceptional antioxidative property (Naguib, 2000). Despite high nutritional value, the use of shrimp oil for food fortification is limited due to the rapid oxidation of its PUFAs. This results in the formation of undesirable off-flavor, especially fishy odor (Gulzar and Benjakul, 2018). Oxidation can be accelerated when oil is exposed to oxygen. Oxidation can also take place with the initiation by prooxidants such as transition metals (Fe, Cu), enzymes (lipoxygenases) and some oxidizing agents. Environmental factors such as heat and light (UV) are also documented to expedite lipid oxidation (Kolanowski *et al.*, 2007).

Encapsulation techniques have been used to conquer the problem related with oxidation in oils. Resulting capsules act as carrier for fortification in foods. Encapsulation of oils and bioactive compounds using nanoliposome technology is one of the powerful technique (Munin and Edwards-Lévy, 2011). Liposomes are spherical vesicles, typically made of a phospholipid bilayer and an aqueous core. The size of liposomes range from nanometers to micrometers, and liposomes with diameter less than 1000 nm are referred to as nanoliposomes (Hallaj-Nezhadi *et al.*, 2013). Nanoliposomes have been used to encapsulate and fortify fish oil in variety of food products (Ghorbanzade *et al.*, 2017). There are several advantages of encapsulating oils in nanoliposomes. Those include better oxidative stability, retention of n-3 fatty acids, etc (Ghorbanzade et al., 2017). The phospholipid bilayer in liposomes forms a protective covering around encapsulated oils, preventing the contact of oils from prooxidants or air, and thus eliminating the chances of oxidation to a large extent. Characteristic fishy odor in marine oils can also be masked by encapsulation in liposomes. Liposomal systems are able to ensure the targeted delivery of encapsulated materials and therefore enhance the bio-accessibility of target compounds during

digestion. *In vitro* digestion of fish oil encapsulated in liposomes revealed that fish oil from liposomes was released in a controlled manner during digestion (Wang *et al.*, 2015).

More recently, shrimp oil microcapsules prepared by using spray drying has been fortified in breads and biscuits (Takeungwongtrakul and Benjakul, 2017; Takeungwongtrakul *et al.*, 2015. However, the use of high temperature during drying poses potential problem via inducing oxidation (Jafari *et al.*, 2008). Also the rehydration property and solubility of shrimp oil powder in liquid foods is questionable. To our knowledge, no study has been conducted on fortification of shrimp oil in liquid food systems. Milk is widely consumed all over the world and shrimp oil fortification in the form of nanoliposome could be a means to enrich skim milk with n-3 fatty acids and astaxanthin. Due to the ease of fortification, better solubility of liposomes, minimum processing requirements, possibility of large scale production etc., nanoliposome loaded with shrimp oil could be a promising fortifying agent.

7.3 Objectives

To characterize skim milk fortified with shrimp oil nanoliposomes at different levels.

To evaluate physicochemical and microbiological stability of the fortified milk during the refrigerated storage.

7.4 Materials and methods

7.4.1 Preparation of nanoliposomes

Shrimp oil was extracted from cephalothorax of Pacific white shrimp (*Litopenaeus vanammei*) following the method of Gulzar and Benjakul (2020). Nanoliposomes were prepared by the modified ethanol injection method. Lecithin was dissolved in ethanol to attain 5% (w/v) and heated up to 45 °C to ensure complete dissolution. Subsequently, 5 mL of shrimp oil (preheated to 30 °C) was dropped gradually into 100 mL of prepared lecithin solution. The mixture was stirred at 1,000

rpm on a hotplate magnetic stirrer at 30 °C for 5 min. Deionized water (100 mL) and glycerol (2% v/v) were added to hydrate the solution and homogenized for 10 min at a speed of 3000 rpm by an IKA Labortechnik homogenizer (Selangor, Malaysia). The liposomal dispersions with a total volume of 200 mL were subjected to ultrasonication (10 min; 1 s on and off pulse) at 25 °C using an ultrasonic processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude. Ethanol was removed using EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 30°C. After preparation, the obtained nanoliposome was placed under nitrogen atmosphere at 25 °C for 1 h for stabilization. Shrimp oil nanoliposome was referred to as 'SONL'.

Particle size distribution, encapsulation efficiency and centrifugal stability of SONL were also calculated. The particle size of SONL ranged between 40 and 284 nm, whereas the mean particle size of SONL was 104.77 nm. Encapsulation efficiency and centrifugal stability of SONL was calculated to be 93.64±0.98% and 94.5±0.56%, respectively.

7.4.2 Fortification of SONL to skim milk.

Freshly prepared SONL was added to pasteurized milk sample with 0% fat (Meiji Pasteurized Skimmed Milk, Bangkok, Thailand) to obtain the final levels of 2, 4, 6, 8 and 10% (v/v) SONL. Approximately ten drops of synthetic pineapple flavor (R&B Food Supply, Bangkok, Thailand) were added to 100 mL of fortified skim milk. The mixtures were stirred for 5 min. The samples were pasteurized at 63 °C for 30 min (Westhoff, 1978) and transferred into aseptic bottles. The lid was closed tightly. Samples were stored at 4 °C and subjected to analyses.

7.4.3 Physicochemical and sensory properties of skim milk fortified with SONL at different levels.

7.4.3.1 Color

Color of skim milk samples fortified with SONL at different levels was measured by a Hunter lab colorimeter (ColorFlex, Hunter Lab Inc., Reston, VA,

USA). L*, a* and b* values indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colorimeter was calibrated with a white standard. Total difference in color (ΔE*) was also calculated according to the following equation (Gennadios *et al.*, 1996):

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

where ΔL*, Δa*and Δb*are the differences between the corresponding color parameter of the sample and that of control (non-fortified skim milk).

7.4.3.2 Viscosity

Viscosity measurement of fortified milk samples was carried out using a Brookfield digital viscometer (model DV II+, Brookfield Eng. Labs Inc., Stoughton, MA, USA) equipped with spindle no. 1, operated at 100 rpm at 4 ºC and 25 ºC. Viscosity of fortified milk samples was determined by comparing with that of the control (unfortified milk) sample and the values were reported as cP.

7.4.3.3 Sensory analysis

Sensory analysis of shrimp oil fortified skim milk samples was done following the method of Meilgaard *et al.* (1999). Each sample was assigned a random 3-digit code and served in white plastic cups at room temperature under the fluorescent day-light type illumination. Sixty non-trained panelists, who were familiar with milk consumption (age 25-35 years), took part in sensory evaluation. The panelists were asked to evaluate for color, texture, odor, flavor and overall likeness of fortified skim milk using 9-point hedonic scale (1, extremely dislike; 9, extremely like). Panelists were asked to rinse their mouths after evaluating each sample.

7.4.4 Storage stability of skim milk fortified with SONL at a selected level

Fortified skim milk, having the satisfactory score for sensory evaluation, in which the highest amount of SONL was fortified (10%), was prepared and stored up to 15 days at 4 °C. Samples were taken every 3 days and determined.

7.4.4.1 Microbiological analysis

Microbiological analysis was carried out following the method of Maqsood and Benjakul (2011) with some modifications. Milk sample (5 mL) was collected aseptically and diluted ten-time with sterile saline solution (0.85%). After homogenizing, a series of 10-fold dilutions were made using a saline solution. Total viable count (TVC) was conducted using plate count agar (PCA) incubated at 37 ºC for 2 days. Psychrophilic bacterial count (PBC) was also enumerated using PCA incubated at 4 ºC for 7 days. Microbial counts were expressed as log colony forming unit per mL of sample (log CFU/mL).

7.4.4.2 Measurement of pH and acidity

pH of milk samples was measured using a Lab 855 pH meter (SI Analytics, Xylem Inc., Rye Brook, NY, USA). Titrable acidity was determined following the AOAC method 947.05 (AOAC, 1990). Twenty mL of milk sample were diluted with hot distilled water, cooled and titrated against 0.1 N NaOH using 1% phenolphthalein indicator. Titrable acidity was expressed as g of lactic acid/100 g

7.4.4.3 Determination of lipid oxidation

Firstly, the oil was extracted from fortified milk using the mixture of chloroform and methanol (2:1) at the sample/solvent ratio of 1:5. Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were used as indicators for lipid oxidation. PV was determined using the ferric thiocyanate method as described by Chaijan *et al.* (2006). TBARS were examined by the method tailored by Buege and Aust (1978).

7.4.4.4 Determination of fatty acid profile

Fatty acid profiles of lipid samples extracted as mentioned above were examined in the form of fatty acid methyl esters (FAMEs) prepared following the method of Gulzar and Benjakul (2019c). Fatty acids were firstly transmethylated by 2 M methanolic sodium hydroxide, along with 2 M methanolic hydrochloric acid. FAMEs were analyzed by gas chromatography (GC) using Agilent

7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. FAME dissolved in hexane was determined using Agilent J&W fused silica capillary column (100 m × 0.25 mm × 0.20 μm) (a split ratio of 1:20). The injection port temperature was kept at 250 °C and detector (flame ionization detector, FID) temperature was 270 °C. The oven temperature ranged from 170 to 225 °C with a flow rate of 1 °C /min. The chromatographic peaks of the samples were identified based on retention times, compared to those of standards. Peak area ratio was used for calculation and the content was expressed as g fatty acid/100 g lipid.

7.4.5 Statistical analysis

All the experiments were done in triplicates and the data were expressed as means ± SD. Completely randomized design (CRD) was used throughout the study. Randomized complete block design (RCB) was used for sensory analysis study. Analysis of variance (ANOVA) was performed and means were compared by Duncan's multiple range test. Analysis of data was carried out using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

7.5 Results and discussion

7.5.1 Effect of fortification of SONL at various levels on characteristics of skim milk

7.5.1.1 Color

Color values, expressed as L*, a*, b* and ΔE*, of skim milk samples fortified with SONL at different levels are shown in Table 10. Control (unfortified milk) had the highest L* (whiteness) ($p<0.05$). Similar color values for skim milk were posted by Kneifel *et al.* (2007). Whiteness of the fortified milk samples decreased sharply with addition of 2% SONL ($p<0.05$). The continuous decrease was attained as SONL levels increased ($p < 0.05$). The a* value (redness) increased, especially with increasing levels of SONL ($p<0.05$). The augmented redness could be attributed to the presence of astaxanthin, a red pigment present in the shrimp oil located as core in SONL. Astaxanthin, a keto-carotenoid, naturally occurring in

microalgae, contributes to the characteristic orange-red color of the crustaceans (Higuera-Ciapara *et al.*, 2006). There was an increase in the b* values (yellowness) of the milk samples with increasing SONL concentration. The increase in yellowness was concomitant with the augmentation of redness. The total color difference (ΔE*) amongst the samples was also found to become higher with increasing amount of SONL ($p<0.05$). Overall, with the increasing SONL levels fortified, there was a reduction in whiteness of fortified milk samples, whereas redness and yellowness of the resulting samples were augmented.

Table 10. Color values of skim milk fortified with SONL at various levels

SONL level (%, v/v)	L*	a*	b*	ΔE*
0 (CON)	92.65 ± 0.04a	-4.88 ± 0.03f	11.87 ± 0.01f	0
2	82.57 ± 0.01b	14.22 ± 0.14e	20.87 ± 0.14e	23.40 ± 0.17e
4	78.82 ± 0.01c	20.02 ± 0.08d	24.95 ± 0.01d	31.34 ± 0.06d
6	76.76 ± 0.01d	22.87 ± 0.05c	28.15 ± 0.08c	35.89 ± 0.09c
8	75.17 ± 0.09e	25.03 ± 0.04b	30.90 ± 0.18b	39.53 ± 0.18b
10	73.45 ± 0.01f	27.01 ± 0.05a	32.83 ± 0.05a	42.72 ± 0.06a

Different lowercase letters in the same column indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome; CON: Unfortified skim milk.

7.5.1.2 Viscosity

Viscosities of skim milk samples fortified with SONL at various concentrations kept at two different temperatures are shown in Figure 37. The viscosity of control sample was lowest ($p<0.05$) and increased gradually with increasing SONL levels ($p<0.05$). The viscosity of skim milk is generally low with Newtonian behavior (Vliet and Walstra, 1980). Viscosity of milk is a function of several factors including pH, temperature, solid content etc., and tends to increase with the increase in fat content (Cox, 1952). In skim milk, the hydrodynamic interactions are contributed only by lactose and protein molecules. However, with the addition of fat, these interactions increase the friction, contributing to higher viscosity

(Phipps, 1969). This was confirmed by the increased viscosity of skim milk fortified with SONL, particularly at high levels. With the addition of oil in milk system, the rheological parameters were changed, and the size of droplets and density largely affected the viscosity (Chanamai and McClements, 2000). SONL was viscous in nature and resulted in the increased viscosity of fortified skim milk. The viscosity of milk samples was increased at low temperature (4 °C). Various studies revealed that viscosity of milk increased with decreasing temperature (Fernández-Martín, 1972). There are several reasons for the rise in viscosity with lowering temperature, depending upon the concentration of milk. For skim milk, the increase in viscosity is mainly due to the increased density of water at low temperature and hydration of casein micelles (Kristensen *et al.*, 1997). Furthermore, the agglomeration of these oil droplets tend to increase the viscosity in the colloidal system, especially at low temperature. Overall, the viscosity of fortified milk was slightly increased with increasing SONL levels and low temperature.

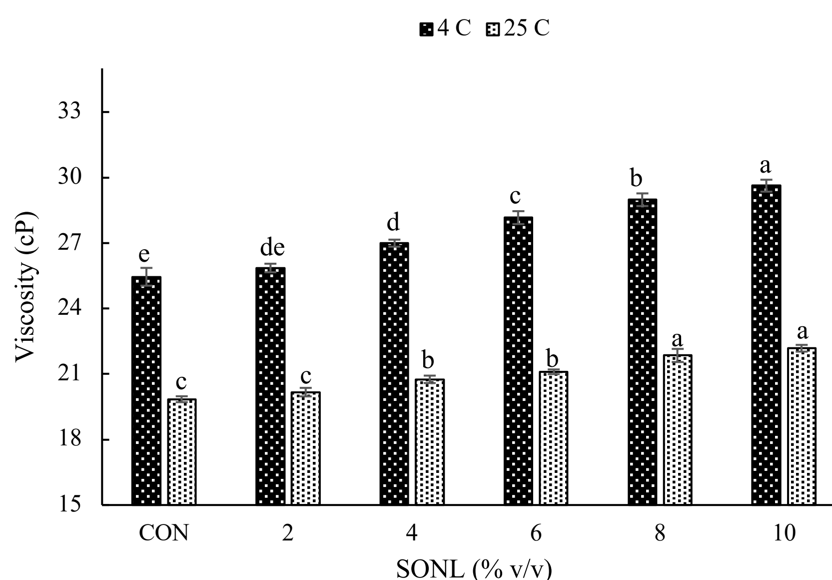

Figure 37. Viscosity of skim milk fortified with SONL at various levels at 4 °C and 25 °C. Bars represent standard deviation (n=3). Different lowercase letters on the bars within the same temperature indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome

7.5.1.3 Acceptance test

Likeness scores of SONL fortified skim milk samples are tabulated in Table 11. The score for color likeness increased gradually when SONL was added ($p<0.05$). This indicated that the reddish color of the fortified milk samples was desirable. Similarly, viscosity of samples increased significantly when SONL was added above 6% ($p<0.05$) indicating that addition of SONL at higher level was desirable in terms of mouth feel. No significant difference was found in odor likeness of samples in which SONL was added up to 10% ($p>0.05$). No fishy odor was reported by any panelist, indicating that nanoliposomes were able to mask the fishy odor of shrimp oil. However, pineapple flavor was added to compensate for and match with the color of fortified milk samples. As a consequence, there was no difference in flavor likeness ($p>0.05$) among all the samples. The likeness scores for taste attribute was found to be decreased when 10% SONL was added. Some panelists pointed out that the samples were slightly bitter in taste. Stephan and Steinhart (1999) reported that modification processes such as hydrolysis of soy lecithin contributed to bitterness. Some studies proposed that oxidation of phospholipids caused bitterness (Sessa *et al.*, 1976; Wieser *et al.*, 1984). Lecithin added to UHT milk was found to impart some bitterness to the milk (Stephan and Steinhart, 2000). This could be plausibly due to the exposure of hydrophobic parts of lecithin to the taste buds. Nevertheless, there was no significant difference in the overall likeness score for all the samples. Addition of SONL above 10% showed negative impact on quality and stability of fortified milk (data not shown). SONL above 10% were agglomerated during pasteurization and excess SONL was obviously separated and floated.

Table 11. Likeness score of skim milk fortified with SONL at various levels

SONL level (%, v/v)	Color	Viscosity	Odor	Flavor	Taste	Overall likeness
0 (CON)	5.91 ± 0.67c	6.41 ± 0.98b	5.77 ± 0.67b	6.73 ± 0.83a	6.59 ± 0.78a	6.86 ± 0.62a
2	6.45 ± 1.23bc	6.59 ± 1.19ab	6.14 ± 1.36a	6.18 ± 0.89b	6.09 ± 1.53ab	6.23 ± 1.35a
4	6.95 ± 1.07ab	6.68 ± 1.14ab	6.27 ± 1.21a	6.09 ± 0.67b	6.05 ± 1.33ab	6.32 ± 1.26a
6	7.00 ± 0.85ab	6.86 ± 0.97ab	6.32 ± 1.46a	6.05 ± 0.82b	6.18 ± 1.27ab	6.14 ± 1.41a
8	7.36 ± 1.02a	7.14 ± 0.76a	6.50 ± 0.99a	6.05 ± 0.71b	5.91 ± 1.00ab	6.27 ± 1.29a
10	7.41 ± 0.65a	7.18 ± 0.83a	6.51 ± 1.09a	6.00 ± 1.00b	5.64 ± 1.30b	6.23 ± 1.05a

Different lowercase letters in the same column indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome; CON: Unfortified skim milk.

7.5.2 Storage stability of skim milk fortified with 10% SONL during storage

Based on the sensory score and maximum SONL incorporation, milk fortified with 10% SONL was chosen for storage at 4 °C up to 15 days.

7.5.2.1 Microbiological quality

Figure 38 shows the total viable count (TVC) of unfortified milk and milk sample fortified with 10% SONL over the storage period of 15 days. TVC of control (unfortified) and fortified milk samples were lowest on day 0 and increased gradually up to day 15 ($p>0.05$). TVC of both samples remained below the standard as defined by Pasteurized milk ordinance (PMO) (FDA, 2015) over the entire storage time. The results indicated that pasteurization was effective and inactivated most of the mesophilic bacteria. Moreover, the intolerance of mesophilic bacteria towards refrigerated storage could also result in lower TVC of milk samples. TVC of control sample was similar to that of fortified one at all storage time tested ($p>0.05$). The results were in agreement with Ziarno *et al.* (2005) who reported that total bacterial count in pasteurized milk during the storage of 7 – 14 days remained low (<5 log CFU/mL) and increased significantly after 21 days. Pasteurization, generally does not destroy or inactivate all the micro-organisms, but reduces their number, at which they do not pose significant health hazard (Burgess, 1994; Hassan *et al.*, 2009). There was no detectable growth of psychrophilic bacteria over the entire storage period and psychrotropic bacterial count (PBC) was not detected. Overall, the milk was found microbiologically stable and fit for consumption, regardless of SONL fortification.

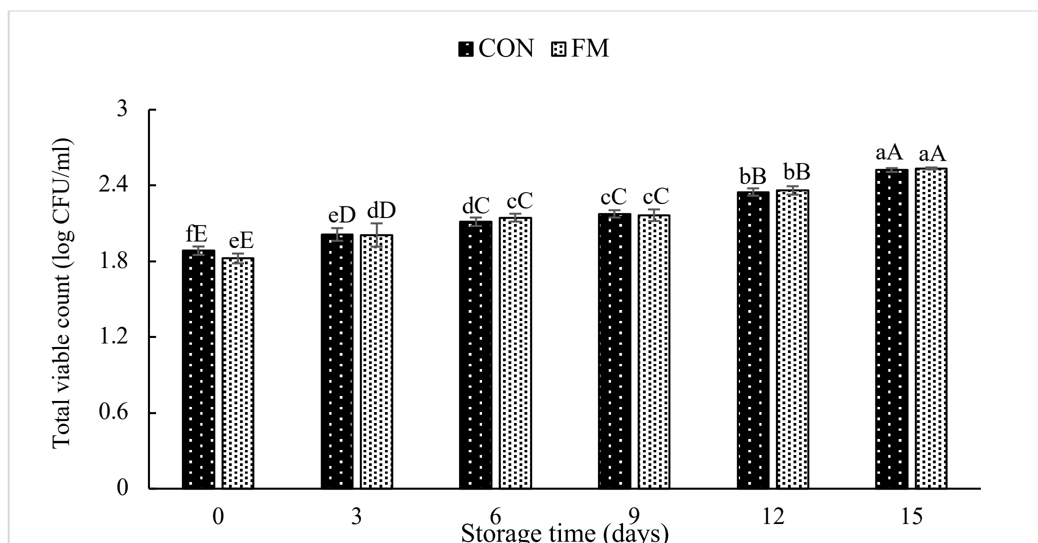

Figure 38. Total viable count (TVC) of control (unfortified skim milk) and milk fortified with 10% SONL during the storage of 15 days at 4 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same sample indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome.

7.5.2.2 pH and acidity

pH and acidity changes of control and fortified milk sample during 15 days of storage at 4 °C are shown in Figure 39A and 39B, respectively. pH values of control sample ranged between 6.55 – 6.45, whereas those of fortified milk varied from 6.54 to 6.43. There was a gradual decline in the pH of the milk samples over the time. The drop in pH could plausibly be due to the conversion of lactose into lactic acid by lactic acid bacteria, which might be present in the milk. The results were in agreement with the findings of Hassan *et al.* (2009) who reported similar pH values of pasteurized milk stored under refrigerated conditions for up to 20 days. On the other hand, acidity values of milk samples slightly increased over time. This was concomitant with the decrease in pH values. Overall, the acidity values of fortified milk remained within the prescribed legal standard limits.

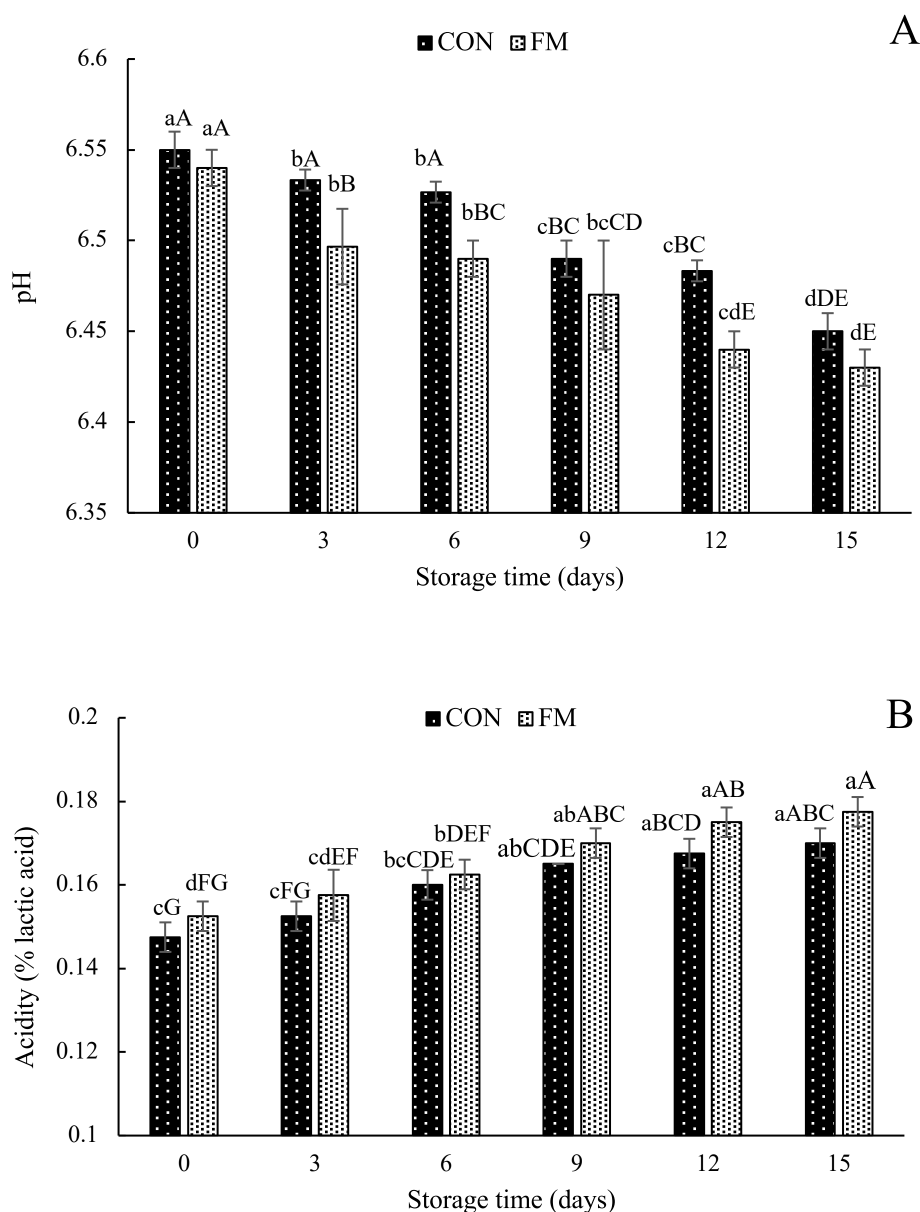

Figure 39. pH (A) and acidity (B) of control (unfortified skim milk) and milk fortified with 10% SONL during the storage of 15 days at 4 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same sample indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome; CON: Unfortified skim milk; FM: Fortified skim milk

7.5.2.3 PV and TBARS

Figure 40A and 40B represents PV and TBARS of shrimp oil extracted from SONL fortified milk stored over the period of 15 days, respectively. Slight increase in PV was observed at day 2 ($p<0.05$). Nevertheless, there was no significant increase in the PV up to the end of storage (15 days) ($p>0.05$). For TBARS values, no difference was attained up to day 12. A slight increase in TBARS was found at day 15 ($p<0.05$). This could be attributed to the protective effect of nanoliposomes that prevents the oxidation of unsaturated fatty acids in shrimp oils localized as core. Nanoliposomes are known to form a protective phospholipid bilayer around the encapsulated oil, thus reducing the contact of oil with prooxidants and catalysts such as transition metals (Fe, Cu), enzymes (lipoxygenases), chemical oxidizers as well as from environmental factors such as light (UV) and oxygen. Ghorbanzade *et al.* (2017) reported that fish oil encapsulated in nanoliposomes and fortified in yogurt showed constant PV during 21 days of storage at 4 °C. Ye *et al.* (2009) claimed that addition of encapsulated fish oil in cheese reduced PV and TBARS values after 35 days of storage at 5 °C. PV and TBARS of shrimp oil extracted from cephalothorax of Pacific white shrimp stored over 6 days in iced storage was found to be higher (Takeungwongtrakul *et al.*, 2012). Shrimp oil microcapsules fortified in biscuits showed low PV and TBARS values during storage at 30 °C for 12 days (Takeungwongtrakul and Benjakul, 2017). The slight increase in PV and TBARS of SONL fortified milk during the storage could be attributed to the unencapsulated or free oil which could undergo oxidation.

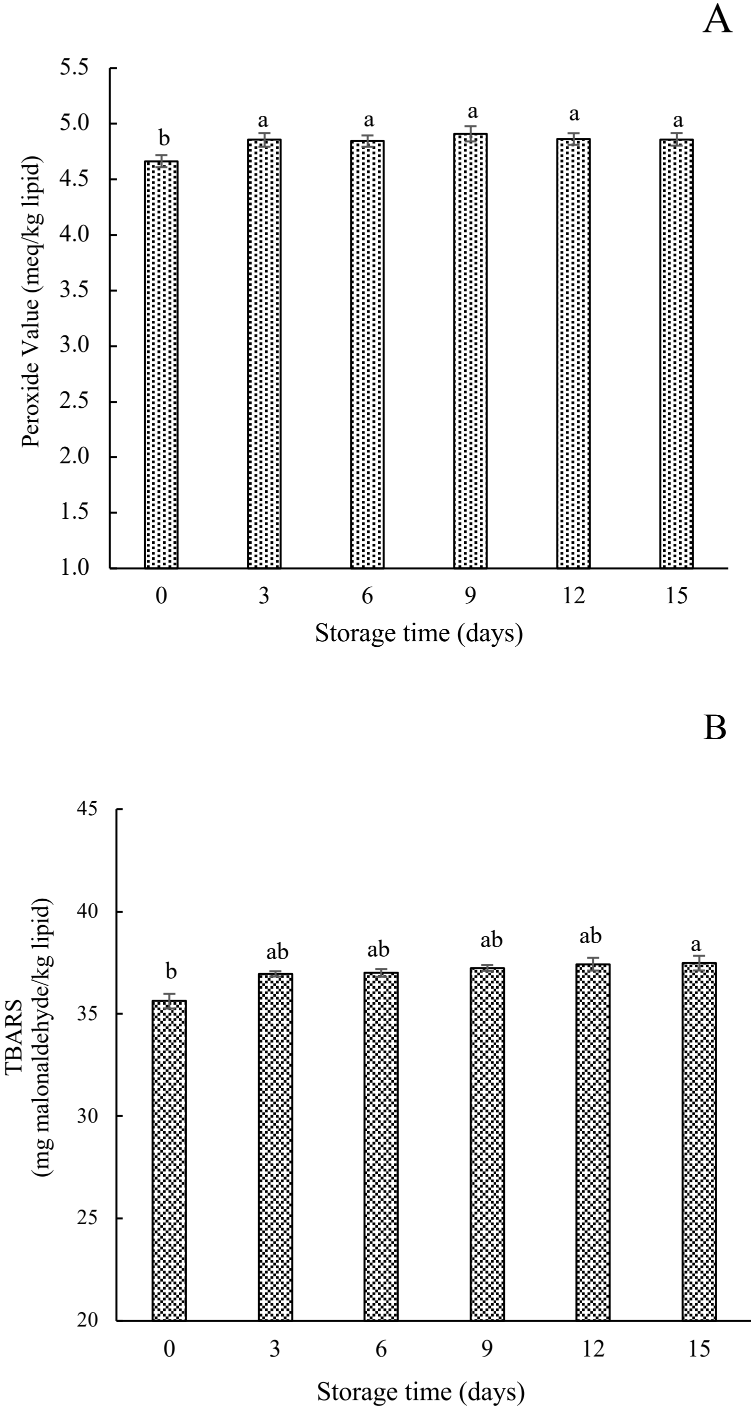

Figure 40. Peroxide value (A) and TBARS value (B) of skim milk fortified with 10% SONL during the storage of 15 days at 4 °C. Different lowercase letters on the bars indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome

7.5.2.4 Fatty acid profiles

Fatty acid profiles of SONL fortified milk during the storage period has been shown in Table 12. Shrimp oil extracted from SONL fortified skim milk contained PUFAs as major fatty acids. Linoleic acid (LA) was found to be the major unsaturated fatty acid, followed by DHA and EPA. There was no significant change in the fatty acids over time. Unsaturated fatty acids were found to be more retained with only slight decrease towards the end of storage time. Nanoliposomes were therefore effective in preventing oxidation of shrimp oil as indicated by the negligible increase in PV and TBARS (Figure 40A and 40B). Shrimp oil extracted from cephalothorax and hepatopancreas stored in iced storage over 6 days experienced loss of PUFAs (Takeungwongtrakul *et al.*, 2012).. However, the PUFAs were more retained when shrimp oil was microencapsulated and fortified in biscuits (Takeungwongtrakul and Benjakul, 2017). There was no change in the saturated fatty acid content of the shrimp oil, which conversely implied that no oxidation of unsaturated fatty acids took place. In general, polar heads of phospholipid projected to aqueous phase, whereas the fatty acids were localized inside. As a result, fatty acids were less prone to oxidation. Polyene index of oil (0.85) was almost constant over the entire storage time. Overall, the encapsulation of shrimp oil in nanoliposomes was effective in protecting against loss of n-3 fatty acids.

7.6 Conclusion

Shrimp oil nanoliposomes (SONL) could be effectively fortified in skim milk up to 10%. Fortified milk was sensorially acceptable, however, some panelists highlighted that the fortified milk was slightly bitter in taste. Microbial load of SONL fortified skim milk was quite stable over the storage period of 15 days at 4 °C. Fortified milk had slight changes in pH and acidity during storage. Nanoliposome encapsulation resulted in the augmented oxidative stability of shrimp oil, in which unsaturated fatty acids, especially EPA and DHA, were retained.

Table 12. Fatty acid profile of skim milk fortified with 10% SONL during storage of 15 days at 4 °C.

Fatty acids (g/100g lipids)	Storage Days					
	0	3	6	9	12	15
C14:0	0.71 ± 0.01b	0.75 ± 0.04a	0.72 ± 0.01b	0.76 ± 0.00a	0.78 ± 0.00a	0.72 ± 0.01b
C14:1	0.21 ± 0.00b	0.24 ± 0.05ab	0.24 ± 0.01ab	0.23 ± 0.00ab	0.25 ± 0.01ab	0.25 ± 0.01a
C15:0	0.41 ± 0.01c	0.44 ± 0.01b	0.45 ± 0.00b	0.49 ± 0.00a	0.40 ± 0.01c	0.42 ± 0.02c
C16:0	22.59 ± 0.02a	22.10 ± 0.00b	22.43 ± 0.28a	22.48 ± 0.21a	22.15 ± 0.16b	22.05 ± 0.05b
C16:1 n-7	1.51 ± 0.1a	1.51 ± 0.12a	1.40 ± 0.01a	1.40 ± 0.00a	1.47 ± 0.03a	1.39 ± 0.02a
C17:0	0.86 ± 0.04a	0.86 ± 0.05a	0.81 ± 0.02a	0.87 ± 0.03a	0.82 ± 0.01a	0.84 ± 0.01a
C17:1	0.15 ± 0.00a	0.16 ± 0.01a	0.15 ± 0.03a	0.18 ± 0.04a	0.17a ± 0.01a	0.18 ± 0.01b
C18:0	9.41 ± 0.12a	9.07 ± 0.07a	9.00 ± 0.01a	9.04 ± 0.06a	9.15 ± 0.15a	9.25 ± 0.36a
C18:1 n-9	14.93 ± 0.08a	14.50 ± 0.17b	14.25 ± 0.32bc	14.01 ± 0.00c	14.10 ± 0.11c	14.07 ± 0.06c
C18:2 n-7	2.86 ± 0.12a	2.81 ± 0.05a	2.89 ± 0.04a	2.74 ± 0.07a	2.81 ± 0.04c	2.37 ± 0.24b
C18:2 n-6	15.01 ± 0.04a	14.07 ± 0.05c	14.11 ± 0.11c	14.2 ± 0.05bc	14.2 ± 0.23bc	14.43 ± 0.20b
C20:1 n-9	1.73 ± 0.03a	1.71 ± 0.01ab	1.73 ± 0.06a	1.60 ± 0.05c	1.67 ± 0.05abc	1.63 ± 0.06bc
C20:2 n-6	1.65 ± 0.16a	1.75 ± 0.06a	1.70 ± 0.08a	1.67 ± 0.02a	1.61 ± 0.01a	1.62 ± 0.02a
C20:4 n-6 (ARA)	0.67 ± 0.08a	0.69 ± 0.02a	0.70 ± 0.01a	0.72 ± 0.02a	0.72 ± 0.01a	0.75 ± 0.02a
C20:5 (EPA)	7.69 ± 0.13a	7.22 ± 0.11bc	7.51 ± 36ab	7.26 ± 0.11bc	7.13 ± 0.11c	7.34 ± 0.11bc
C22:0	1.54 ± 0.13a	1.34 ± 0.07b	1.40 ± 0.10ab	1.40 ± 0.01ab	1.43 ± 0.03ab	1.41 ± 0.02ab
C22:1	0.45 ± 0.01a	0.42 ± 0.03a	0.33 ± 0.19a	0.46 ± 0.02a	0.48 ± 0.01a	0.48 ± 0.02a
C22:2 n-6	0.34 ± 0.01bc	0.32 ± 0.02bc	0.31 ± 0.01c	0.35 ± 0.03b	0.39 ± 0.01a	0.35 ± 0.02bc
C22:6 n-3 (DHA)	11.68 ± 0.04ab	11.37 ± 0.11ab	11.77± 0.19a	11.27 ± 0.33b	11.71 ± 0.38ab	11.38 ± 0.16ab
C23:0	3.69 ± 0.14b	3.23 ± 0.11c	4.04 ± 0.06ab	4.13 ± 0.04a	4.05 ± 0.08ab	4.21 ± 0.23a
C24:1	0.79 ± 0.03a	0.77 ± 0.06ab	0.78 ± 0.01ab	0.70 ± 0.04c	0.70 ± 0.01c	0.73 ± 0.01bc
Saturated fatty acid (SFA)	39.23 ± 0.34a	38.45 ± 0.47a	38.86 ± 0.42a	39.17 ± 0.31a	38.79 ± 0.24a	38.89 ± 0.26a
Monounsaturated fatty acid (MUFA)	19.77 ± 0.21a	19.32 ± 0.15b	18.88 ± 0.18c	18.57 ± 0.11d	18.83 ± 0.17cd	18.74 ± 0.11cd
Polyunsaturated fatty acid (PUFA)	39.92 ± 0.36a	38.97 ± 0.21b	38.98 ± 0.56b	38.21 ± 0.23b	38.56 ± 0.55b	38.22 ± 0.64b

Different lowercase letters in the same row indicate significant differences ($p<0.05$).

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CHAPTER 8

IMPACT OF β-GLUCAN ON DEBITTERING, BIO-ACCESSIBILITY AND STORAGE STABILITY OF SKIM MILK FORTIFIED WITH SHRIMP OIL NANOLIPOSOMES

8.1 Abstract

Shrimp oil was encapsulated in nanoliposomes and fortified into skim milk. Shrimp oil nanoliposomes (SONL) were thermodynamically stable when added into skim milk at 10% (v/v). Mild bitterness in fortified skim milk caused by the SONL was masked by adding β-glucan at various levels (0.05 – 0.2%, w/v). With the addition of SONL, fortified skim milk appeared more reddish in color due to the presence of astaxanthin. Addition of β-glucan resulted in the increase in viscosity of the fortified milk by forming network of junction zones. During the storage of skim milk fortified with SONL and 0.1% β-glucan at 4 °C for 15 days, no major quality changes took place. Simulated *in vitro* digestion studies revealed that 45.41% eicosapentaenoic acid (EPA) and 48.86% docosahexaenoic acid (DHA) from shrimp oil were bioaccessible for absorption in the gut after digestion.

8.2 Introduction

Strong evidences that polyunsaturated fatty acids (PUFAs) have several health promoting benefits on human body have been documented. They have been effective against numerous ailments including coronary heart diseases, hypertension, Alzheimer's disease, etc. (Cole et al., 2005; Sidhu, 2003). PUFAs are majorly obtained from marine sources including fish and crustaceans. Shrimp oil is one of crustacean oils rich in both PUFAs and carotenoids (Higuera-Ciapara *et al.*, 2006). Shrimp oil is extracted from cephalothorax or hepatopancreas (Takeungwongtrakul *et al.*, 2012), the leftover from shrimp processing industry. Shrimp oil is a major source of astaxanthin, a pigment known to have an exceptional antioxidative property (Gómez-Estaca *et al.*, 2017; Naguib, 2000).

One of the major problems associated with shrimp oil, that limits its use in food fortification, is the intense oxidation, which results in the development of

undesirable off-flavor, especially fishy odor (Gulzar and Benjakul, 2018). Occurrence of oxidation in shrimp oil is attributed to the presence of high amount of PUFAs, which are highly susceptible to oxidation. Other factors promoting oxidation of oils include prooxidants such as transition metals (Fe, Cu), enzymes (lipoxygenases) and some oxidizing agents. Environmental factors such as heat and light (UV) also accelerated lipid oxidation of oils (Kolanowski *et al.*, 2007). Various conventional and novel methods have been used to the tackle the menace of oxidation in oils. Those include modified atmospheric packaging, use of antioxidants, micro-encapsulation (Raei and Jafari, 2013; Takeungwongtrakul *et al.*, 2014), etc. Encapsulation method has been proven to be effective in retarding or suppressing oxidation process and masking undesirable odors and flavors of oils (Takeungwongtrakul et al., 2014; Tonon *et al.*, 2011).

One of the most recent and novel methods of encapsulation for bioactive compounds in food systems is the liposome technology (Munin and Edwards-Lévy, 2011). Liposomes are spherical vesicles consisting of a phospholipid bilayer and an aqueous core. Colloidal particles with the size less than 1000 nm are referred as nanoparticles (Hallaj-Nezhadi *et al.*, 2013). The phospholipid bilayer in liposomes forms a protective covering around encapsulated oils, preventing the contact of oils from prooxidants or air. As a result, the oxidation of core is retarded. Nanoliposomes offer better advantages than other encapsulation techniques such as better oxidative stability by avoiding high temperature during drying (Jafari *et al.*, 2008), controlled and targeted release of core material, and increased bioavailability of bioactive components (Chaudhry *et al.*, 2008). Those include better oxidative stability, retention of n-3 fatty acids, etc. (Ghorbanzade *et al.*, 2017). The phospholipid bilayer in liposomes forms a protective covering around encapsulated oils, preventing the contact of oils from prooxidants or air, and thus eliminating the chances of oxidation to a large extent. Characteristic fishy odor in marine oils can also be masked by encapsulation in liposomes. In dairy industry, liposomes have been used for fortification of vitamins to increase their nutritional value as well as enhance the bioaccessibility (Mozafari *et al.*, 2008). Vitamin D from cheeses encapsulated in

liposomes had the highest recovery, compared to commercially prepared ones and solubilized forms (Banville *et al.*, 2000).

β-glucan is a polysaccharide of D-glucose molecules joined by β-(1→3) and β-(1→4) linkages. It can be obtained from plant sources such as oat and barley, where it is present as cell wall polysaccharide, or from the yeast (*Saccharomyces cerevisiae*) (Böhm and Kulicke, 1999). β-glucan is known to possess a wide range of health benefits such as serum cholesterol reduction, blood sugar regulation, antioxidant activity, and immunomodulatory activity (Rahar *et al.*, 2011). Intake of β-glucan is also known to be effective in the treatment of microbial infections cancer, diabetes and hypercholesterolemia (Chen and Seviour, 2007). β-glucan has been used in liposomal systems to protect them from hydroxyl radical invasion in the aqueous systems (Babincová *et al.*, 2002). Glucan-liposome combination has been used synergistically to enhance the potency of drug delivery by protecting the degradation of drug (Machová *et al.*, 2000). β-glucan was reported to inhibit lipid peroxidation in phosphatidylcholine liposomes (Babincová *et al.*, 1999).

Characteristic fishy odor present in shrimp oil could also be masked by encapsulation in liposomes (Gulzar and Benjakul, 2020a). However, bitterness could be detected to some degree when nanoliposomes loaded with shrimp oil was fortified in skim milk. Bitterness issue in the lecithin based liposomes is caused by the oxidation of the phospholipid resulting in the formation of bitter compounds (Sessa *et al.*, 1976). Addition of soy lecithin in UHT milk promoted increase in bitterness during storage (Stephan and Steinhart, 2000). Coating of liposomes with β-glucan could be an approach to lower bitterness, while enhancing the oxidative stability of liposome, both phospholipid and core.

8.3 Objectives

To investigate the impacts of β-glucan at various levels on characteristics of skim milk fortified with shrimp oil nanoliposomes.

To examine physical and microbiological stability of the fortified milk during the refrigerated storage and investigate the bioaccessibility of EPA and DHA by simulated *in vitro* digestion of fortified skim milk

8.4 Materials and methods

8.4.1 Preparation of nanoliposomes

Shrimp oil was extracted from cephalothorax of Pacific white shrimp (*Litopenaeus vanammei*) following the method of Gulzar and Benjakul (2020b). Nanoliposomes were prepared by the modified ethanol injection method of Gulzar and Benjakul (2020a). Lecithin was dissolved in ethanol to attain 5% (w/v) and heated up to 45 °C to ensure complete dissolution. Subsequently, 5 mL of shrimp oil (preheated to 30 °C) was dropped gradually into 100 mL of prepared lecithin solution. The mixture was stirred at 1,000 rpm on a hotplate magnetic stirrer at 30 °C for 5 min. Deionized water (100 mL) and glycerol (2% v/v) were added to hydrate the solution. The mixture was homogenized for 10 min at a speed of 3000 rpm by an IKA Labortechnik homogenizer (Selangor, Malaysia). The liposomal dispersions with a total volume of approximately 200 mL were subjected to ultrasonication (10 min; 1 s on and off pulse) at 25 °C using an ultrasonic processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude. Ethanol was subsequently removed using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 30°C. After preparation, the obtained nanoliposome was placed under nitrogen atmosphere at 25 °C for 1 h for stabilization. Shrimp oil nanoliposome was referred to as ‘SONL’.

Particle size distribution, encapsulation efficiency and centrifugal stability of SONL were calculated using the method of Gulzar and Benjakul (2019a). Particle size distribution and mean particle size of nanoliposome samples, both US and MF, were determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA). Five ml of each sample were diluted with 20 mL of 1% (v/v) sodium dodecyl sulfate (SDS) solution to dissociate the flocculated vesicles. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the nanoliposome vesicles were measured.

For measuring the centrifugal stability, 5 ml of nanoliposomes were subjected to centrifugation at 3,500 g for 15 min. Nanoliposome stability (NS) was calculated as follows:

$$NS = \frac{f_{ev}}{i_{ev}}$$

where f_{ev} is the final volume of bottom phase and i_{ev} is the initial volume of liposomal dispersion.

Encapsulation efficiency (%EE) was calculated according to the following equation

$$\%EE = \frac{(OD_{468}\text{ total oil}) - (OD_{468}\text{ unencapsulated oil})}{(OD_{468}\text{ total oil})} \times 100$$

where OD_{468} total oil is the absorbance at 468 nm for total shrimp oil extracted from nanoliposomal dispersion using chloroform/methanol (2:1) mixture and OD_{468} unencapsulated oil is the absorbance at 468 nm for non-encapsulated shrimp oil collected as supernatant after centrifugation of nanoliposomal dispersion at 4200 g for 15 min and extracted using chloroform/methanol (2:1) mixture.

8.4.2 Fortification of skim milk with SONL in combination with β-glucan

Freshly prepared SONL was added to pasteurized milk sample (Meiji Pasteurized Skim Milk, Fat 0%, CP Meiji Co., Ltd., Bangkok, Thailand) to obtain the final concentration of 10% (v/v) nanoliposomes in milk. β-glucan powder was added to SONL fortified milk to obtain final levels of 0.05, 0.1, 0.15 and 0.2%. Approximately ten drops of synthetic pineapple flavor (R&B Food Supply, Bangkok, Thailand) were added to 100 mL of fortified skim milk. The mixtures were homogenized for 2 min at 5,000 rpm to ensure complete dissolution of β-glucan in milk. The samples were pasteurized at 63 °C for 30 min (Westhoff, 1978) and transferred into aseptic bottles. The lid was closed tightly. Samples were stored at 4 °C and directly subjected to analyses.

8.4.3 Physical properties and bitterness of skim milk fortified with 10% SONL in combination of β-glucan at different levels

8.4.3.1 Color

Color of SONL fortified milk samples added with different levels of β-glucan was measured was measured by a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA) following the method of Mad-Ali, Benjakul, Prodpran, and Maqsood (2016).

8.4.3.2 Viscosity

Viscosity measurement of SONL fortified milk samples containing β-glucan at different levels at two different temperatures (4 ºC and 25 ºC) was carried out using a Brookfield digital viscometer (model DV II+, Brookfield Eng Labs Inc., Stoughton, MA, USA.) equipped with spindle no. 1, at 100 rpm. Viscosity of all the fortified milk samples was determined by comparing with control (unfortified milk) sample and the values were reported as cP.

8.4.3.3 Bitterness

Bitterness of SONL fortified milk samples added with β-glucan at different levels was examined following the method of Sinthusamran *et al.* (2018) using standard reference solution of caffeine. Ten panelists aged between 25 and 33 were trained using caffeine as a standard for a period of 1 month, twice a week. Standard solutions at different concentrations (0, 25, 50 and 75 ppm) were presented. Distilled water was used to represent a score of 0, while 75 ppm caffeine represented a score of 15. For the evaluation, a 15-cm line scale ranging from “none” to “intense” was used. Fortified milk samples with different levels of β-glucan were served at room temperature (25 – 26 °C) coded with three-digit random numbers, together with a standard reference solution of caffeine. The panelists then evaluated the bitterness of the samples, compared to the reference sample using a 15-cm line scale. Between samples, the panelists were asked to eat a piece of un-salted cracker and rinse their mouths thoroughly with distilled water.

8.4.4 Characterization and storage stability of skim milk fortified with SONL and β-glucan at the selected level

Skim milk fortified with 10% (v/v) SONL and β-glucan at the level yielding the least bitterness was characterized. Another portion was subjected to storage at 4 °C for 15 days. Samples were taken every 3 days and determined.

8.4.4.1 Characterization of fortified milk

8.4.4.1.1 Microstructure analysis

Microstructures of 10% (v/v) SONL fortified milk sample added without and with 0.1% (w/v) β-glucan addition were analyzed using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, the Netherlands) following the method of Buamard and Benjakul (2018).

8.4.4.1.2 *In vitro* gastrointestinal digestion

To determine the bio-accessibility of EPA and DHA in shrimp oil, loaded in liposome *in vitro* release of shrimp oil in stimulated gastrointestinal tract system was performed following the method of Sae-Leaw *et al.* (2016) with some minor modifications. To mimic the gastric phase of human digestion, freshly prepared pepsin (0.04 g/mL in 0.1 N HCl) was added into fortified milk sample at the enzyme/sample ratio of 1:10. The pH of the mixture was adjusted to 2.0 using 0.1 N HCl followed by incubation at 37 °C for 1 h in an orbital shaking water bath (Grant OLS200; Grant Instruments, Cambridge, UK) at 95 rpm. For duodenal digestion, the pH was raised to 6.8 using 0.9 M sodium bicarbonate, followed by the addition of freshly prepared bile salts (0.04 g/mL glycodeoxycholate, 0.04 g/mL taurocholate, and 0.025 g/mL taurodeoxycholate) and porcine pancreatin including lipase, trypsin, amylase, ribonuclease, and protease (0.08 g/mL). Subsequently, the mixture was incubated for 2 h at 37 °C in an orbital shaking water bath. Immediately after digestion, oil was extracted from the digest using the mixture of chloroform and methanol (2:1) at the sample/solvent ratio of 1:5 and the oil obtained was subjected to fatty acid analysis. Oil from fortified milk before gastrointestinal digestion was also extracted and analyzed for fatty acid profile.

Fatty acid profile was analyzed as fatty acid methyl esters (FAMEs) using gas chromatography (GC) following the method of Gulzar and Benjakul, (2019d). Fatty acids were firstly transmethylated by 2 M methanolic sodium hydroxide, along with 2 M methanolic hydrochloric acid. FAMEs were analyzed by gas chromatography (GC) using Agilent 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. FAME dissolved in hexane was determined using Agilent J&W fused silica capillary column (100 m × 0.25 mm × 0.20 µm) (a split ratio of 1:20). The injection port temperature was kept at 250 °C and detector (flame ionization detector, FID) temperature was 270 °C. The oven temperature ranged from 170 to 225 °C with a flow rate of 1 °C /min. The chromatographic peaks of the samples were identified based on retention times, compared to those of standards. Peak area ratio was used for calculation and the content was expressed as g fatty acid/100 g lipid.2.4.2 Storage study of fortified milk

8.4.4.2.1 Microbiological analysis

Microbiological analysis was carried out following the method of Maqsood and Benjakul (2011) with some modifications. Milk sample (5 mL) was collected aseptically and diluted ten-time with sterile saline solution (0.85%). After homogenizing, a series of 10-fold dilutions were made using a saline solution. Total viable count (TVC) was conducted using plate count agar (PCA) incubated at 37 ºC for 2 days. Psychrophilic bacterial count (PBC) was also enumerated using PCA incubated at 4 ºC for 7 days. Microbial counts were expressed as log colony forming unit per mL of sample (log CFU/mL).

8.4.4.2.2 pH and acidity

pH of milk samples was measured using a Lab 855 pH meter (SI Analytics, Xylem Inc., Rye Brook, NY, USA). Titrable acidity was determined following the AOAC method 947.05 (AOAC, 1990). Twenty mL of milk sample were diluted with hot distilled water, cooled and titrated against 0.1 N NaOH using 1% phenolphthalein indicator. Titrable acidity was expressed as g of lactic acid/100 g

8.4.4.2.3 Determination of lipid oxidation

Firstly, oil in nanoliposome was extracted from fortified milk using the mixture of chloroform and methanol (2:1) at the sample/solvent ratio of 1:5. Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were determined using the ferric thiocyanate method as described by Chaijan, Benjakul, Visessanguan, and Faustman (2006), and by the method tailored by Buege and Aust (1978), respectively.

8.4.5 Statistical analysis

All the experiments were done in triplicates and the data were expressed as means ± SD. Randomized complete block design (RCB) was implemented for sensory analysis study. Analysis of variance (ANOVA) was performed and means were compared by the Duncan's multiple range test. Data analysis was carried out using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

8.5 Results and discussion

8.5.1 Effect of β-glucan at various levels on characteristics of SONL fortified skim milk

8.5.1.1 Color

Table 13 shows L*, a*, b* and ΔE* values of skim milk samples fortified with 10% SONL in the presence of β-glucan at different levels. L* (whiteness) (93.03) was highest in the control (unfortified) sample ($p<0.05$). Whiteness of milk samples (75.45) decreased sharply with the addition of SONL ($p<0.05$). Addition of β-glucan further decreased the whiteness of the samples in the dose-dependent manner ($p<0.05$). The decrease in whiteness of milk samples with the addition of β-glucan could be attributed to the light brown color of the β-glucan. β-glucan added in skim and whole milk resulted in darker color of samples (Bangar, 2011). Whiteness of Indian low-fat yogurt (*dahi*) decreased significantly with increasing β-glucan concentration (Bhaskar *et al.*, 2017). Addition of β-glucan above 0.3% reduced L* values of set-yogurt (Singh *et al.*, 2012). The a* (redness) of skim milk increased drastically when SONL was added. The increase in redness could be

owing to the presence of astaxanthin, a red pigment present in the shrimp oil located as the core of SONL. Astaxanthin, a keto-carotenoid, naturally occurring in microalgae, contributes to the characteristic orange-red color of the crustaceans (Higuera-Ciapara *et al.*, 2006). With the increasing level of β-glucan, redness was further increased ($p<0.05$). The increase in redness could be associated with the brownish color of β-glucan powder. Redness of set-style yogurt was found to increase with increasing concentration of β-glucan added (Singh *et al.*, 2012). The b* value (yellowness) of the samples was increased markedly with the addition of SONL in skim milk and further increased gradually as β-glucan levels were augmented ($p<0.05$). The increase in yellowness was concomitant with the augmentation of redness. The total color difference (ΔE*) among the samples was also noted to increase with increasing amount of β-glucan ($p<0.05$). Overall, skim milk fortified with SONL had the reduction in whiteness with the increment of redness and yellowness when the amounts of β-glucan added were augmented.

Table 13. Color values and bitterness score of skim milk fortified with 10% (v/v) SONL in the presence of β-glucan at various levels

β-glucan level (%)	L*	a*	b*	ΔE*	Bitterness score[#]
CON	93.03± 0.05a	4.79 ± 0.09f	11.73 ± 0.04f	0	0
0	75.45 ± 0.01b	26.95 ± 0.07e	32.83 ± 0.05e	42.84 ± 0.10e	10.50 ± 1.40a
0.05	73.24 ± 0.04c	27.85 ± 0.19d	33.31 ± 0.04d	43.85 ± 0.15d	5.90 ± 1.50b
0.10	72.58 ± 0.05d	28.88 ± 0.04c	34.28 ± 0.05c	45.39 ± 0.06c	0.50 ± 0.50c
0.15	71.13 ± 0.04e	29.61 ± 0.05b	35.81 ± 0.09b	47.35 ± 0.11b	0.60 ± 0.70c
0.20	70.14 ± 0.05f	30.33 ± 0.03a	36.36 ± 0.06a	48.62 ± 0.09a	0.50 ± 0.50c

Different lowercase letters in the same column indicate significant differences ($p<0.05$). CON: Unfortified skim milk. [#]0: No bitterness; 15: Extremely bitter.

8.5.1.2 Viscosity

Viscosities of skim milk samples fortified with 10% SONL in combination with β-glucan kept at two different temperatures are shown in Figure 41. Viscosity of skim milk increased with the addition of SONL ($p<0.05$). Skim milk generally observes a Newtonian behavior (Vliet and Walstra, 1980) and its viscosity

is mediated by the hydrodynamic interactions between lactose and protein molecules. With the increase in fat content, viscosity is generally increased due to the higher interactions, resulting in the increase in the internal friction (Phipps, 1969). Viscosity of SONL fortified skim milk samples increased as β-glucan at increasing concentrations was added ($p<0.05$). Various studies have revealed that β-glucan increased the viscosity and contributed to the denser texture of milk and milk products (Bhaskar *et al.*, 2017; Vasiljevic *et al.*, 2007). β-glucan added to milk at 0.622% increased the viscosity by four-fold, measured at 10 °C (Bangar, 2011). Increase in viscosity could be attributed to the network formation by β-glucan, which might aggregate by association of cellotriose units, and formation of junction zones (Böhm and Kulicke, 1999). In addition to the formation of junction zones, the increased viscosity can also be caused by the increase in the solids content of skim milk (Vasiljevic *et al.*, 2007). When comparing viscosity of milk samples stored at both temperatures, significantly higher viscosity was found at low temperature. Many studies have suggested that the increase in viscosity of skim milk at low temperature is mainly due to the increase in density of water and hydration of casein micelles (Kristensen *et al.*, 1997). Increased viscosity at low temperature also depends on the concentration of milk. Furthermore, the agglomeration of oil droplets tends to increase the viscosity of colloidal system, especially at low temperature. Overall, the viscosity of fortified milk was largely affected by the increasing levels of β-glucan and low temperature.

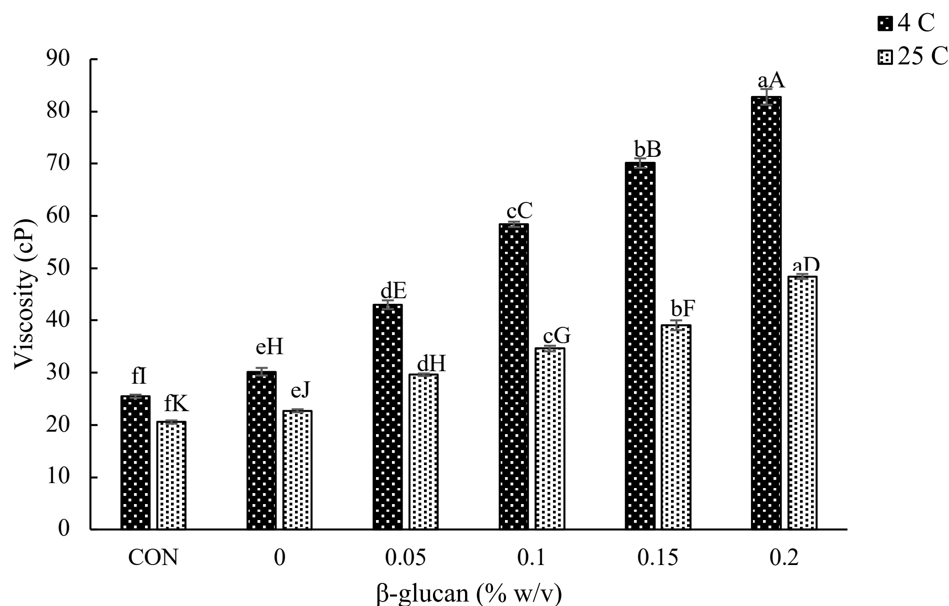

Figure 41. Viscosity at 4 °C and 25 °C of skim milk fortified with 10% SONL in the presence of β-glucan at various levels. CON: Unfortified skim milk; SONL: Shrimp oil nanoliposome. Bars represent standard deviation (n=3). Different lowercase letters on the bars within the same temperature indicate significant differences ($p<0.05$). 4 °C: Viscosity measured at 4 °C; 25 °C: Viscosity measured at 25 °C

8.5.1.3 Bitterness

The bitterness scores of 10% SONL fortified skim milk added with 0, 0.05, 0.1, 0.15 and 0.2% β-glucan were 10.15, 5.90, 0.50, 0.60 and 0.50, respectively (Table 13). Standard solution of caffeine (75 ppm) had an average score of 12.44. Bitterness of fortified skim milk samples reduced significantly with the addition of β-glucan up to 0.1% ($p<0.05$). There was no further change in the bitterness of fortified skim milk samples when β-glucan above 0.1% was incorporated ($p>0.05$). Bitterness of SONL fortified skim milk could plausibly be caused by lecithin used as the wall. Soy lecithin was documented to impart bitterness to UHT milk (Stephan and Steinhart, 2000). Additionally, oxidation of phospholipids could cause the increased bitterness (Sessa *et al.*, 1976). Bitterness of lecithin could possibly be due to the exposure of its hydrophobic parts to the taste buds, particularly those which were not localized as bilayer but were present as free form in aqueous phase. Hydrophobicity is a major cause of bitterness in products such as protein hydrolysates (Thiansilakul *et*

al., 2007). However, addition of β-glucan could possibly have covered the hydrophobic parts of phospholipids, or some parts of nanoliposome, thus minimizing their interaction with the taste buds. Therefore, β-glucan had a significant impact on the reduction of bitterness in the fortified skim milk samples. Due to the most effectiveness of β-glucan at 0.1% on lowering bitterness and had less impact on viscosity, it was selected for fortification along with SONL into skim milk.

8.5.2 Characteristics of skim milk fortified with SONL and 0.1% β-glucan

8.5.2.1 Microstructure

The particle size of SONL ranged between 40 and 284 nm, whereas the mean particle size of SONL was 104.77 nm. Encapsulation efficiency and centrifugal stability of SONL was calculated to be 93.64±0.98% and 94.5±0.56%, respectively. Figure 42 shows the microstructure of 10% SONL fortified skim milk with and without 0.1% β-glucan, as visualized by SEM. The micrographs revealed the change in the morphology of the nanoliposome in the skim milk before and after the addition of β-glucan. The nanoliposomes could be clearly seen as individual capsules before the addition of β-glucan with size ranging from few hundred nanometers to a few microns. However, with the addition of β-glucan, a long chain polysaccharide was found to localize surrounding the nanoliposome capsule. The micrographs also illustrated a bunch of nanoliposomes clumped together by β-glucan chain, indicating that β-glucan more likely interacted with the nanoliposome to form a relatively large cluster. β-glucan is believed to form a strong nonspecific binding to the lipid bilayer, rendering a shield around the liposomes, thus protecting it from the attack of hydroxyl radicals produced in the surrounding aqueous phase (Babincová *et al.*, 2002). These micrographs also explained the increase in the viscosity of SONL fortified skim milk samples when added with β-glucan (Figure 41). The large clusters formed were related with the augmented resistance to flow than individual nanoliposomes.

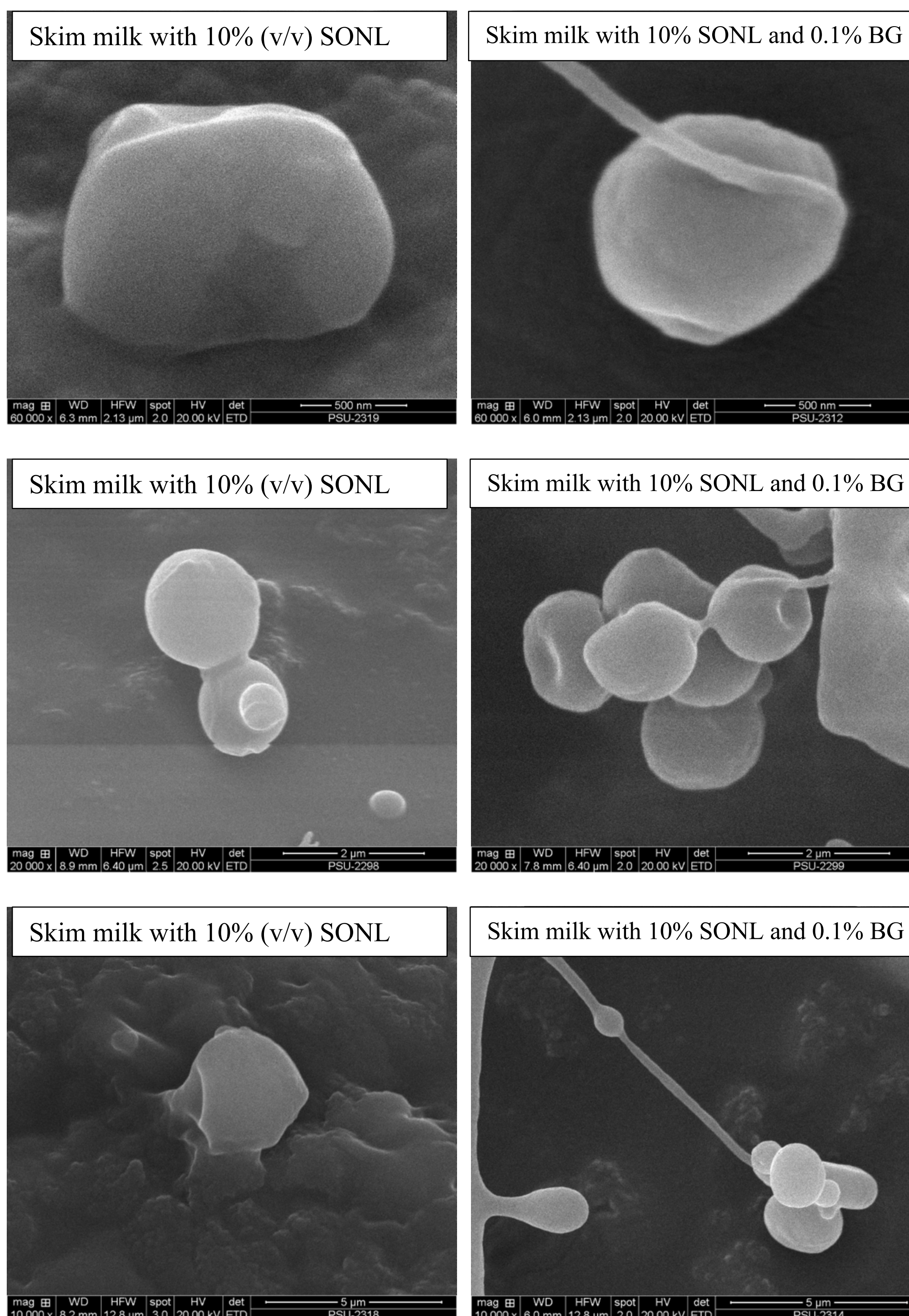

Figure 42. Scanning electron micrographs of 10% SONL fortified skim milk samples with and without 0.1% β-glucan. SONL: Shrimp oil-loaded nanoliposome. BG: β-glucan. Magnification: 60,000x, 20,000x, 10,000x

Reduction of bitterness by the addition of β-glucan might be due to the covering of liposome surface by β-glucan. As proposed by many studies, bitterness perceived by humans is associated with the binding of bitter compounds by bitter taste receptors located in the taste buds (Fu *et al.*, 2019). These receptors have a specific 'pocket' size to attach the bitter compound and create a neural stimulus (Ishibashi *et al.*, 1988). However, with the formation of nanoliposome clusters, their average size became too large for the bitter taste receptors to attach and create a neural response. Coincidentally, the hydrophobic domains were also masked by β-glucan. Therefore, the bitterness was not perceived by the taste buds (Table 13).

8.5.2.2 *In vitro* gastrointestinal digestion

Fatty acid profiles of shrimp oil extracted from SONL fortified in skim milk before and after *in vitro* digestion of SONL and β-glucan fortified skim milk sample are tabulated in Table 14. Oil from undigested sample contained PUFAs as major fatty acids, in which linoleic acid constituted as the most abundant unsaturated fatty acid, followed by DHA and EPA, respectively. On the other hand, digested oil contained the higher proportion of saturated fatty acids, indicating the loss of PUFAs taken place during *in vitro* digestion process ($p<0.05$). Fortified milk sample was subjected to several hydrolytic enzymes including lipases, which hydrolyzed the oil in the nanoliposome core, thus liberating free fatty acids. When free fatty acids, particularly the PUFAs, were released in the system, they were prone to oxidation. Oxidation of PUFAs resulted in the loss of their unsaturation. The results indicated that more than half of the EPA and DHA were lost from the oil loaded in SONL and the remaining EPA (45.41%) and DHA (48.86%) were bioaccessible for absorption into the gut after digestion. Our results were in agreement with Ilyasoglu and El (2014) who documented that bioaccessibility values for EPA and DHA from nanoencapsulated fish oil were 36.25% and 56.16%, respectively. Milk fortified with n-3 PUFAs in the form of nanoliposomes were more stable and encountered less loss of PUFAs (5.6 – 5.9%) compared to microencapsulated form. The fortified milk had sensory attributes close to control sample without any fishy or unpleasant flavor (Rasti *et al.*, 2017). No significant loss in linoleic acid content was observed in the oil ($p>0.05$). However, slight increase in oleic acid was noticeable after digestion.

Therefore, n-3 fatty acids with higher degree of unsaturation loaded in nanoliposome were still prone to oxidation, as indicated by the lowered level detected after *in vitro* digestion.

Table 14. Fatty acid profile of shrimp oil extracted from 10% SONL and 0.1% β-glucan fortified skim milk before and after *in vitro* digestion.

Fatty acids (g/100 lipids)	CON	*in vitro*
C14:0	0.59 ± 0.03b	0.77 ± 0.06a
C14:1	0.31 ± 0.02a	0.25 ± 0.04b
C15:0	0.48 ± 0.01a	0.41 ± 0.01b
C16:0	23.43 ± 0.33b	25.37 ± 0.44a
C16:1 n-7	1.63 ± 0.10a	1.51 ± 0.06a
C17:0	0.86 ± 0.02a	0.80 ± 0.05a
C17:1	0.17± 0.01b	0.27 ± 0.06a
C18:0	8.62 ± 0.37b	9.38 ± 0.27b
C18:1 n-9	15.09 ± 0.24b	15.60 ± 0.31a
C18:2 n-7	2.89 ± 0.22a	2.78 ± 0.15a
C18:2 n-6	16.62 ± 0.26a	16.93 ± 0.31a
C20:1 n-9	1.57 ± 0.05a	1.69 ± 0.19a
C20:2 n-6	1.58 ± 0.14b	1.78 ± 0.06a
C20:4 n-6 (ARA)	0.77 ± 0.05a	0.37 ± 0.01b
C20:5 (EPA)	6.87 ± 0.04a	3.12 ± 0.05b
C22:0	1.20 ± 0.08b	1.38 ± 0.02a
C22:1	0.38 ± 0.04b	0.57 ± 0.05a
C22:2 n-6	0.51 ± 0.02a	0.36 ± 0.02b
C22:6 n-3 (DHA)	10.58 ± 0.09a	5.17 ± 0.13b
C23:0	3.91 ± 0.02b	4.23 ± 0.21a
C24:1	0.94 ± 0.04a	0.76 ± 0.03b
Saturated fatty acid (SFA)	39.09 ± 0.21b	42.36 ± 0.53a
Monounsaturated fatty acid (MUFA)	19.77 ± 0.21a	20.66 ± 0.40a
Polyunsaturated fatty acid (PUFA)	39.92 ± 0.36a	30.56 ± 0.16b

Different lowercase letters in the same row indicate significant differences ($p<0.05$).
CON: Undigested shrimp oil; *in vitro*: digested shrimp oil (*in vitro*)

8.5.3 Storage stability of skim milk fortified with SONL and 0.1% β-glucan during storage

8.5.3.1 Microbiological quality

Total viable count (TVC) of control (unfortified skim milk) and skim milk fortified with 10% SONL and 0.1% β-glucan over the storage period of 15 days is illustrated in Figure 43. TVC of both milk samples increased gradually up to day 15 ($p<0.05$). However, the TVC values of both the samples did not exceed 2.5 log CFU/mL, which is much lower than the standard limit (5 log CFU/mL) set by the Pasteurized milk ordinance (PMO) (FDA, 2015). The results were in agreement with Ziarno *et al.* (2005) who reported that total bacterial count in pasteurized milk during the storage of 7 – 14 days remained low (<5 log CFU/mL) and increased significantly after 21 days. Lower levels of microbial load indicated that pasteurization of fortified milk was effective and most mesophilic bacteria were killed or inactivated. Pasteurization process destroys or inactivates majority of the micro-organisms including the pathogenic micro-organisms, which can pose a severe health problem or outbreak. Over the entire storage period, no growth of psychrotropic bacteria was observed and the psychrotropic bacterial count (PBC) remained undetected. Those aforementioned micro-organisms were more likely killed during pasteurization. Overall, the microbial quality of milk was acceptable and appropriate for consumption, regardless of fortification of SONL and β-glucan.

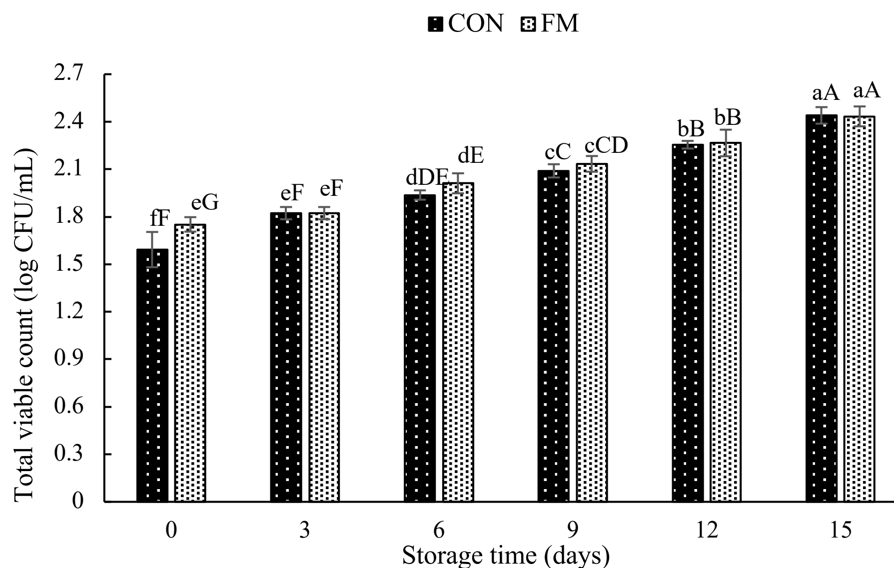

Figure 43. Total viable count (TVC) of control (unfortified skim milk) and skim milk fortified with 10% SONL and 0.1% β-glucan during the storage of 15 days at 4 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same sample indicate significant differences (p<0.05). Different uppercase letters on the bars within the same storage time indicate significant differences (p<0.05). SONL: Shrimp oil-loaded nanoliposome; CON: Unfortified skim milk; FM: Fortified skim milk

8.5.3.2 pH and acidity

Changes in pH and acidity of the control and 10% SONL fortified milk sample containing 0.1% β-glucan during the storage of 15 days are shown in Figure 44A and 44B, respectively. pH of control and fortified samples had no significant change within the first 6 days of storage (p>0.05). Afterwards, pH of both the samples decreased continuously up to the end of storage (p<0.05). The decrease in pH could be possibly due the conversion of lactose into lactic acid by lactic acid bacteria, which might be present in the milk. Similar values of pasteurized milk stored under refrigerated conditions for up to 20 days were posted by Hassan *et al.* (2009). The pH values of control sample dropped more sharply than fortified milk (p<0.05). This could be probably due to lower viscosity of control sample, resulting in the greater mobility of ions available for micro-organisms. Concomitantly, the acidity of both the

milk samples gradually increased, after day 6 ($p<0.05$). However, for control sample, acidity was increased after 3 days of storage ($p<0.05$). Overall, the acidity values of fortified milk were still within the prescribed legal standard limits (not more than 0.18) (JETRO, 2010)

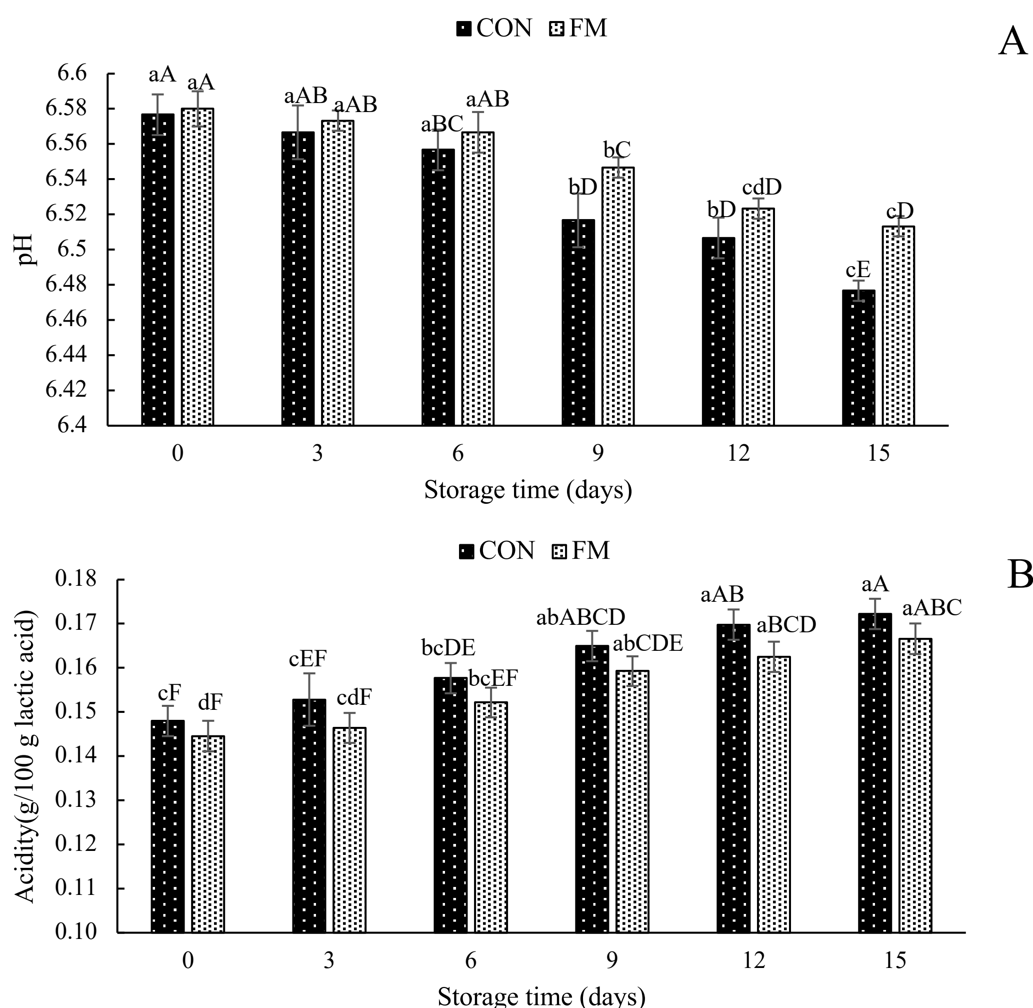

Figure 44. pH (A) and acidity (B) of control (unfortified skim milk) and skim milk fortified with 10% SONL and 0.1% β-glucan during the storage of 15 days at 4 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same sample indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). SONL: Shrimp oil nanoliposome; CON: Unfortified skim milk; FM: Fortified skim milk

8.5.3.3 PV and TBARS

PV and TBARS values of shrimp oil extracted from 10% SONL fortified skim milk containing 0.1% β-glucan stored over the period of 15 days are depicted in Figure 45A and 45B, respectively. No increases in PV or TBARS were observed in the extracted shrimp oil from the SONL fortified milk over the entire storage period ($p>0.05$), indicating that no oxidation took place in the shrimp oil. Nanoliposomes form a protective barrier of phospholipid bilayer around the oil, which was localized as core. This barrier prevented the oxidation of unsaturated fatty acids present in the oil by reducing the contact of oil with prooxidants and catalysts such as transition metals (Fe, Cu), enzymes (lipoxygenases), chemical oxidizers as well as from environmental factors such as light (UV) and oxygen (Gulzar and Benjakul, 2019b). Encapsulation of fish oil in nanoliposomes resulted in the reduced oxidation and better retention of unsaturated fatty acids during the storage (Ghorbanzade *et al.*, 2017; Ye *et al.*, 2009). Biscuits fortified with shrimp oil microcapsules showed low PV and TBARS during the storage at 30 °C for 12 days (Takeungwongtrakul and Benjakul, 2017). The PV and TBARS of n-3 PUFAs encapsulated in nanoliposomes and fortified in milk was much lower than the microencapsulated n-3 PUFAs (Rasti *et al.*, 2017). The major advantage of nanoliposomes over other microencapsulation technologies is the stability of nanoliposomes in high water activity systems, whereas other encapsulation techniques impart stability to food ingredients in the dry state but release the core material readily in high water activity systems, giving up all protection properties (Gouin, 2004). Moreover, the encapsulation efficiency of shrimp oil in nanoliposome was found to be much higher (~95%) (Gulzar and Benjakul, 2019a), compared to the encapsulation efficiency of microencapsulation (~50%) (Takeungwongtrakul *et al.*, 2014). As a result of lower encapsulation efficiency of microencapsulation method, higher surface oil present on the microcapsules undergoes rapid oxidation. Overall, the encapsulation of shrimp oil in nanoliposomes was effective in protecting against oxidation of fatty acids in shrimp oil localized as the core.

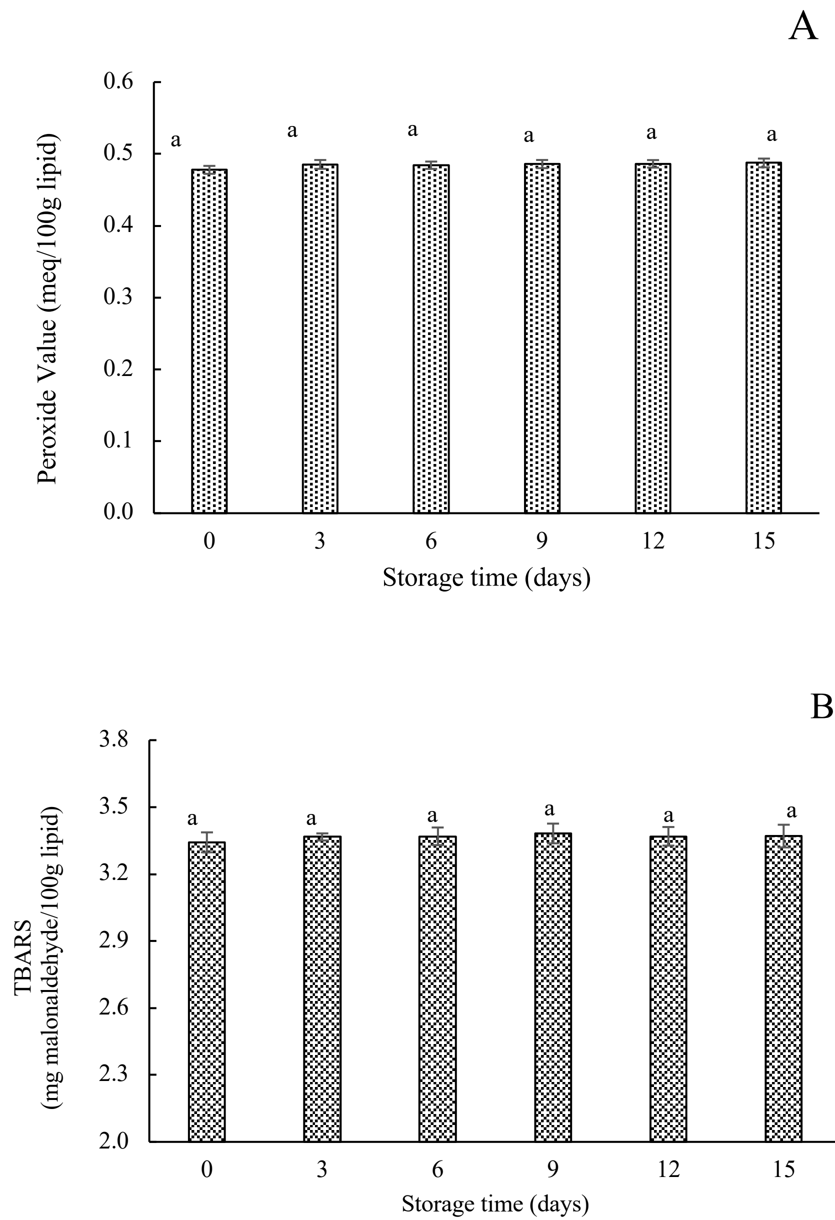

Figure 45. Peroxide value (A) and TBARS value (B) of skim milk fortified with 10% SONL and 0.1% β-glucan during the storage of 15 days at 4 °C. Different lowercase letters on the bars indicate significant differences ($p<0.05$). SONL: Shrimp oil-loaded nanoliposome.

8.6 Conclusion

Fortification of shrimp oil nanoliposomes (SONL) in skim at 10% resulted in high bitterness in taste. Addition of β-glucan at 0.1% to the SONL fortified skim milk almost completely removed the bitterness of the sample. However, β-glucan, particularly at high level, resulted in the increases in viscosity of fortified skim milk samples. Nanoliposomes were covered or connected by β-glucan added, forming cluster and being related with increased viscosity. *In vitro* digestion studies revealed that almost half of EPA and DHA were bioaccessible for adsorption by the body after the release of shrimp oil from the nanoliposomes in the gastrointestinal tract. Microbial load of SONL fortified skim milk was quite stable over the storage period of 15 days at 4 °C. Slight changes in pH and acidity of fortified milk samples were observed. Shrimp oil encapsulated in nanoliposomes had enhanced oxidative stability throughout the storage at 4 °C.

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CHAPTER 9

EFFECT OF CARBOXYMETHYL CELLULOSE AND FUMED SILICA ON THE CHARACTERISTICS AND STABILITY OF SHRIMP OIL NANOLIPOSOME POWDER PREPARED BY FREEZE-DRYING AND SPRAY-DRYING

9.1 Abstract

Shrimp oil nanoliposomes (SONL) dried by freeze-drying and spray-drying were characterized, in which carboxy methyl cellulose (CMC) and fumed silica (SiO_2) at various proportions were used as wall material and anti-caking agent. Spray-dried powder (SDP) was spherical in shape with average particle size of 5.33±2.55 μm, while the freeze-dried powder (FDP) having average size of 243.6±256.7 μm was irregular. SDP showed better flowability than FDP, which was more porous with much lower bulk density. Encapsulation efficiency and solubility of SDP were greater than FDP ($p<0.05$). However, the wettability of FDP was higher with shorter reconstitution time. FDP exhibited lower oxidation of total encapsulated oil and better retention of n-3 fatty acids ($p<0.05$). Overall, both FDP and SDP were thermodynamically stable and the aforementioned powders had the prolonged storage time.

9.2 Introduction

Shrimp cephalothorax, an inedible by-product generated from processing of whole shrimps, have been used to produce oil containing bioactive compounds, especially polyunsaturated fatty acids (PUFAs) and astaxanthin (Takeungwongtrakul *et al*, 2012). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the abundant n-3 fatty acids (n-3-FA) present in shrimp oil. EPA and DHA exhibited lowering cardio-vascular diseases (CVD) and CVD risk factors, such as hypertension and elevated plasma lipids (Harper and Jacobson, 2001). n-3-FAs are also able to prevent cancer, suppress Alzheimer's disease, help in reducing rheumatoid arthritis etc. (Cole *et al*., 2005; Sidhu, 2003). Shrimp oil is also a major source of astaxanthin, a red carotenoid found in crustaceans, known to have exceptional antioxidative

property (Naguib, 2000). Astaxanthin had singlet oxygen scavenging activity higher than coenzyme Q10 by 1000 times (Mori *et al.*, 2013). Apart from the use of astaxanthin as an anti-inflammatory, it is also used to treat UV induced skin ailments and maintain healthy skin (Ito *et al.*, 2018).

Despite the presence of several bioactive compounds, shrimp oil has remained underutilized in food and nutraceutical applications. The major reason is the augmented oxidation of shrimp oil, mostly due to high amount of PUFAs, causing the development of off-flavor, particularly fishy odor (Gulzar and Benjakul, 2018). Additionally, prooxidants such as transition metals (Fe, Cu), enzymes (lipoxygenases), heat and light (UV) facilitate the oxidation of oils (Kolanowski *et al.*, 2007). Encapsulation has been successful in conquering the problems associated with quality deterioration of oils, particularly during processing or storage (Takeungwongtrakul *et al.*, 2014). Encapsulation process also ensures controlled release of target compounds, in addition to masking of undesirable odors and flavors (Gulzar and Benjakul, 2019a). Various encapsulation methods have been employed to encapsulate the oils for fortification in foods as nutraceutical or functional ingredient. A novel and widely used method of encapsulating bioactive compounds in the food and nutraceutical industry is the nanoliposome technology (Munin and Edwards-Lévy, 2011).

Liposomes are spherical microscopic phospholipid vesicles with a bilayer membrane and an aqueous core. Liposomes form a protective wall around encapsulated oils. Nanoliposomes, the liposomal vesicles with size of around 100 nm or lesser, have been used to encapsulate shrimp oil. Encapsulation in nanoliposomes resulted in the prevention of oxidation and complete masking of fishy odor (Gulzar and Benjakul, 2019a). Nanoliposomes offer advantages over other encapsulation techniques, such as better oxidative stability, targeted release, and retention of bioactive compounds etc. (Ghorbanzade *et al.*, 2017). Encapsulation of unsaturated fatty acids into liposomal vesicles creates a barrier, which prevents the oxidation of these highly susceptible compounds (Hadian, 2016). Encapsulation of shrimp oil nanoliposomes and fortification in skim milk resulted in better retention of EPA and DHA, in addition to complete masking of fishy odor (Gulzar and Benjakul, 2019e).

Although, nanoliposomes are thermodynamically stable during storage, phospholipids as wall is oxidized or hydrolyzed over time. Hydrolysis can be minimized by removing water as much as possible (Gibbs *et al.*, 1999). A gradual decline in EPA and DHA contents was attained in shrimp oil encapsulated in nanoliposomes stored at room temperature for 8 weeks (Gulzar and Benjakul, 2019a). Therefore a more stable system can be achieved by removing the water from the nanoliposomal dispersions by drying process. A few studies have shown that liposomes retain their stability and narrow size distribution in the powder form (Chun *et al.*, 2017).

Many drying methods have been employed to prepare liposomal dry powder formulations (Courrier *et al.*, 2002). Ideal powder characteristics such as good flow properties, lower reconstitution time and efficient delivery of target compounds are dependent on several factors including drying methods, interparticle friction, cohesive forces, particle size, etc. (Khoe *et al.*, 1991). Most food powders are susceptible to moisture adsorption from the ambient atmosphere, which significantly impacts the flow behavior of powder (Fitzpatrick, 2005). The process of powder particles sticking together governed by either strong interparticle cohesion or formation of solid bridges among particles, namely caking (Fitzpatrick, 2005). Caking is an undesirable powder attribute and is majorly ascribed to the van der Waals forces between the powder particles. Anticaking agents or flow additives are used in food powders to conquer the problems related to agglomeration and to provide better powder flowability.

Carboxy methyl cellulose (CMC) is a water-soluble cellulose of significant technical importance. It is used as a food thickener and has been widely used in the encapsulation of drugs (Milani and Maleki, 2012). One of the major reason of using CMC as an encapsulating agent (wall material) is high water solubility and is able to develop the fine and dense network upon dehydration (Benchabane and Bekkour, 2008). Wall materials should not allow lipid separation during dehydration and should be able to readily absorb the water upon reconstitution (Gharsallaoui *et al.*, 2007). No research has been conducted on the dehydration of aqueous nanoliposomal shrimp oil.

9.3 Objective

To examine the effect of wall material and anti-caking agent along with the drying methods on properties of shrimp oil nanoliposome powder.

9.4 Materials and methods

9.4.1 Preparation of nanoliposomes

Shrimp oil was extracted from cephalothorax of Pacific white shrimp (*Litopenaeus vanammei*) (Gulzar and Benjakul, 2019c). Modified ethanol injection method as tailored by Gulzar and Benjakul (2019a) was adopted for preparation of nanoliposomes. Briefly, 2 g of phosphatidyl choline (PC) was dissolved in 100 mL of ethanol and heated to 45 °C for complete solubilization. Subsequently, 2 mL of shrimp oil (preheated to 30 °C) was dropped gradually into 100 mL of prepared PC solution. The mixture was stirred at 1,000 rpm on a hot plate magnetic stirrer at 30 °C for 5 min. Deionized water (1000 mL) and glycerol (2% v/v) were added to hydrate the solution. Carboxy methyl cellulose (CMC) and fumed silica (SiO_2) (CAB-O-SIL® M-5P, Cabot, Inc.) were added to the prepared liposomal dispersion at the ratios of PC:CMC:SiO_2 1:5:5, 1:5:10, 1:10:5 and 1:10:10 (w/w/w). All the samples were homogenized for 10 min at a speed of 3000 rpm by an IKA Labortechnik homogenizer (Selangor, Malaysia). The liposomal dispersions with a total volume of approximately 1100 mL were ultrasonicated (10 min; 1 s on and off pulse) at 25 °C using an ultrasonic processor (Vibra-Cell™ VC 750, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude. Ethanol was removed using EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan) at 30°C. The shrimp oil nanoliposomes obtained were referred to as 'SONL'.

9.4.2 Drying of SONL

9.4.2.1 Freeze-drying

Firstly, the SONL samples were frozen at -40ºC for 6 h, followed by lyophilization using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at -50ºC for 72 h. The freeze-dried samples were ground after dehydration using a blender (National Model MK-K77, Tokyo, Japan) for 2 min. Freeze-dried powder

(FDP) with the wall material ratios of PC:CMC:SiO_2 at 1:5:5, 1:5:10, 1:10:5 and 1:10:10 (w/w/w) were labelled as FD1, FD2, FD3 and FD4, respectively.

9.4.2.2 Spray-drying

Different SONL samples were dried using a laboratory scale spray-dryer (LabPlant Ltd., LabPlant SD-05, Huddersfield, UK). The sample was fed to the drying chamber using a peristaltic pump at the feed rate of 5 mL/min. The inlet temperature was kept at 180±2 °C with a flow rate of 4.3 m/s and the outlet temperature was fixed at 105±2 °C. Spray-dried powder (SDP) with the wall material ratios of PC:CMC:SiO_2 at 1:5:5, 1:5:10, 1:10:5 and 1:10:10 (w/w/w) were labelled as SD1, SD2, SD3 and SD4, respectively.

9.4.3 Characterization of SONL powders

9.4.3.1 Particle size

Mean particle size and particle size distribution of FDP and SDP were measured using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) following the procedure of Castellani *et al.* (2006). Five gram of each sample were added with 20 mL of 1% (v/v) sodium dodecyl sulfate (SDS) solution in order to dissociate the flocculated vesicles. Surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the powder samples were determined.

9.4.3.2 Microstructure

Microstructures of FDP and SDP were analyzed using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, the Netherlands). The samples were finally mounted on individual bronze stubs and sputter-coated with gold layer (Sputter coater SPI-Module, West Chester, PA, USA). A scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) was used for visualization of specimen at an acceleration voltage of 20 kV. Magnifications from 10,000 to 300,000x were used.

9.4.3.3 Density and flowability

Flowability of FDP and SDP were characterized by using Carr index (Ci) calculated by the following equation:

$$Ci = \frac{(\rho t - \rho u)}{\rho t} \times 100$$

where ρt is tapped density of the powder samples; ρu is untapped density of powders. Tapped density is measured by filling a graduated cylinder with the powder and mechanically tapping the cylinder up and down a minimum of 50 times against the table (Carstensen, 1973). Volume of the powder is calculated from the graduations of the cylinder after no further change in volume takes place with tapping of cylinder to calculated tapped density. Untapped density is the normal bulk density of powder, calculated as mass per volume of powder without any tapping.

Ci of <15, 15–20, 20–35, 35–45 and >45 were classified as very good, good, fair, bad, and very bad, respectively (Turchiuli *et al.*, 2005).

9.4.3.4 Encapsulation efficiency

Encapsulation efficiency (EE) of powders were measured by the method as tailored by Takeungwongtrakul *et al.* (2015). Surface oil and total oil from FDP and SDP were recovered and measured. Surface oil was recovered by mixing 2 g powder with 15 mL hexane and shaking by a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY) for 2 min at room temperature, followed by filtering through a Whatman No. 1 filter paper. Twenty mL of hexane were used for mixing the collected powder for three times. The filtrate was transferred to a round bottom flask and evaporation of solvent was done till the weight was constant.

The total oil was measured by dissolving 2 g powder in 25 mL of 0.88% (w/v) KCl solution + 25 mL of methanol and 50 mL chloroform. The mixtures were homogenized for 5 min at 15,000 xg and transferred to a separating funnel. The chloroform layer was collected and removed by evaporation. EE was then calculated with the following equation.

$$EE = \frac{TO - SO}{TO} \times 100$$

where TO and SO are total oil content and surface oil content, respectively.

9.4.3.5 Wettability and solubility

Wettability of powders was determined by measuring the time to completely sink samples (5 g) in 100 mL water was recorded to determine the wettability of powders (Barkouti *et al.*, 2013). For measuring solubility, powder (0.1 g) was dispersed in 24.9 mL of distilled water. The mixtures were stirred at 25 °C for 15 min, followed by centrifugation at 3350 xg for 20 min. Supernatant (10 g) was dried at 105 °C in an oven until constant weight was achieved (Li *et al*., 2014). The solubility was calculated as follows:

$$Solubility\ (\%) = \frac{m \times 2.5}{w} \times 100$$

where *m* and *w* are dry matter content of dried supernatant and total mass of powder, respectively.

9.4.4 Characterization of selected SONL powder

FDP and SDP with the optimum concentration of CMC and SiO_2 that yielded the desirable flowability, encapsulation efficiency, wettability and solubility were selected for characterization

9.4.4.1 Lipid oxidation

Surface oil and total oil obtained from FDP and SDP samples stored for 0-6 weeks at 30 °C were extracted and examined for lipid oxidation. Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were determined using the ferric thiocyanate method as described by Chaijan *et al.* (2006) and the method as tailored by Buege and Aust (1978), respectively.

9.4.5 Fatty acid profile

Fatty acid profile reported as fatty acid methyl esters (FAMEs) using gas chromatography (GC) was examined as detailed by Gulzar and Benjakul (2019b).

Transmethylation was done by 2 M methanolic NaOH, along with 2 M methanolic HCl. The resulting FAMEs were analyzed by gas chromatography (GC) using Agilent 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) connected with a flame ionization detector. The chromatographic peaks of the standards were used to identify types of fatty acid. Calculation was made based on peak area ratio and the content was expressed as g fatty acid/100 g lipid.

9.4.6 Zeta potential

The zeta (ζ) potential of powder samples was determined with a zeta potential analyzer (ZetaPALs, Brookhaven Instruments Co., Holtsville, NY, USA). Each powder sample (1 g) was added with 20 mL of distilled water and transferred to an auto titrator (model BI-ZTU, Brookhaven Instruments Co.). The pH of solutions were adjusted to 2–6 by addition of either 1.0 M nitric acid or 1.0 M KOH. The zeta potential was then recorded.

9.4.7 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter (Perkin Elmer, Model DSC7, Norwalk, CA, USA) (Gulzar and Benjakul, 2019a). The powder samples were rehydrated by adding deionized water to the samples (1:40 w/v) and allowed to stand for 2 days at 4 °C. The samples were accurately weighed into aluminium pans and sealed. The samples were scanned at 10 °C/min over the range of -40 to 200 °C. An empty pan was used as the reference. Temperature calibration was run using the Indium standard.

9.4.8 FTIR spectra

Powder samples (200 mg) were placed in the horizontal attenuated total reflectance trough plate crystal cell (45 ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies, Inc., Madison, WI, USA) and mounted to FTIR spectrometer (Bruker Co., Ettlingen, Germany). Spectra of mid-infrared region (4000–500 cm^{-1}) were collected in 16 scans at a resolution of 4 $cm^{-}1$. They were resized towards a background spectrum attained from the clean empty cell at 25 °C.

Spectral data analysis was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

9.4.9 Statistical analysis

All the experiments were carried out in triplicates and the data were expressed as means ± SD. Completely randomized design (CRD) was used throughout the study. Analysis of variance (ANOVA) was performed and means were compared by Duncan's multiple range test (Steel and Torrie, 1980). Analysis of data was carried out using SPSS Statistics by IBM (SPSS version 23.0, IBM, Armonk, NY, USA).

9.5 Results and discussion

9.5.1 Characteristics of SONL powder prepared by freeze-drying and spray-drying methods

9.5.1.1 Particle size and morphology

Size distribution of both SONL powders, FDP and SDP is shown in Figure 46. The size of freeze-dried powder particles varied between 4 and 1800 µm with the mean size of 243.3±256.7 µm, whereas the size of spray-dried powder particles lied between 0.1 to 12 µm with the mean size of 5.33±2.55 µm. Freeze-drying generally resulted in the larger particle size of powder since there was no size reduction process involved unlike spray-drying, in which the particle size was reduced by atomization followed by dehydration (Ray *et al.*, 2016). The mean size of shrimp oil nanoliposomes prepared by ultrasonication method was found to be 104.77 nm (Gulzar and Benjakul, 2019a). However, the addition of polysaccharide (CMC) as wall material resulted in the agglomeration of nanoliposomes into microcapsules during the drying process.

The SEM images of FDP and SDP are shown in Figure 47. Structurally, SDP were spherical while the FDP particles were irregular flake-like. Drying processes greatly influence the shape of final product. Spray dried products are spherical since the drying occurs in tiny atomized droplets at very high temperature, thus resulting in rapid moisture migration and constant rate drying at the surface (Gouaou *et al.*, 2019).

However, some spray-dried powders can have a collapsed structure due to differential shrinkage rate caused by uneven drying at surface and core (Nishad *et al.*, 2017). Therefore, the wall materials play a major role in maintaining the spherical shape of powder particles (Takeungwongtrakul *et al.*, 2015). In case of freeze-dried powder, the irregular shape was mediated by low temperature and vacuum that pressed the particles together making them layered or flake-like (Karthik and Anandharamakrishnan, 2013). The images also indicated the presence of silica particles with the size of approximately 40 nm on the wall of both powders.

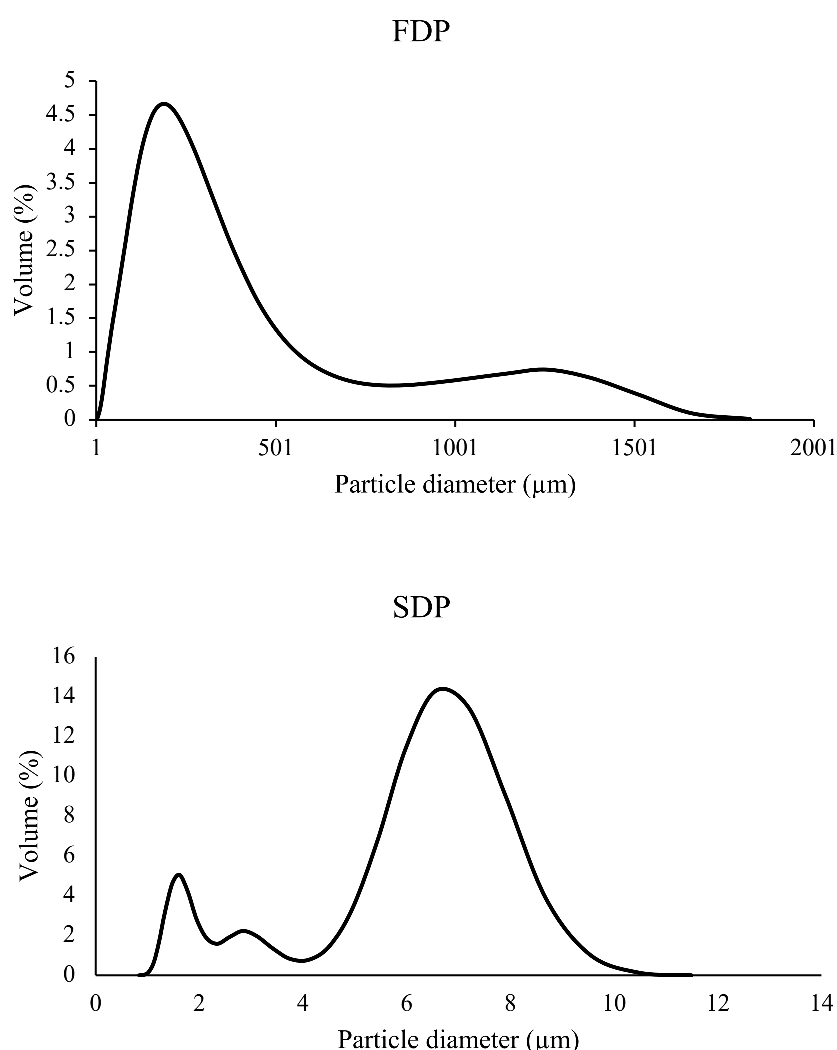

Figure 46. Particle size distribution (d_{43}) of shrimp oil nanoliposome powder prepared by different drying methods. FDP: Shrimp oil nanoliposome powder prepared by freeze-drying; SDP: shrimp oil nanoliposome powder prepared by spray-drying

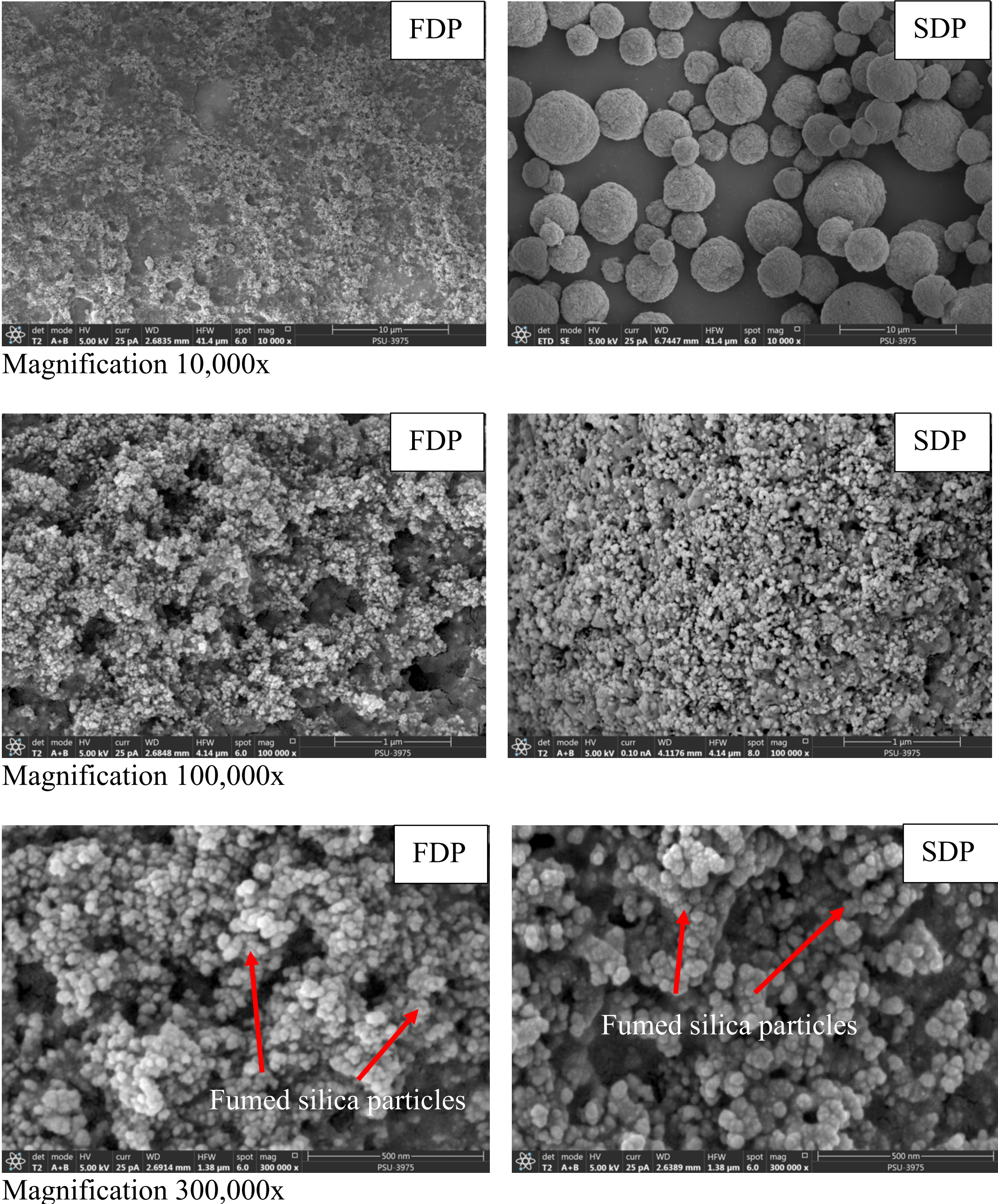

Figure 47. Scanning electron micrographs of shrimp oil nanoliposome powder prepared by different drying methods. FDP: Shrimp oil nanoliposome powder prepared by freeze-drying; SDP: shrimp oil nanoliposome powder prepared by spray-drying

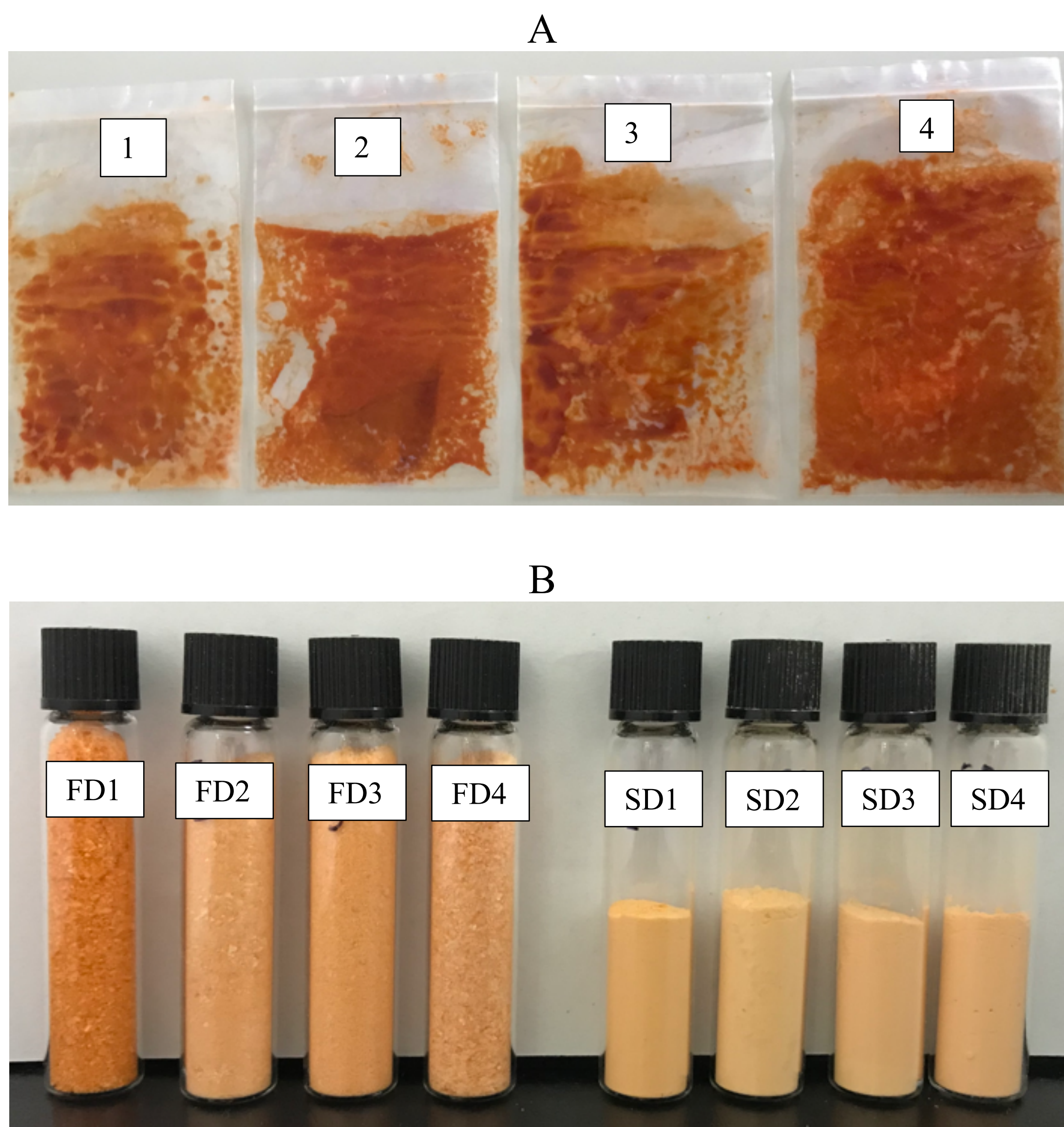

Figure 48. Pictures of freeze-dried shrimp oil nanoliposome powder without the addition of fumed silica (A) and shrimp oil nanoliposome powder prepared by different drying methods (B). 1, 2, 3 and 4 represent the freeze-dried SONL powder samples with PC:CMC (w/w) at 1:2.5, 1:5, 1:7.5 and 1:10, respectively. FD1, FD2, FD3 and FD4 represent the freeze-dried SONL powder samples with the wall material ratios of PC:CMC:SiO_2 (w/w/w) at 1:5:5, 1:5:10, 1:10:5 and 1:10:10, respectively. SD1, SD2, SD3 and SD4 represent the spray-dried SONL powder samples with the wall material ratios of PC:CMC:SiO_2 (w/w/w) at 1:5:5, 1:5:10, 1:10:5 and 1:10:10, respectively.

9.5.1.2 Density and flowability

The tapped and untapped (bulk) densities of FDP and SDP are shown in Table 15. The density of FDP was much lower than SDP ($p<0.05$). FDP were porous with void structure that caused profound effect on lowering the bulk density ($p<0.05$). Moreover, FDP were flake-like and larger in size, resulting in very little difference in untapped and tapped densities. On the other hand, SDP had fairly higher density and less porous structure due to smaller size of particles. The difference in the untapped and tapped density of SDP was higher than that of FDP, indicating more ordered structure of SDP particles. Low densities of FDP and SDP resulted from the presence of fumed silica in the powder. Fumed silica, which has a very low bulk density (approx. 40 kg/m^3) affected the overall bulk density of the powder. Higher moisture content of FDP was attained than SDP ($p<0.05$). Moisture content also affected the bulk density of the powders. Due to the high moisture content, particles tended to stick together, creating more interstitial spaces and consequently increasing the bulk density (Goula and Adamopoulos, 2005).

Flowability of SONL powders was described by carr index, which is one of numerous indices used to measure flowability of powders in many industries (Li *et al.*, 2004). Carr index of freeze-dried powders lied in the range of 20.40–22.50, whereas that of spray dried powders varied between 27.64 and 29.43. Flowability of powders having carr index between 20–35 is considered as fair (Barkouti *et al.*, 2013). FDP was more free-flowing than SDP, as confirmed by lower carr index. There was no agglomeration observed in the SONL powders prepared by both drying methods. Free flowing property of SONL powders could be attributed to the anti-caking property of fumed silica. Anticaking agents disrupt the cohesive interactions between powder particles by placing themselves between powder particles and reducing the friction (Fitzpatrick, 2005). Fumed silica (SiO_2) is one of the widely used anti-caking agents used in pharmaceutical and food industries. Fumed silica is hydrophilic and has a large number of hydroxyl groups on the surface, which can readily interact through hydrogen bonds forming a three dimensional network (Ahmad *et al.*, 2006). Hygroscopic fumed silica works by absorbing moisture from the environment without liquefying and reducing friction between the particles.

Without the addition of fumed silica, the freeze-dried samples formed a paste-like structure with very high hygroscopicity (as shown in Figure 48A), whereas the spray-dried samples could not be collected, since it got stuck to the drying chamber during drying. Addition of fumed silica resulted in free flowing powders (Figure 48B). Overall, the flow behavior of SONL powders was fairly good and no caking or lump formation was observed when fumed silica was incorporated.

9.5.1.3 Encapsulation efficiency

Encapsulation efficiency (EE) of SONL powder prepared using freeze-drying and spray-drying methods is shown in Table 15. EE of SONL powders varied between 51.18±3.81% and 68.52±1.85%. Spray-dried SONL powder had higher encapsulation efficiency compared to freeze-dried counterpart ($p<0.05$). EE is the index of protection offered by the wall material to encapsulated oil or bioactive compounds (Takeungwongtrakul *et al.*, 2015). EE between 0 and 95% have been documented (Baik *et al.*, 2004; Hardas *et al.*, 2000; Kha *et al.*, 2010; Klinkesorn *et al.*, 2006). EE of microencapsulated shrimp oil powder having whey protein concentrate and sodium caseinate as wall material varied between 14.65 and 52.05% (Takeungwongtrakul *et al.*, 2014). With increasing CMC concentration, EE was increased irrespective of the drying methods used ($p<0.05$). Silica concentration however did not have any pronounced impact on EE ($p>0.05$). The results were in agreement with Takeungwongtrakul *et al*. (2014) who documented the increase in EE as core/wall material ratio was increased from 1:2 to 1:4. Encapsulation efficiency of oils is also a function of drying method employed for encapsulation. SDP had higher EE than FDP ($p<0.05$), indicating the presence of more surface oil on FDP. Quispe-Condori *et al.* (2011) demonstrated that freeze drying resulted in lower EE of flax oil, compared to spray drying. EE of DHA oil using spray drying was higher (82.16%) than that using freeze-drying method (73.08%) (Karthik and Anandharamakrishnan, 2013). This could be possibly due to the dehydration of emulsifiers/wall materials, promoting droplet-droplet interactions and reducing stability (Choi *et al*., 2007). Since freeze-drying took a longer time, the removal of ice crystals during drying process resulted in the formation of pores and voids, causing the release of oil from the core (Chen *et al*., 2013).

Table 15. Untapped density, tapped (bulk) density, carr index, encapsulation efficiency, reconstitution time, solubility and moisture content of freeze dried and spray-dried shrimp oil nanoliposome powder with different wall material formulations.

Sample	Wall materials	Untapped density (kg/m^3)	Tapped density (kg/m^3)	Carr index	%EE	Reconstitution Time (min)	Solubility	Moisture content
FD1	PC:CMC:Sil (1:5:5, w/w/w)	48 ± 2f	61 ± 3f	21.36 ± 1.64cd	52.94 ± 2.93d	15.2 ± 0.9b	69.61 ± 1.9d	3.98 ± 0.23a
FD2	PC:CMC:Sil (1:5:10, w/w/w)	55 ± 3e	71 ± 4e	22.3 ± 0.70c	51.18 ± 3.81d	15.8 ± 0.8b	71.25 ± 1.3d	4.02 ± 0.18a
FD3	PC:CMC:Sil (1:10:5, w/w/w)	89 ± 3d	115 ± 5d	22.5 ± 0.50c	53.85 ± 2.09d	13.4 ± 1.0c	74.15 ± 1.1c	4.16 ± 0.29a
FD4	PC:CMC:Sil (1:10:10, w/w/w)	108 ± 4c	135 ± 5c	20.39 ± 0.60d	52.17 ± 2.76d	14.1 ± 1.2bc	75.12 ± 1.8c	3.92 ± 0.24a
SD1	PC:CMC:Sil (1:5:5, w/w/w)	222 ± 5a	310 ± 7a	28.34 ± 1.65a	61.29 ± 3.05c	21.9 ± 0.9a	78.70 ± 1.9b	2.84 ± 0.13b
SD2	PC:CMC:Sil (1:5:10, w/w/w)	212 ± 6ab	297 ± 8ab	28.65 ± 1.35a	65.52 ± 1.96b	22.1 ± 0.8a	80.15 ± 1.6b	2.94 ± 0.16b
SD3	PC:CMC:Sil (1:10:5, w/w/w)	208 ± 7b	287 ± 8b	27.63 ± 1.07ab	68.51 ± 1.85a	20.7 ± 1.1a	84.72 ± 1.5a	2.98 ± 0.19b
SD4	PC:CMC:Sil (1:10:10, w/w/w)	221 ± 7a	313 ± 9a	29.42 ± 0.57a	69.23 ± 1.76a	21.4 ± 0.7a	85.22 ± 1.4a	2.88 ± 0.17b

Caption see Figure 48. Different lowercase letters in the same column indicate significant differences ($p<0.05$).

9.5.1.4 Solubility and wettability

Solubility has been considered as the major criterion to reflect the overall quality of powders. Solubility of both FDP and SDP is shown in Table 15. FDP had lower solubility than SDP ($p<0.05$). The lower solubility of FDP could be caused by the presence of higher hydrophobic surface oil. Solubility of powders in water depends on the hydrophilic and hydrophobic components in the dried powder. CMC is a water soluble polysaccharide used as a tablet binder and for stabilizing emulsions (Guo *et al.*, 1998). CMC had a positive impact on improving the solubility of the powders. Both FDP and SDP samples with high CMC proportion had higher solubility ($p<0.05$), as seen in the FD3, FD4, SD3 and SD4 samples. Fumed silica is hydrophilic with large number of surface hydroxyl groups, and is fairly soluble in water.

Reconstitution time (RT) largely depends on the wettability of individual powder particles and the agglomeration upon rehydration. Reconstitution time of FDP was significantly lower than that of SDP ($p<0.05$) (Table 15). Lower reconstitution time of powders indicated more wettability of powders. Reconstitution time of powders can vary from few seconds to hours, depending on the structure, porosity, presence of hydrophobic/hydrophilic groups, etc. (Seth *et al.*, 2017). Due to larger size of FDP, its wettability was greater than SDP. Moreover, the porous structure of freeze-dried samples enables the particles to absorb water like a sponge (Chen *et al*., 2013). During freezing of emulsions, some water molecules remain unfrozen which are bonded to protein or carbohydrate by hydrogen bonds. When these unfrozen water molecules are removed during the drying process, they leave behind the active sites, previously occupied by them, were exposed. Upon rehydration, these powders readily absorb the water, particularly at these active sites (Shefer and Shefer, 2003). The process of rehydration is also augmented by the open structure and high surface area of the FDP. Conversely, in case of SDP, the rapid drying results in the shrinkage and lower hydrogen bonding between water and carbohydrates or proteins. This might reduce the dissolution rate of the SDP (Chen *et al.*, 2013). Nevertheless, the presence of polysaccharides enhances the solubility of powders (Guo *et al.*, 1998). Samples with higher CMC proportion had lower reconstitution time ($p<0.05$). Increased silica

concentration, however, increased the reconstitution time due to its poor wettability and smaller size.

9.5.2 Stability of SONL powder prepared by freeze-drying and spray drying method

Based on the physical properties of the powders, optimum wall material concentration of PC:CMC:SiO_2 (1:10:5 w/w/w) was used for preparation of FDP and SDP for further characterization and stability studies

9.5.2.1 Oxidative stability

Oxidative stability of total oil and encapsulated oil in the selected FDP and SDP was monitored by determination of PV and TBARS values over the storage of 6 weeks at 30 °C. Changes in the PV of the total oil and encapsulated oil recovered from FDP and SDP over 6 weeks are shown in Figure 49. PV of total oil from SDP was higher than FDP ($p<0.05$) and continued to increase over time. This could be attributed to high temperature conditions used for spray drying, that might induce lipid oxidation of the surface oil. Although freeze-dried samples had higher surface oil, compared to spray-dried powder, the oil suffered less oxidation because the vacuum and low temperature conditions were used in freeze-drying. High temperature in the presence of oxygen can accelerate lipid oxidation (Frankel, 2005). Moreover, the spherical shape of spray-dried powder particles resulted in higher surface/volume ratio, which could favor the oxidation of oil. PV of total oil recovered from both the powder samples continued to rise over the storage period mainly due to non-encapsulated oil, rendering it prone to oxidation. Nevertheless, PV of encapsulated oil was lower ($p<0.05$), with no difference between FDP and SDP ($p>0.05$). Chen *et al.* (2013) showed that total oil from freeze-dried and spray-dried fish oil microcapsules underwent more oxidation than encapsulated fish oil. PV of surface oil from microencapsulated fish oil was 10-time higher than the encapsulated counterpart (Baik *et al.*, 2004). Nanoliposomal encapsulation is known to prevent the lipid oxidation by forming the barrier around the oil and protecting it from prooxidants (Hadian, 2016). Encapsulation of SONL resulted in enhanced oxidative stability and lower PV when stored over a period of 8 weeks (Gulzar and Benjakul, 2019a). Drying

of SONL to powder form could further prevent the oxidation as the mobility of prooxidants such as enzymes (lipoxygenases), some transition metals (Cu, Fe) etc., is reduced (Kolanowski *et al*., 2007).

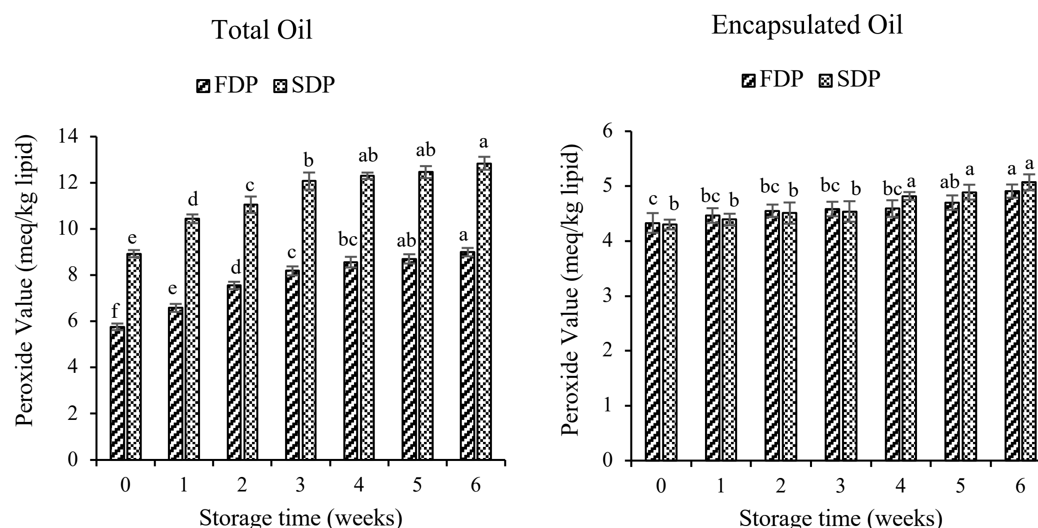

Figure 49. Peroxide value of total oil and encapsulated oil from shrimp oil nanoliposome powder prepared by different drying methods during storage of 6 weeks at 30 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars in the same samples denote significant difference ($p<0.05$). Caption see Figure 46.

Figure 50 represents the change in TBARS values of total oil and encapsulated oil recovered from FDP and SDP over 6 weeks of storage. Total oil had higher TBARS value than encapsulated oil and continued to augment over the storage period ($p<0.05$). Increase in TBARS values followed the similar trend as that of PV. However, sharp rise in TBARS values was seen after week 4 for total oil, indicating the increased formation of secondary lipid oxidation products. Hydroperoxides formed as the primary oxidation products are further decomposed to produce an off-flavor volatile compounds (Frankel, 2005). Encapsulation in nanoliposomes resulted in higher oxidative stability over the extended storage time.

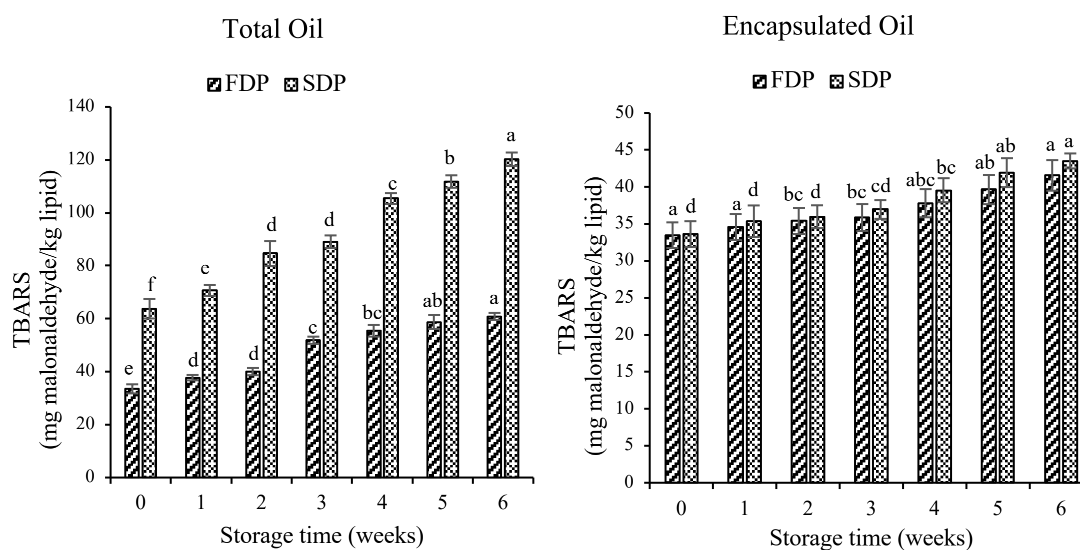

Figure 50. TBARS value of total oil and encapsulated oil from shrimp oil nanoliposome powder prepared by different drying methods during storage of 6 weeks at 30 °C. Bars represent the standard deviation (n=3). Different lowercase letters on the bars in the same samples denote significant difference ($p<0.05$). Caption see Figure 46.

9.5.2.2 Fatty acid profiles

Fatty acid profile of total oil recovered from FDP and SDP is illustrated in Table 16. Shrimp oil from both powder samples contained saturated fatty acids (SFA) as major fatty acids, followed by PUFAs. Linoleic acid was the major unsaturated fatty acid, followed by DHA and EPA. Shrimp oil recovered from SDP showed a decreased content of PUFA, particularly EPA and DHA ($p<0.05$), which could be attributed to the oxidation of PUFAs, as confirmed by the increased PV and TBARS (Figure 49 and 50). Loss of PUFAs led to increase in SFA content of shrimp oil in SDP. Oxidation of PUFAs resulted in the decrease in the polyene index (ratio of DHA + EPA/C16:0) of oils (Gulzar and Benjakul, 2019b). Polyene index of shrimp oil recovered from SDP decreased by 34.9%, compared to shrimp oil recovered from FDP, confirming better oxidative stability of the latter. Due to the oxidation of shrimp oil, DHA and EPA were decreased, while palmitic acid (C16:0) remained mostly unchanged. This lowered the polyene index (Belhaj *et al.*, 2010).

Linoleic acid and oleic acid remained unchanged ($p>0.05$), probably because of their lower degree of unsaturation, whereas EPA and DHA were significantly reduced ($p<0.05$) as they are highly susceptible to oxidation (Miyashita *et al.*, 1993). Encapsulation of shrimp oil in nanoliposomes resulted in higher retention of PUFAs, especially EPA and DHA (Gulzar and Benjakul, 2019a). However, oxidation of surface oil in SDP could reduce the overall PUFA content of total shrimp oil.

Table 16. Fatty acid profile of total encapsulated shrimp oil and shrimp oil nanoliposomes prepared by different drying methods.

Fatty acids (g/100 g lipids)	FDP	SDP
C14:0 (Myristic)	2.66 ± 0.03a	2.38 ± 0.02b
C15:0 (Pentadecanoic)	1.34 ± 0.01a	1.24 ± 0.01b
C16:0 (Palmitic)	21.26 ± 0.67a	21.06 ± 0.70a
C16:1 (Palmitoleic)	1.81 ± 0.01a	1.61 ± 0.02b
C17:0 (Heptadecanoic)	1.97 ± 0.03a	1.83 ± 0.04b
C18:0 (Stearic)	7.25 ± 0.13b	7.60 ± 0.22a
C18:1 (Oleic)	10.69 ± 0.06a	10.85 ± 0.33a
C18:2 (Linoleic)	14.90 ± 0.15a	14.77 ± 0.09a
C20:0 (Arachidic)	2.18 ± 0.02b	3.12 ± 0.02a
C20:0 (Docosanoic)	2.26 ± 0.01b	4.16 ± 0.01a
C20:1 (Eicosenoic)	1.60 ± 0.06a	1.33 ± 0.01b
C20:2 (Eicosadienoic)	1.91 ± 0.00a	1.62 ± 0.03b
C20:5 (EPA)	5.16 ± 0.07a	4.34 ± 0.11b
C21:0 (Heneicosanoic)	2.03 ± 0.09a	2.19 ± 1.48a
C22:6 n-3 (DHA)	8.79 ± 0.05a	4.65 ± 0.15b
C23:0 (Tricosanoic)	3.28 ± 0.05a	2.80 ± 0.06b
C24:0 (Lignoceric)	2.21 ± 0.01a	2.10 ± 0.01b
C24:1 (Nervonic)	1.26 ± 0.00	ND
Unidentified peak	3.67 ± 0.03b	6.33 ± 0.02a
Saturated fatty acid (SFA)	46.44 ± 0.94b	48.49 ± 0.39a
Monounsaturated fatty acid (MUFA)	15.36 ± 0.12a	13.79 ± 0.36b
Polyunsaturated fatty acid (PUFA)	30.76 ± 0.27a	25.38 ± 0.38b

Different lowercase letters in the same row indicate significant differences ($p<0.05$). Caption see Figure 46.

9.5.2.3 Differential scanning calorimetry

DSC thermograms of FDP and SDP are shown in Figure 51. The transition from orderly (crystalline) to disorderly state takes place as thermal energy is applied to any system, and the glass transition temperature (T_g) is recorded with calorimetry. The phase transition of liposomes occurs at a defined temperature. With the addition of thermal energy to the system, the phospholipid bilayers transit from a gel (orderly) state to a fluid (disorderly) state. This affects the Van der Waals interactions between the hydrocarbon chains and increases their mobility (Oh *et al.*, 2012). Most of the phospholipid bilayers have a transition temperature between 40 and 70 °C (Liu *et al.*, 2015). SONL prepared by ultrasonication method had a T_g of 57.3 °C (Gulzar and Benjakul, 2019a). However with the addition of a plasticizing agent, the T_g was significantly lowered. Due to higher availability of water on the surface of fumed silica, it lowered the T_g of polycarbonate composites (Yadav *et al.*, 2017). As the water content of liposomes was augmented, the T_g was decreased due to enhanced segmental movements. Polysaccharides such as CMC also have higher water binding capacity which can also result in reducing the T_g of powder. T_g is also dependent on the chain length and saturation of acyl chains. Therefore the higher T_g of SDP was attributed to the presence of higher saturated fatty acids (Table 16). Although the transition of states does not reflect the overall stability of the product, it provides the information on the nature of thermodynamic and energetic changes associated with the lipid transformation from one state to another (Biltonen and Lichtenberg, 1993).

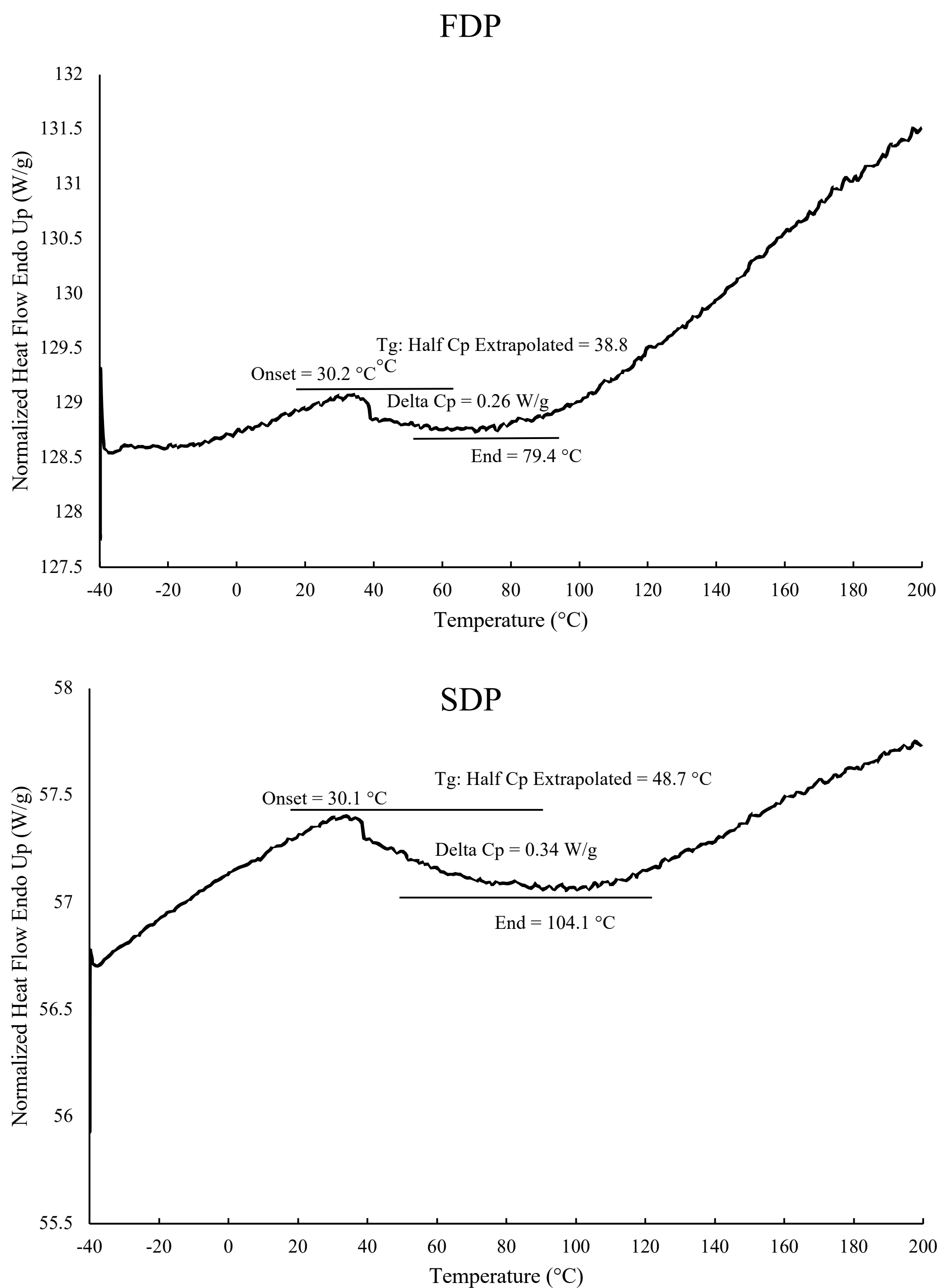

Figure 51. Differential scanning calorimetry thermograms of shrimp oil nanoliposome powder samples prepared by different drying methods. Caption see Figure 46.

9.5.2.4 ζ-Potential

Zeta-potential is an index of stability of emulsions and liposomal dispersions in which the potential difference between the particles in the dispersion medium is measured. ζ-potential of freeze-dried SONL powder was -68.86 mV, whereas that of spray-dried SONL powder was -58.04 mV. Negative values higher than -35 mV in liposomes were considered to be stable (Chun *et al*., 2017). Higher negative charge induces sufficient electrostatic repulsion to prevent flocculation and coalescence of emulsion upon reconstitution. High value of ζ-potential, either positive or negative, means the higher stability governed by the mutual repulsion between the electrical double layers of macromolecules (Acedo-Carrillo *et al.*, 2006). Higher negative values also reflect the enhanced solubility of the powders upon reconstitution in water. The negative charge on the particles was mostly contributed by the phosphate group of the phospholipids and negatively charged residues on the oil. Silica is known to induce negative charge on the surface of particles (Nolan *et al.*, 1981). ζ-potential of fumed silica nanofiber based composite was changed from -31.53 to -40.82 mV when silica content was increased from 8 to 12 wt% (Jung *et al.*, 2009). Higher negative value of FDP could be attributed to the larger particle size than SDP. Studies have shown gradual increase in absolute ζ-potential with augmenting particle size (Ofir *et al.*, 2007).

9.5.3 FTIR spectra

FTIR spectra of FDP and SDP are presented in Figure 52. Broad peaks at wavenumber of ~3600-3200 cm^{-1} representing —OH groups was observed in both the samples which could be attributed to the hydroxyl groups present in fumed silica and some hydroperoxides formed as a result of lipid oxidation. Shrimp oil extracted from shrimp cephalothorax using nitrogen flushing method had negligible peaks in the absorbance bands of 3600-3200 cm^{-1} (Gulzar and Benjakul, 2019c), indicating that shrimp oil has lower hydroxyl or hydroperoxide content. However, with the oxidation of oil, the peaks at the specified bands were increased (Gulzar and Benjakul, 2018). The peak at 2920 cm^{-1} assigned to the stretching vibration of —CH_2— group is used for the determination of saturation of fatty acid mixtures (Chapman, 1965) and the peaks at ~2850 cm^{-1} corresponding to the aliphatic —CH

vibrating bands are also employed to determine degree of unsaturation of oil (Guillén and Cabo, 1997). The peak at ~1730 cm^{-1} represents the C=O stretching located between the hydrophilic head and hydrophobic tail of lecithin molecules (Tantipolphan *et al.*, 2007), confirming the presence of lecithin, present as the wall material of nanoliposomes. The absorption bands at ~1590 cm^{-1} have been known to represent free fatty acid (FFA) of the oils (Guillén and Cabo, 1997). Shrimp oil contained 9.11 – 34.9% FFA, depending on the method of oil extraction employed (Gulzar and Benjakul, 2018; Takeungwongtrakul *et al.*, 2012). The peak at 1000–1200 cm^{-1} corresponds to the presence of carbohydrate (Ahmed *et al.*, 2013), implicating the larger presence of CMC in the powders. The absorbance at ~460 cm^{-1} represents the phospholipid backbone of the lecithin, as well as the shrimp oil. Nevertheless, similar peaks were observed in the FTIR spectra of shrimp oil containing 45.98% phospholipids, as reported by Gulzar and Benjakul (2018). Overall, FTIR spectra were useful in identifying and confirming the presence of various compounds in SONL powder and monitoring the changes taken place during processing.

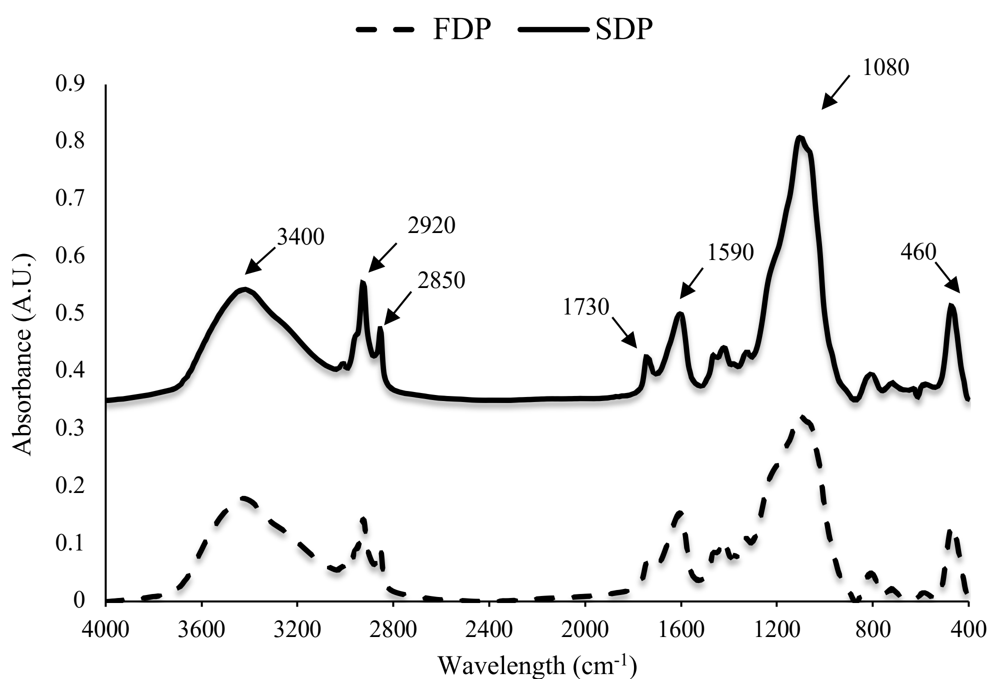

Figure 52. FTIR spectra of shrimp oil nanoliposome powder samples prepared by different drying methods. Caption see Figure 46.

9.6 Conclusion

Freeze-drying and spray-drying were employed to dehydrate shrimp oil nanoliposomes. Wall matrix of shrimp oil nanoliposomes consisted of CMC as emulsion stabilizer and fumed silica as anti-caking agent. The concentration of CMC and fumed silica had significant effect on physicochemical properties of SONL powder prepared using freeze-drying and spray-drying. Both the powders were free-flowing without any significant agglomeration or hygroscopicity. Spray dried powder had higher bulk density, encapsulation efficiency and solubility, whereas freeze-dried powder showed higher oxidative stability and better retention of EPA and DHA. Overall, the powders prepared by either methods were stable and could be used to fortify in to a variety of foods as excellent source of n-3 fatty acids and astaxanthin.

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CHAPTER 10

CONCLUSION AND SUGGESTION

10.1 Conclusion

1. Ultrasonic assisted extraction (UAE) significantly increased the yield of lipids and carotenoids extracted from Pacific white shrimp cephalothorax (SC), particularly with the continuous mode. Nevertheless, UAE resulted in enhanced oxidation and hydrolysis of lipids.

2. Pre-heating of SC at 95 °C in the presence of tannic acid (TA) (0.1%) resulted in the enhanced extraction yield and better oxidative stability of lipids, when UAE was used. TA inhibited lipase, thus suppressing hydrolysis of extracted lipids.

3. Pulsed electric field (PEF) pre-treatment of SC prior to lipid extraction by UAE process increased the extraction yield of lipids and was beneficial in suppressing lipid oxidation. Consequently, PEF helped in retaining more PUFAs and carotenoids in the extracted lipids.

4. Nitrogen flushing during ultrasonication, pre-treatment of SC by vacuum-microwave along with the addition of TA had beneficial effects on the quality of lipids extracted by UAE process. The lipids showed highest retention of PUFAs and carotenoids, particularly astaxanthin.

5. Shrimp oil was encapsulated into liposomes by modified ethanol injection and ultrasonication method, in which nanoliposomes were thermodynamically stable. The resulting nanoliposomes prevented the oxidation of encapsulated shrimp oil while masking the characteristic fishy odor of the oil.

6. Skim milk fortified with shrimp oil nanoliposomes (SONL) up to 10% were acceptable sensorially. Fortified milk was microbially stable during the storage period. However, mild bitterness was perceived by some panelists in sensory evaluation.

7. Skim milk fortified with SONL in presence of β-glucan at 0.1% had negligible bitterness. Nonetheless, there was slight increase in the viscosity of fortified skim milk samples.

8. SONL dehydrated using freeze-drying and spray-drying containing carboxymethyl cellulose and fumed silica were free-flowing powder without any significant agglomeration or hygroscopicity.

10.2 Suggestions

1. Studies on extraction of shrimp oil using other novel extraction techniques such as supercritical fluid extraction and high pressure extraction in combination with UAE should be taken in to consideration.

2. Encapsulation efficiency of SONL powders should be improved by further development of other wall materials.

3. Stability of astaxanthin in free and encapsulated forms of shrimp oil should be enhanced.

4. Fortification of shrimp oil nanoliposomes in other food systems such as fruit juice beverages and bakery products should be carried out.

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List of Publication and Proceedings

Publication

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Patent

1. Gulzar, S. and Benjakul, S. The process of extracting oil from shrimp heads that provides high quality and yield. Request number 1903002454.